Aamir Raina · Mohammad Rafiq Wani · Rafiul Amin Laskar · Nasya Tomlekova · Samiullah Khan *Editors*

# Advanced Crop Improvement, Volume 2

## Case Studies of Economically Important Crops

Springer

Advanced Crop Improvement, Volume 2

Aamir Raina • Mohammad Rafiq Wani
Rafiul Amin Laskar
Nasya Tomlekova • Samiullah Khan
Editors

# Advanced Crop Improvement, Volume 2

## Case Studies of Economically Important Crops

Springer

*Editors*
Aamir Raina
Mutation Breeding Laboratory
Department of Botany
Aligarh Muslim University
Aligarh, Uttar Pradesh, India

Botany Section, Women's College
Aligarh Muslim University
Aligarh, Uttar Pradesh, India

Rafiul Amin Laskar
Department of Botany
Pandit Deendayal Upadhyaya Adarsha
Mahavidyalaya (PDUAM), Eraligool
Karimganj, Assam, India

Samiullah Khan
Mutation Breeding Laboratory
Department of Botany
Aligarh Muslim University
Aligarh, Uttar Pradesh, India

Mohammad Rafiq Wani
Department of Botany
Abdul Ahad Azad Memorial Degree College
Bemina, Cluster University
Srinagar, Jammu and Kashmir, India

Nasya Tomlekova
Molecular Biology Laboratory
Maritsa Vegetable Crops Research Institute
Agricultural Academy
Plovdiv, Bulgaria

ISBN 978-3-031-26668-3 ISBN 978-3-031-26669-0 (eBook)
https://doi.org/10.1007/978-3-031-26669-0



This Springer imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

*Dedicated to Sir Syed Ahmad Khan, the Founder of Aligarh Muslim University, Aligarh, India. Sir Syed Ahmad Khan, born on 17 October 1817, in Delhi, has played a critical role in shaping the modern India. Sir Syed was a great social reformer, educationist, philosopher, and a pioneer in emphasizing the vital role of education in the empowerment of Muslim community. He worked selflessly in educating and igniting the minds of Muslims. He was the first to realize the need of imparting formal education to Muslims and acquiring proficiency in the English language and modern sciences. He established Scientific Society in 1863 to inculcate a scientific temperament into the Muslims and to make the Western knowledge accessible to Indians. Dr. Sir Mohammad Iqbal observes: "The real greatness of Sir Syed consists in the fact that he was the first Indian Muslim who felt the need of a fresh orientation of Islam and worked for it – his sensitive nature was the first to react to modern age."*
*On 24 May 1875, Sir Syed established the Madarsatul Uloom in Aligarh following the*

*patterns of Oxford and Cambridge universities. His aim was to shape a college in line with the British education system but without compromising its Islamic values. During Sir Syed's own lifetime, The Englishman, a renowned British magazine of the nineteenth century, remarked in a commentary on 17 November 1885: "Sir Syed's life strikingly illustrated one of the best phases of modern history." He died on 27 March 1898, and lies buried next to the main mosque at AMU.*

# Preface

With the twin pressures of climate change and burgeoning population, achieving sustainable development goals in general and food security in particular is a daunting task. The human population is increasing at a faster rate in developing nations, and food and nutrition available to feed the sky-high population is challenging task to scientists. As expanding arable land is not possible, the viable approach to enhance the food production is to create varieties with higher yielding potential and wide adaptability. So far, both conventional and new plant breeding approaches have contributed in terms of developing plant varieties with high yield and better resistance to biotic and abiotic stresses and in enhancing the food production. In both approaches, genetic variation is a prerequisite for improving yield and yield-attributing traits in crops. The major drawback of conventional plant breeding approaches is that more time is required for achieving the goals. However, new plant breeding technologies such as molecular breeding complement the conventional breeding approaches to obtain the desired food production. Recently developed tools and techniques such as molecular markers, genome wide association studies, Omics, TILLING, Eco-TILLING, and gene editing have made significant contributions in the crop improvement programs. These approaches have brought preferred set of traits in the varieties particularly high yielding potential, stress tolerance, nutrient quality and adaptability.

This book provides insights into the concept, limitations, and role of conventional breeding approaches as well as latest developments in the modern plant breeding field. This book consists of two volumes: Volume 1 subtitled Theory and Practice and Volume 2 subtitled Case Studies of Economically Important Crops. This first volume comprises 18 chapters and the range of topics covered encompasses mutation breeding, molecular breeding, nanotechnology, transgenics, crop biofortification, forward and reverse genetics, RNA interference technology, doubled haploid production, TILLING and ECO-TILLING, genome-wide association study, genome editing, CRISPR/CAS, and Next-Generation Sequencing. The second volume consists of 19 chapters and covers detailed aspects of different crops such as capsicum, potato, carrot, buckwheat, cowpea, mung bean, lentil, chickpea, faba bean, maize, sunflower, and sorghum in addition to several techniques such as

Raman spectroscopy, molecular markers, in vitro embryo rescue techniques, genome-wide association study, and CRISPR/CAS. Each chapter contains different sections such as introduction providing background, present progress, and a detailed discussion and explanations. Each chapter ends with a conclusion and future directions and a comprehensive list of references to facilitate further reading. In addition, each chapter is supported by good-quality figures and tables. This book shall prove useful to researchers who intend to expand their plant breeding techniques. Besides, it will help practicing plant breeders working in government and private sectors. Moreover, the book shall be helpful for undergraduate and postgraduate students pursuing specialization of plant breeding, plant genetics, and plant biotechnology.

Chapters were drafted by internationally recognized scientists, and each chapter was reviewed multiple times to ensure high-quality content and scientific integrity and accuracy. In this book, the experienced writers have put in a lot of effort in converting their vast experience and knowledge into useful guidelines for students, teachers, plant breeders, geneticists, policymakers, and other stakeholders. We are thankful to our contributors for nicely drafting the chapters and facilitating the publication of this two-volume book representing about 144 scientists from 19 countries. Despite our careful editing and reviewing, we might have missed some errors for which we seek reader's indulgence and feedback. We the editors are proud in completing this book by working day and night amid tough times of Covid-19 pandemic. Lastly, we are thankful to Springer for providing us an opportunity to compile this book. Moreover, we are grateful to all the other staff members of Springer, particularly Kenneth Teng, Arun Siva Shanmugam, Alicia Richard, Vinesh Velayudham and Kate Lazaro for helping us in accomplishing the publication of two volumes of this book.

Aligarh, Uttar Pradesh, India — Aamir Raina
Srinagar, Jammu and Kashmir, India — Mohammad Rafiq Wani
Karimganj, Assam, India — Rafiul Amin Laskar
Plovdiv, Bulgaria — Nasya Tomlekova
Aligarh, Uttar Pradesh, India — Samiullah Khan

# Acknowledgments

We acknowledge the financial support from International Atomic Energy Agency, grant number BUL/5/016 and RER/5/024. ERA-NET CORE Organic “Diversifying organic crop production to increase resilience” (DIVERSILIENCE) (МОН – КП06ДО-02/7). We acknowledge all original contributors whose works are cited in the chapters.

# About the Book

In the current scenario of an increased rate of urbanization and expanding cities, urban food security and nutrition is a great challenge. The human population is growing at a faster rate in developing nations, and that puts a lot of pressure on food systems. Besides sky high human population, food production is reduced due to climate change, dwindling arable lands, exhausting water resources, narrow genetic diversity, and shift in agriculture toward cultivation of few food crops. Food and Agriculture Organization predicted that the human population will exceed 9.6 billion by 2050 and this means 70% more food would be required to feed the burgeoning population. As the expansion of land is not possible, the food production can be increased by developing crop varieties with more yielding potential. In this context, breeding approaches have played a pivotal role in developing crops with desired traits. Conventional breeding approaches are arduous and tedious; therefore, newer plant breeding techniques like omics and gene editing should be used to supplement the already existing breeding strategies in achieving the desired food production. New plant breeding strategies should be designed and implemented to accelerate the crop development and to bring preferred set of traits in crops of economic importance. In any breeding approach for crop improvement programs, genetic variability is an important prerequisite. The book entitled *Advanced Crop Improvement* is divided into two volumes with emphasis on role of breeding approaches in enhancing genetic variability in important crops. The first volume of the book covers topics such as mutation breeding, molecular breeding, nanotechnology, transgenics, crop biofortification, forward and reverse genetics, RNA interference technology, doubled haploid production, TILLING and ECOTILLING, genome-wide association study, genome editing, CRISPR/CAS, and Next-Generation Sequencing. The second volume covers detailed aspects of different crops such as capsicum, potato, carrot, buckwheat, cowpea, mung bean, lentil, chickpea, faba bean, maize, sunflower, and sorghum.

The basic concept of this book is to provide a broader view of collective role of breeding approaches (both conventional and modern) in advancing the crop

improvement programs. The book provides a detailed background of breeding approaches and recent advancements as well as the role and land mark achievements in accelerating the crop development. This book may prove as a complete guide for plant breeders, geneticists, researchers engaged in plant breeding programs in addition to post- and undergraduate students specializing in the field of mutation breeding.

# Contents

# About the Editors

**Aamir Raina** is currently working as Assistant Professor in the Department of Botany, at Aligarh Muslim University, Aligarh, India. Dr. Raina obtained his Masters in Botany in 2013 with specialization in "Genetics and Plant Breeding" from the University of Kashmir, Srinagar, Jammu and Kashmir, India. Dr. Raina earned the Degree of Doctorate in 2018 for his research work on "Induced Mutagenesis in Cowpea" from the Aligarh Muslim University, Aligarh, India. His current research interests are the selection for novel mutations induced by mutagens in pulses, medicinal and aromatic plants and elucidation of physiological and molecular mechanisms in response to radiations and looking for suitable germplasm donors for breeding purpose. Working on the mutagenesis of plants, Dr. Raina has found a significant role of mutagens in the regulation of plant growth and development and has suggested that radiations could play an important role in improving the yield and yield attributing traits of crops. Recognizing the contribution of Dr. Raina in the field of mutation breeding, International Atomic Energy Agency (IAEA), Vienna, Austria, appointed Dr. Raina as Technical Corporation Expert and Lecturer to conduct national-level mutation breeding training in Sudan. The successful conduct of Dr. Raina in different IAEA projects as an expert supported his research in mutation breeding of pulse crops and proved his ability to work in a multicultural environment. Considering all his achievements and outstanding contributions in the field of Plant Science, he has been conferred with

various research fellowships including CSIR JRF, DBT-SRF, GATE, ICAR NET, JKSET and Nuffic OKP (from Dutch Ministry of foriegn Affairs, The Netherlands) and awards including National Environmental Science Academy (NESA) Young scientist and Bharat Vikas Award. He has been recognized as Young Scientist of the Year 2018, receiving the award from President of NESA, India. Dr. Raina has published more than 40 journal articles and 25 book chapters. Dr. Raina has edited special issue on research topic entitled "Legume Breeding in Transition: Innovation and Outlook" in *Frontiers in Genetics and "Emerging Talents in Horticulture Breeding and Genetics 2022" in Frontiers in Horticulture*. He is among the editorial board members of many scientific journals such as *Frontiers in Plant Science*, *Frontiers in Genetics*, *Plos One Scientific Reports*, and *BMC Plant Biology*. Dr. Raina has participated and presented several research papers in different national and international conferences. Dr. Raina has also attended many workshops, training, and short courses under scholarships from national and international funding agencies.

**Mohammad Rafiq Wani** is currently working as Assistant Professor (Selection Grade) in the Department of Botany at Abdul Ahad Azad Memorial Degree College Bemina, Cluster University Srinagar, Jammu and Kashmir, India. Dr. Wani did his Masters' degree in Botany in 2003 with a specialization in "Genetics and Plant Breeding" from Aligarh Muslim University (AMU), Aligarh, Uttar Pradesh, India. After obtaining the Degree of Doctorate in 2008 for his research work on "Chemical Mutagenesis in Mungbean" from the same university, he joined the Department of Higher Education, Jammu and Kashmir Government in 2009. He teaches a range of bioscience-related subjects at undergraduate and postgraduate levels. Dr. Wani's research interests are primarily focused on induced mutagenesis and molecular biology in food crops more particularly in pulse crops. As a part of his research endeavor, Dr. Wani has extensively researched and written on the issues of induced mutagenesis in food crops, with special reference to pulses. He has published more

than 65 research papers in peer-reviewed journals and 20 book chapters in various research-oriented volumes. Dr. Wani, while constantly working for his academic and research interests, has so far edited six volumes of books on the subjects of Plant Stress Physiology, Induced Plant Mutagenesis, and Crop Improvement with reputed international publishers like Springer and Wageningen Academic Publishers, the Netherlands.

**Rafiul Amin Laskar** is an Assistant Professor of Plant Sciences affiliated with the Department of Botany, Pandit Deendayal Upadhyaya Adarsha Mahavidyalaya (PDUAM), Eraligool, Karimganj, Assam, India. He received his M.Sc. in Botany with specialization in Genetics and Plant Breeding in 2012 and DBT-PDTC in Plant Tissue Culture and Micropropagation in 2013 from the Aligarh Muslim University, Aligarh, India. Dr. Laskar has obtained his Ph.D. in 2018 from the Faculty of Life Sciences, Aligarh Muslim University, Aligarh, India, for his work on Induced Mutagenesis. After completing his Ph.D., he worked as a Guest Faculty at Nagaland University, Lumami for over 1 year and as an Assistant Professor at Bahona College, Jorhat for over 3 year. Dr. Laskar is actively engaged in research on mutation breeding in grain legumes for genetic improvement of qualitative and quantitative traits. He is a life member of the various educative societies and associations and had been active member of the interdisciplinary societies. To his credit are edited book, 10 book chapters published by reputed publishing companies like Springer and more than 50 other publications as research papers, editorials, popular science articles, review papers, and other reports. He has edited a special issue entitled “Legume Breeding in Transition: Innovation and Outlook” in *Frontiers in Genetics* and is serving as Editorial Board Member of many journals. He has presented a number of research papers in various national and international conferences and symposiums. He has attended many workshops and short courses under scholarship from national and international bodies. He has acted as the co-coordinator of the Institutional Biotech Hub, Bahona College funded by Department of

Biotechnology, India. Dr. Laskar has served as the Co-Principal Investigator for the project titled "DBT-NER Institutional Biotech Hubs at Bahona College, Jorhat, Assam with a Focused Area of 'Development of Infrastructure and Skilled Human Resources Through Science & Technology Intervention". Currently he is maintaining an active student research program on induced mutagenesis of food crops, cytogenetics, and in vitro tissue culture of economically important plants focusing on plant genetic resources of North East India.

**Nasya Tomlekova** is a Professor and Head of Molecular Biology Laboratory in the Department of Breeding at the Maritsa Vegetable Crops Research Institute, Plovdiv, Bulgaria. She graduated from Sofia University "St. Kliment Ohridski" in Biology and did her Masters with specialization in genetics and biochemistry. She has been employed in the Institute in 1990; trained in the area of her research interests by 12 international fellowships; and she has a long postgraduate experience in research on the application of induced mutagenesis, molecular and in vitro techniques, and other relevant biotechnologies related to crop improvement. Mrs. Tomlekova obtained the Degree of Doctorate at the Commission on Plant-Growing and Biotechnologies and her Habilitation was at the Commission on Breeding and Seed Production. She has published 5 books, including a methodological recommendation, and 11 book chapters, 121 scientific papers in peer reviewed journals, and participated in 43 international conference proceedings. Prof. Tomlekova teaches on several topics related to biotechnology and she has trained many students and fellows from different countries at Ph.D. and post- and undergraduate levels. She conducted dozens expert missions as a lecturer in different countries from Asia and Africa. Dr. Tomlekova acted as lead editor of two books by Wageningen Publisher, and she is a member of three journal editorial boards, a reviewer of more than 30 international bioscience-related journals and projects, and a member of 5 professional scientific societies, like EUCARPIA, International Carotenoid Society, and International Pepper Network. The

successful participation of Prof. Tomlekova in nine different IAEA and an EU projects as coordinator/scientific holder supported her research in mutation breeding of vegetable crops and potato and proved her ability for a work in a multicultural and multilanguage environment from and outside Europe. Apart from that, she has managed projects and participated in many research teams of national and international projects. Her research focuses on the field of molecular biology with applications in agriculture, induced mutagenesis, genetics, biochemistry, and molecular marker selection.

**Samiullah Khan** is currently working as a Professor and Head of Genetics and Plant Breeding, and Plant Biotechnology sections in the Department of Botany at the Aligarh Muslim University, Aligarh, India. Dr. Khan obtained his Ph.D. in 1990 from Aligarh Muslim University, specializing in Crop Genetics and Plant Breeding. He has 29 years of teaching experience in Embryology of Angiosperms, Genetics, and Plant Breeding and supervised a number of Ph.D., M. Phil., and M.Sc. projects. He has edited several books and published more than 100 research papers in journals of national and international repute. He has attended many conferences in India and abroad including XVIth International Congress of Genetics at Toronto, Canada. He is a member of editorial board of many scientific journals. He is a fellow of the Indian Society of Genetics and Plant Breeding.

# Plant Genetic Resources: Conservation, Evaluation and Utilization in Plant Breeding


**Parmeshwar K. Sahu, Richa Sao, Ishu Kumar Khute, Samrath Baghel, Ravi Raj Singh Patel, Antra Thada, Deepika Parte, Yenkhom Linthoingambi Devi, Sunil Nair, Vinay Kumar, Suvendu Mondal, B. K. Das, and Deepak Sharma**

**Abstract** Conservation, evaluation and utilization of plant genetic resources (PGRs) in plant breeding are crucial for sustaining the ecology among living organisms of the planet and for being self-sufficient in crop production and improvement programmes. PGRs comprise an important component of agro-biodiversity, which sustains humankind by meeting its demands for food, fodder, fibre and fuel. Besides food security, more PGR-related activities, environmental protection and revival of local and distinct resources are also fuelling the development of new industrial products as well as new research breakthroughs. Currently, threat to biodiversity has been increased due to continuous degradation on global land resources, and within agricultural systems, production pressures continue to drive out traditional varieties, landraces and other genetic resources. In situ conservation of PGRs alone cannot assure long-term security in the genetic reserve due to chances of losing the germplasm through environmental hazards. The genetic materials can be preserved as gene banks for long-term storage under suitable conditions using in vitro cultures (plant cells, tissues or organs). Furthermore, advances in biotechnology, in in vitro culture techniques and molecular biology marked out as an appropriate alternative for genetic conservation by cryopreservation where seed banking was not achievable. Moreover, evaluation of PGRs with traditional and advanced breeding and biotechnological approaches is necessary to identify the suitable germplasm with a target trait for their further utilization in crop improvement programmes. Till now,

P. K. Sahu (✉) · R. Sao · I. K. Khute · S. Baghel · R. R. S. Patel · A. Thada · D. Parte
Y. L. Devi · S. Nair · D. Sharma
Department of Genetics and Plant Breeding, Indira Gandhi Krishi Vishwavidyalaya, Raipur, India

V. Kumar
ICAR-National Institute of Biotic Stress Management, Raipur, India

S. Mondal · B. K. Das
Nuclear Agriculture and Biotechnology Division, Bhabha Atomic Research Centre, Mumbai, India

Homi Bhabha National Institute, Training School Complex, Mumbai, India



A. Raina et al. (eds.), *Advanced Crop Improvement, Volume 2*,
https://doi.org/10.1007/978-3-031-26669-0_1

immense efforts have been made for collection; conservation and evaluation of PGRs pay off only with their efficient utilization for enhanced crop productivity and profitability. Utilization of PGRs varies from direct release of potential germplasm, development of new improved varieties, improving the breeding materials through pre-breeding, development of new novel crops (climate-resilient, biotic and abiotic stress-tolerant, biofortified crops), new gene discovery and allele mining studies. Furthermore, there is a concerted global need to be made to streamline and regulate the accessibility of PGRs at international level. In this chapter, holistic efforts have been made to review the status of collection, conservation, evaluation and utilization of PGRs with the help of conventional and advanced breeding approaches.

**Keywords** Biodiversity · Plant genetic resources · Genetic diversity · Crop improvement · Breeding

## 1 Introduction

Plant genetic resources (PGRs) comprise a whole spectrum of genetic resources including modern cultivars, obsolete cultivars, mutants, indigenous landraces, wild and weedy relatives along with the diversity of agro-ecosystems which may be exploited in several ways for agriculture and forestry and the complex set of human interactions. It forms the foundation of agricultural research and development. PGRs are a hope for the future of global food and nutritional security as these diverse germplasms are the storehouse of valuable traits, viz., yield-attributing, biotic and abiotic stress-tolerant, nutritional traits, entailed by the researchers and breeders to swiftly respond to new types or enhanced levels of biotic and/or abiotic stresses induced by climate changes and malnutrition as well (Lee et al., 2020; Halewood et al., 2018).

With the increased pressure of burgeoning human population, degraded land resources and industrial modernization, the loss of precious PGRs and biodiversity is being the most urgent and crucial issue which prompted an international action. Therefore, the conservation and sustainable use of PGRs for food and agriculture has evolved as a scientific discipline and several gene banks and other conservation utilities have been established in many countries (Kaviani, 2011). In this way, scientists have made huge efforts on the collection, conservation, maintenance and evaluation of the PGRs for its sustainable utilization for the welfare of human civilization. At the moment, the International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA) has made great contribution by providing the mechanism for germplasm sharing and effective ex situ conservation of 760,467 accessions for a range of plants through international gene banks of the Consultative Group on International Agricultural Research (CGIAR) (Lee et al., 2020). In India, the ICAR-National Bureau of Plant Genetic Resources (NBPGR), New Delhi, encompasses the National Gene Bank Network which conserves more than

0.40 million accessions of various crops. NBPGR works in service mode for effective utilization of PGR in crop improvement programmes which depends mainly on its systematic characterization and evaluation and identification of potentially useful germplasm (Singh et al., 2020). Management of PGR comprises various activities, viz., ensuring the representation of maximum diversity in ex situ collections; pest-free conservation; seed increase/regeneration; characterization, evaluation and maintenance of active collections; and documentation (Wambugu et al., 2018).

Due to raising concern of conservation, the scope of characterization and evaluation of genetic resources has been widened. Characterization, evaluation and regeneration are considered as the most important and essential activities for better management of PGRs. Moreover, characterization is the process of describing the genotypes with a certain group of descriptors and highly heritable characters which may be used in establishing taxonomic identity. However, in the process of evaluation, potentials for yield parameters, quality parameters and resistance to various biotic and abiotic stresses of a genotypes are being accessed or observed with the help of certain biostatical methods (Gollin, 2020). In this way, the process of characterization and evaluation make possible the exploitation of PGRs in further breeding programmes. Regeneration/maintenance of PGRs without losing genetic integrity at clonal repositories, field gene banks, herbal gardens, etc. also played a critical role in conserving PGRs (Singh et al., 2020).

The evaluation of PGRs for identification of donor genotypes for specific traits is essential for their further utilization. Evaluation should be undertaken in germplasm accessions which are already characterized and where enough quantity of planting material is available. It is a multidisciplinary approach to be done in collaboration with the plant breeder, germplasm curator, biochemist, physiologist, entomologist, pathologist and other experts (Rao, 2004; Jaramillo & Baena, 2000). It will be fruitful when germplasm lines might be evaluated in the area of their adaptation or under similar environmental conditions considering the breeding behaviour and biological status of the germplasm. The evaluation of PGRs is the primary step towards 'prebreeding' or 'germplasm enhancement' in which attempts are made by scientists, to eliminate undesirable traits or unfavourable gene(s) through backcrossing and selection for various generations with the cultivated parent (Singh et al., 2020). Furthermore, PGRs having useful genes for pest and pathogen resistance, nutritive traits, quality traits and yield-attributing traits may also be transferred through various breeding approaches to a new genotype for developing a new variety.

In recent years, new challenges have arisen for the conservation, utilization and exploitation of useful genes for crop improvement. Currently, threat to biodiversity has been increased due to continuous degradation on global land resources, industrialization and high production pressures made out of the PGRs from the channel. For this concern, traditional as well as modern technologies including biotechnological tools have been proven beneficial. With the aforementioned views, this chapter deals more about the plant genetic resources and its potential, utilization, conservations and enhancement and modern tools applicable for better preservation and maintenance of genetic resources.

## 2 Genetic Diversity and Plant Genetic Resources

The sum total of the genetic variability present in a population or in a species which is a combination of all the genes and their alleles represents the genetic diversity of that population. Genetic resources include all the variability which is present in the form of cultivars, wild relatives, breeding lines, races, etc. These resources are the ultimate source of variability for any plant breeding programme (Rao, 2004).

Genetic diversity available within the PGRs is very important for survival of any species as they play a major role in the process of evolution. The diversity present in the genetic constitution allows the species to adapt to the changing environmental conditions. Mutations, natural selection, genetic drift, sexual reproduction, etc. lead to various combinations of genes which generate diversity among a population (Singh, 2017; Raina et al., 2020a, b, ; 2022a, b, c, d; Khursheed et al., 2018a, b, c; Laskar et al., 2018a, b; Goyal et al., 2021a, b; Rasik et al., 2022; Sellapillai et al., 2022). The interaction of a genotype with the environment results in a phenotype of an individual. Only some individuals in a population will be able to cope up with the changing environments, outbreaks of pests and diseases, deficiency or toxicity of mineral elements, etc. The rest of the genotypes will get eliminated in the process of evolution (Rao, 2004). Thus, the diversity present in a population helps in the survival of the species and prevents them from extinction.

In the present scenario, global warming, pollution and use of chemicals have led to an increment in potential threats of insects, pests and diseases among the agricultural crops. Changing climatic conditions has forced us to work on climate-smart agriculture (CSA) in order to cope up with the upcoming situations. In any crop improvement programme, the first and the most important step is to identify the trait of interest which may be present in the population or in wild relatives (Singh, 2017; Ramya et al., 2014). Plant genetic resources can be used to create different gene combinations which meet the required objective. The aim can be improvement in qualitative traits or resistance against biotic or abiotic stresses.

In conventional plant breeding programmes, the existing diversity and plant genetic resources were utilized to create crops which carry desirable genes and characteristics. The advancement in science and technology has provided scientists with ample opportunities to generate genetic diversity according to the requirement. The lack or absence of diversity can be overcome by creating novel genes or transgenic crops with the use of tools of biotechnology (Singh et al., 2020).

To conclude, plant genetic resources are an imperative component to maintain a healthy ecosystem and to cope up with the negative modifications of our surroundings. PGRs are the irreplaceable assets of nature which should be conserved and utilized in a sustainable manner.

## 3 Conservation, Evaluation and Enhancement of PGRs

Plant genetic resource is considered to be the pillar of world food security and the agriculture system. A rich plant genetic resource can be used in the fields of evolutionary biology, cytogenetic, physiological, phylogenetic, biochemical, ecological, pathological and breeding researches (Ulukan, 2011). The ability of a particular species to withstand a changing environment depends on the richness of the genetic resources of that particular species. So, PGR provides the plant genetic pool of a particular species. Genetic erosion has a negative impact on genetic diversity present in the gene pool, so to avoid it, conservation of PGR is necessary for the protection of genetic material loss from genetic erosion (Sharma, 2007). Care must be taken while conserving the PGR and also it needs to be performed wisely as it is being threatened by several factors (Ramya et al., 2014). In the early days, only natural forces are being threatened in the conservation and food security like changes in the weather and climate system, even now the natural forces are there, but along with it, more threats are from artificial activities like habitat destruction, replacement of diverse and landrace cultivars by more genetically uniform ones, deforestation, pollution, urbanization, globalization, overgrazing, fragmentation and degradation (Kaviani, 2011).

So, conservation of genetic variability is essential and can be done through in situ and ex situ conservation. In situ conservation conserves the plant germplasm in its natural habitat like on-farm conservation, reserves and protected areas, while ex situ conservation deals with the movement of plant genetic resource from their habitat and shifting to the artificial storage condition. Farmers need to actively participate in the conservation of traditional and landrace cultivars. All the research work studies can be done on the ex situ collection germplasm through which the nature, property and characteristics of the germplasm can be understood through those studies (Ramya et al., 2014). Seed storage methods like seed banks, botanical garden and field gene bank are part of ex situ conservation. But there are some plant parts which cannot be conserved and stored by either in situ and ex situ method, so for those plants another conservation practice called cryopreservation helps in its storage. Furthermore, biological materials like plant organs and seeds like recalcitrant are used in this conservation method. In vitro conservation provides medium-term storage facility along with reducing the risk of germplasm loss from insect attack, disease attack, nematode attack or any other natural disasters. It is also commonly used in vegetatively propagated plants (Ogbu et al., 2010). Cryopreservation refers to the storage at ultra-low temperature with liquid nitrogen at the temperature of −196 °C. Living biological tissues can be stored through this method as the storage system can arrest the metabolic activity and cell division of the live cell. In cryopreservation, no change in genetic make-up, vigourness and viability of the conserved material is obtained (Cruz-Cruz et al., 2013). Crop evolution study along with taxonomic study, as well as genetic diversity study, helps in understanding more of what to conserve in the genetic population.

The collected accessions need to be evaluated for its characterization and properties. Therefore, a thorough evaluation should be done for all the genotypes with different properties. But sometimes the genotypic collection is large enough that the evaluation methods became difficult to handle. Evaluation is a complex process and they present a serious backlog in most collections. A large number of samples to be handled and a large number of traits to be studied make evaluation a difficult task. Thus, nowadays, improved evaluation methods are being started (Ramya et al., 2014). One utilized methodology was the use of core collection for evaluation. Core collection provides the breeders a manageable number of accessions to evaluate the whole collection or to search for a new character or character combinations. But still in some cases even the core collection was too large as the germplasm collection was quite large, so to tackle such situation, the concept of mini-core collection was again started. A mini-core collection has only 1% of the entire collection and 10% accessions in the core collection which represent the diversity of the entire core collection (Wambugu et al., 2018).

Enhancement in the PGR is required through proper collection and efficient evaluation methods (Hajjar & Hodgkin, 2007; Bains et al., 2012). Enhancement in the utilization of PGRs can be done through introgression and incorporation of desirable genes or traits in the agronomically superior genotype (Spoor & Simmonds, 2001). Introgression and incorporation mainly focused on freely recombining donors from the primary gene pool, while pre-breeding mainly focused on utilizing the crop wild relatives. Recent biotechnological approaches are being used for these methods. By using these processes, Kuraparthy et al. (2007a) introgressed a new major gene (*Yr40/Lr57*) into bread wheat for resistance to stripe rust as well as leaf rust using *Ph* locus manipulation from *Ae. ovata*. Furthermore, a novel major gene (*Lr*58) for leaf rust resistance was introgressed from *Ae. triuncialis* to elite wheat lines using cytogenetic and molecular techniques (Kuraparthy et al., 2007b). Moreover, Rawat et al. (2009) identified the few lines of *Aegilops kotschyi* and *Ae. tauschii* with high iron and zinc content in the grain which are being used as donors. Similarly, Pal et al. (2010) identified a major gene for high protein and enhanced micronutrient content (*DpcB1*) in *T. dicoccoides* which were transferred to a wide range of wheat genotypes through marker-assisted selection.

## 4 Conservation of Plant Genetic Resources Through Various Techniques

The conservation of plant genetic resources is a crucial assertion concerning the human population worldwide. In the current situations, the fast growth in human populations with increased needs caused severe threat to biodiversity. The expanding agricultural frontier has contributed towards the genetic erosion of valuable biodiversity. Such reductions have serious implications for food and nutritional security in the long term (Rao, 2004). The conservation of PGRs is extremely important to meet the present and future needs of various crop improvement programmes and to build reserves of breeding materials that have nutritional or industrial potential.

The genetic diversity present in the PGRs provides opportunities to the plant breeders and farmers as well for developing new and more productive crop varieties which are resistant to biological and environmental stresses through selection and breeding (Rao, 2004; Singh, 2017). However, it would be worthwhile to preserve all the diversity in nature rather than move it into an artificial environment for conservation, because of the peril of genetic erosion in the original location and the need for easy access for exploitation in ex situ conservation approaches which are significant for crop species. In situ conservation alone cannot assure long-term security in the genetic reserve or on-farm for a specific species. One important advantage of ex situ conservation is the easy availability of genetic material to the plant breeder (Hawkes et al., 2012).

Generally, germplasms that are conserved in the germplasm banks are kept as seeds but the major risk of in situ conservation is losing germplasm due to environmental hazards (Singh, 2017). Therefore, the genetic materials may be preserved in gene banks using in vitro cultures (plant cells, tissues or organs) for long duration with suitable conditions. However, fair knowledge of the genetic structure of plant species and the techniques associated in sampling, regeneration, preservation of gene pools, etc. are essential for the successful establishment of gene banks. Advancement and expansion in the field of biotechnology marked out as an appropriate alternative for conservation of PGRs by cryopreservation where seed banking was not achievable. The most commonly used cryopreservation technique is employing liquid nitrogen (−196 °C) where the cells remain in a completely inactive state so that they can be conserved for long periods (Kaviani, 2011).

There are two approaches for conservation of PGRs as mentioned in Article 2 of the Convention on Biological Diversity: (1) in situ conservation and (2) ex situ conservation.

## *4.1 In Situ Conservation (Natural Ecosystem)*

It is defined as the conservation of plant genetic resources at their natural habitat by establishing reserves. These approaches comprise maintenance and recovery of genetic variation at the location where it is found and, in case of cultivated species, where they have developed their peculiar properties in their habitat, either in the wild or in conventional farming strategies. The two major strategies of in situ conservation are discussed briefly in subsequent paragraphs.

### 4.1.1 Genetic Reserve Conservation

The genetic reserves are the protected areas that have been set aside to preserve the genetic diversity of target species with certain protocols (Maxted et al., 1997; Heywood, 2005). This technique is the most applicable for conservation of the bulk of wild species, enables multiple taxon conservation in a single reserve and favours

continued evolution of the species (Edagbo et al., 2011). It is usually referred to as the cornerstone of in situ conservation.

### 4.1.2 On-Farm Conservation

On-farm conservation of plant species is carried out by the farmers. In on-farm, they often adopt conservation to grow, use and conserve landraces, native varieties and other local materials, within their original landscapes and traditional farming systems. Under this conservation system, the diversity is always subjected to evolutionary changes and adaptations (Edagbo et al., 2011). The roles of the farmers in understanding and managing the crop diversity in their field have been considered as indispensable for the on-farm maintenance of PGRs. The landrace and farmers' varieties that are adapted to the local environment and having locally adapted alleles may be significantly effective for specific breeding programmes.

## *4.2 Ex Situ Conservation*

It is defined as the conservation of plant genetic resources outside their natural habitats. It involves seed storage, in vitro storage, DNA storage, pollen storage, field gene banks and botanical gardens.

This method became a choice for scientists and breeders due to easy access to the materials conserved in the ex situ facilities. Ex situ conservation of PGRs has various approaches which may be selected based on the biology of plant species, their method of reproduction, the purpose of conservation and the future usage of the conserved materials (Dulloo et al., 2017; FAO, 2013). Standard protocols have been developed for collection of germplasm under the ex situ environment.

### 4.2.1 Seed Storage Conservation

Usually, seeds are the most suitable and widely used form for the conservation of PGRs. This approach is only confined to seed propagating plants especially for 'orthodox seeds' that can be dried to low moisture content and can tolerate low temperatures (between −20 °C and +4 °C). In this method, seeds are stored at refrigerators, freezers or cold rooms with suitable conditions depending on how long the seeds need to be conserved (Rao et al., 2006). Therefore, seed bank collections are classified into three groups which are given in Table 1.

**Table 1** Types of seed storage conservations and features

| Measures | Base collection | Active collection | Working collection |
|---|---|---|---|
| Conservation duration | Long term (50–100 years) | Short to medium duration (10–15 years) | Short duration (3–5 years) |
| Storage temperature | about −20 °C | About 0 °C | 5–10 °C |
| Use | Normally restricted in distribution and acts as a backup to an active collection | Generally used for evaluation, multiplication and distribution of the PGRs | Frequently used in crop breeding programme |
| Seed moisture content | 5% | 5–8% | ~10% |

### 4.2.2 In Vitro Conservation

This conservation technique involves the maintenance of explants in a sterile, pathogen-free environment and is best suited for recalcitrant or short-lived seed and clonally propagated crops. Depending on species, the explants need to be re-cultured every 6 months to 2 years. This poses a dreadful risk of genetic changes and may cause genetic erosion because in vitro conservation can only be considered for short- to medium-term conservation (Reed, 2004). It involves the establishment of tissue cultures of accessions and their storage under controlled conditions for short periods. However, cryopreservation offers long-term conservation, where plant material is stored in liquid nitrogen at −196 °C. Therefore, plants can also be conserved as tissues, embryos or cells in vitro. Furthermore, there are certain disadvantages of in vitro conservation techniques including the risk of somaclonal variations, need to develop individual maintenance protocols for most species and the relatively high-level technology and cost required which should be addressed carefully or effective conservation of recalcitrant or short-lived seed and clonally propagated crops (Maxted et al., 1997). In vitro conservation offers an alternative to field gene banks in a non-viable form.

### 4.2.3 Slow Growth

Slow growth procedures allow clonal plant material to be conserved for 1–15 years under tissue culture conditions with periodic sub-culturing, depending on species (Rao, 2004). Mostly, to limit the growth, a combination of low temperature with low light intensity or even darkness is used under the storage chamber. Generally, a temperature of 0–5 °C is being maintained for cold-tolerant species, whereas 15–20 °C is maintained for conservation of tropical plant species. In some cases, plant growth may be limited by modifying the culture media (Withers & Engelmann, 1997). Shoots are the best part for storage under sow growth and successfully employed in potato (Muthoni et al., 2019), *Musa* species, yam, *Allium* species, sweet potato, cassava, temperate tree species (Rao, 2004) and malanga (*Colocasia esculenta* L. Schott) (Mancilla-Álvarez et al., 2019).

### 4.2.4 Cryopreservation

It is a highly reliable method for long-term preservation of PGRs for those crop species which are normally unable to produce seed, viz., tuber crops and roots. Plant tissues and organs can be frozen and stored in liquid nitrogen at −196 °C, since at this temperature, cell division and all metabolic activities remain suspended and the material can be preserved with no morphological, biochemical, genetic and karyotypic changes. Also for international exchange of genetic materials, cryopreserved tissues are preferred because they are safe, clean and disease-free (Feng et al., 2011).

### 4.2.5 Pollen Conservation

Initially, the concept of pollen storage started with controlling the pollination of asynchronous flowering genotypes, especially in fruit tree species. However, nowadays, it is also used for the conservation of PGRs. Pollen can be easily collected and cryopreserved in large quantities in a relatively small space. Likewise, it may be worthwhile in the ultimate future to regenerate haploid plants from pollen cultures, but still no generalized schedules have been established. It has the advantage that it is a relatively low-cost option, but the disadvantage is that only paternal material would be conserved and regenerated (Maxted et al., 1997).

### 4.2.6 Field Gene Bank Conservation

This technique is traditionally useful for recalcitrant species or the type of plants which does not easily produce seeds, or seed is highly heterozygous and preferable to store clonal material. Plant species like rubber, cassava, cocoa, banana, coconut, mango, coffee, yam, sweet potato, sugarcane (Rao, 2004) and taro (Mancilla-Álvarez et al., 2019) belong to this category. However, field gene banks are easily accessible for utilizing and evaluating the material being conserved but restricted in terms of genetic diversity because of their susceptibility to pests, disease and vandalism and involves extensive areas of land. It cannot reflect genetic diversity in a field gene bank.

### 4.2.7 Botanical Garden Conservation

Conservation on botanical gardens has the freedom to focus on wild species that may not be given sufficient priority for conservation. These gardens do not have the same constraints as institutes to focus on their activities on crop or crop-related species.

### 4.2.8 Plant Herbarium

Herbariums also can preserve variability of crop plants, their wild relatives and other important species represented as dried plant specimens and seed samples.

## 5 Role of Biotechnology in the Conservation of Plant Genetic Resources

Biotechnology has flared up with a new technique at the molecular level. Biotechnological methods are reliable and can provide continuously safe, higher-quality natural products similarly (Nalawade et al., 2003; Julsing et al., 2007). Biotechnological approaches have been applied for better conservation and utilization of genetic resources thorough in vitro clonal propagation, analysis of genetic diversity to identify the promising parents and genetic fingerprinting for removal of duplicates, elimination of pathogens for safe distribution and conservation of germplasm and speeding up of breeding through identification and selection of potential progenies and searching of new sources of genes in germplasm. Application of biotechnological tools have made it possible to conserve PGR using in vitro culture and cryopreservation, especially of species that are difficult to conserve as seeds (Rao, 2004). The following approaches are used for conservation and utilization of plant genetic resources using biotechnological approaches.

### *5.1 Cryopreservation*

The basics of the cryopreservation approach have already been discussed earlier in this chapter. Cryopreservation is performed for the preservation of recalcitrant seed and vegetatively propagated plants. It has also an added advantage of storage of a higher number of genetic resources in very little space, protection of material from external contaminations and cost-effective storage option for maintenance of germplasm. There are various approaches of cryopreservation; some are based on the classic methods involving freeze-induced dehydration of cells as well as newer methods based on vitrification (Engelmann, 2000).

The vitrification process involves the treatment of material/samples with cryoprotective agents, dehydration with highly concentrated solutions, rapid freezing and thawing, removal of cryoprotectants and recovery. The cryoprotectant molecules provide the optimum cellular cryoprotection environment of plant cells.

Cryoprotectants ethylene glycol, propylene glycol and glycerol and DMSO are added to the freezing mixtures to increase osmotic potential and maintain membrane integrity in the external medium. Using cryopreservation approaches, the germplasm of various crops has been preserved and this approach is widely used to

conserve the germplasm of other species as well. In mulberry the ideal plant part for cryopreservation was found to be winter buds, though embryonic axes, pollen and synthetic seeds have also been used (Niino & Sakai, 1992; Niino et al., 1993; Vijayan, 2020) and globe artichoke using vitrification (Bekheet et al., 2020).

## 5.2 Development of Pathogen-Free Planting Material

Pathogen-free stock plants and plant material is essentially required as propagation material in nurseries and pathogen-free/healthy material is an essential criterion for germplasm exchange between countries or region through quarantine programmes. Several biotechnological approaches have been developed and widely used to eliminate viruses from infected plants, namely, meristem culture, chemotherapy, thermotherapy or cryotherapy and cryopreservation (Kaya, 2021). Cryopreservation has also been used for eradication of viruses from affected plants by cryotherapy and cryopreservation of obligate plant pathogens (Bettoni et al., 2016; Bettoni et al., 2021).

## 5.3 Removal of Sexual Barrier for Germplasm Uses

A number of techniques have been developed to overcome problems of sexual incompatibility that lead to hybrid sterility or lack of genetic recombination in wide crosses involving distant wild relatives and cultivated species: (a) *Embryo rescue*: In vitro culture techniques that are used to assist in the development of plant embryos that might not survive to become viable plants. In embryo rescue, an immature hybrid embryo is shifted to a medium where viable plants may be regenerated and then backcrossed to the cultivated species to introduce the desired trait. Embryo rescue has been successful in wide crosses, and obtaining plants from inherently weak embryos (Sharma et al., 1996). (b) *Somatic hybridization*: The traits can also be transferred from one species to another using the fusion of protoplast This approach has been successful in overcoming the pre-zygotic sexual incompatibilities and facilitate plant regeneration from the heterokaryons (Fahelson et al., 1994). This technique is highly useful for transferring beneficial characteristics/genes from wild crops to the cultivated crop species, breaking the sexual barrier for gene transfer and accelerating plant breeding programmes.

## 5.4 Characterization of Genetic Diversity

Molecular markers are increasingly used for the screening of germplasm to study genetic diversity, identify redundancies in the collections, test accession stability and integrity and resolve taxonomic relationships. Exploring geographic or

ecological patterns of distribution of diversity in many different crops and their wild relatives that include banana (Pillay et al., 2001) and sweet potato (Gichuki et al., 2003) is useful. Molecular markers have been used to analyse the genetic diversity of medicinal plants like ashwagandha (Kumar et al., 2018). Molecular markers are also used for genetic fingerprinting in germplasm to provide unique identity as well as remove duplicate entry.

### *5.5 DNA Bank or Preservation of DNA*

DNA banks are meant for conservation of genomic DNA/tissue samples for the purpose of DNA extraction. The main aim of DNA bank is to accelerate downstream research using advanced molecular biology tools and facilitate strategic conservation, characterization and enhanced utilization of germplasm. It involved preservation of isolated DNA or direct storage of cells and tissues under low temperature (−80 °C). Preserved DNA can be used for the molecular marker analysis and genetic fingerprinting of PGRs (de Vicente & Andersson, 2006).

## 6 Major Centres for Conservation and Maintenance of Plant Genetic Resources of Various Crops in India and the World

With the rise in urbanization, more and more land are acquired each day for the purpose of farming or for infrastructure. This has led to a sharp decline in biodiversity and loss of valuable genetic resources. This is a great threat to our biodiversity which may lead to unbearable consequences in the future. To avoid this, many national and international organizations have been established in order to collect, maintain and utilize these genetic resources. In India, the main activities of germplasm collection, exploration, introduction and maintenance are performed by the ICAR-National Bureau of Plant Genetic Resources, New Delhi, and at the international level, it has been carried out by Bioversity International, Rome, Italy. Furthermore, a list of some major national and international centres for conservation and maintenance of PGRs is given in Tables 2, 3, 4 and 5.

## 7 Status of Germplasm of Different Crops at Indira Gandhi Krishi Vishwavidyalaya, (IGKV), Raipur, India

Indira Gandhi Krishi Vishwavidyalaya (IGKV), Raipur 492012 (Chhattisgarh), is well known for its biodiversity conservation in agricultural fraternity, mainly for conserving 23,250 rice germplasm accessions followed by 1964 grass pea (*Lathyrus*

**Table 2** Institutions involved in the conservation, maintenance and utilization PGRs in India

| S. no. | Organization | Objectives |
|---|---|---|
| 1. | ICAR-National Bureau of Plant Genetic Resources, New Delhi | The major objective is ex situ conservation of PGRs in gene banks comprising of long-term seed storage, in vitro repository and clonal field gene banks |
| 2. | Department of Environment, Forests and Climate Change, New Delhi | The major objective is in situ conservation of wild species, wild relatives, endangered plant species, rare plants and forest resources |

**Table 3** List of major places where in situ conservation of PGRs is ongoing in India

| S. no. | Place/region | Name of categorized biosphere reserve | Total area ($km^2$) | State |
|---|---|---|---|---|
| 1. | Bengalian rainforest | Sundarban Biosphere | 9630 | West Bengal |
| 2. | Malabar rainforest | Nilgiri | 5520 | Karnataka, Kerala and Tamil Nadu |
| 3. | Bengalian rainforest | Manas | 2837 | Assam |
| 4. | Himalayan valley | Nanda Devi | 1560 | Uttarakhand |
| 5. | Andaman and Nicobar Islands | Great Nicobar | 885 | Andaman and Nicobar |
| 6. | Coromandel coast region | Gulf of Mannar | 555 | Tamil Nadu |
| 7. | Burma monsoon forests | Nokrek | 80 | Meghalaya |

**Table 4** National active germplasm sites (NAGS) of different crops in India

| S. no. | Crop | Name of sites/institutions | No. of accessions conserved |
|---|---|---|---|
| 1. | Wheat | ICAR-Indian Institute of Wheat and Barley Research (IIWBR), Karnal (Haryana) 132001 | 17,000 |
| 2. | Rice | ICAR-National Rice Research Institute (NRRI), Cuttack (Orissa) 753006 | 20,000 |
| 3. | Maize | ICAR-Indian Institute of Maize Research (ICAR-IIMR), Ludhiana (Punjab) 141004 | 25,000 |
| 4. | Barley | ICAR-Indian Institute of Wheat and Barley Research (IIWBR), Karnal (Haryana) 132001 | – |
| 5. | Sorghum | ICAR-Indian Institute of Millets Research, Rajendranagar, Hyderabad (AP) 500030 | 2767 |
| 6. | Pearl millet | ICAR-Indian Institute of Millets Research, Rajendranagar, Hyderabad (AP) 500030 | – |
| 7. | Minor Millets | ICAR-Indian Institute of Millets Research, Rajendranagar, Hyderabad (AP) 500030 | 8572 |
| 8. | Pulses | ICAR-Indian Institute of Pulses Research, Kanpur (UP) 208024 | 9310 |
| 9. | Soybean | ICAR-Indian Institute of Soybean Research, Indore (MP) 452001 | 2500 |

(continued)

**Table 4** (continued)

| S. no. | Crop | Name of sites/institutions | No. of accessions conserved |
|---|---|---|---|
| 10. | Oilseeds | ICAR-Indian Institute of Oilseeds Research, (ICAR-IIOR), Hyderabad (AP) 500030 | 15,629 |
| 11. | Rapeseed and mustard | ICAR-Directorate of Rapeseed-Mustard Research, Bharatpur (Rajasthan) 321303 | 15,082 |
| 12. | Groundnut | ICAR-Directorate of Groundnut Research, Junagadh (Gujarat) 362105 | 6432 |
| 13. | Sugarcane | ICAR-Sugarcane Breeding Institute, Coimbatore (TN) 641007 | 3979 |
| 14. | Cotton | ICAR-Central Institute for Cotton Research, Nagpur (Maharashtra) 440001 | 6896 |
| 15. | Jute and allied fibres | ICAR-Central Institute for Jute and Allied Fibres, Barrackpore (WB) 743101 | 3226 |
| 16. | Vegetables | ICAR-National Bureau of Plant Genetic Resources, Pusa Campus, New Delhi 110012 | 16,139 |
| 17. | Potato | ICAR-Central Potato Research Institute, Shimla (HP) 171001 | 2375 |
| 18. | Forages | ICAR-Indian Grassland and Fodder Research Institute (Forage Crops), Jhansi (UP) 284003 | 6267 |
| 19. | Spices | ICAR-Indian Institute of Spices Research, Calicut (Kerala) 673012 | 2847 |
| 20. | Tobacco | ICAR-Central Tobacco Research Institute Rajahmundry (AP) 533105 | 1500 |
| 21. | Plantation crops | ICAR-Central Plantation Crops Research Institute, Kasaragod (Kerala) 673024 | 307 |
| 22. | Medicinal and aromatic plants | All India Coordinated Research Project on Medicinal and Aromatic Plants, NBPGR, Pusa Campus, New Delhi 110012 | 375 |
| 23. | Agroforestry plants | ICAR-Indian Grassland and Fodder Research Institute, Jhansi (UP) 284003 | 40 |
| 24. | Fruits (semi-arid) | All India Coordinated Project (Semi-Arid Fruits), Hisar (Haryana) 125004 | 541 |
| 25. | Fruits (subtropical and temperate) | NBPGR Regional Station, Phagli, Shimla (HP) 171004 | 454 |
| 26. | Fruits | ICAR-Indian Institute of Horticultural Research, Bangalore (Karnataka) 560080 | 13,118 |
| 27. | Citrus | ICAR-Central Citrus Research Institute, Nagpur (Maharashtra) 440006 | 51 |
| 28. | Mango | ICAR-Central Institute for Subtropical Horticulture, Lucknow (UP) 226016 | 587 |
| 29. | Tuber crops | ICAR-Central Tuber Crops Research Institute, Sreekariyam, Trivandrum (Kerala) 695017 | 3586 |
| 30. | Pseudo-cereals | NBPGR Regional Station, Shimla (HP) 171004 | 3682 |
| | | Total holdings | 188,262 |

Source: https://www.bioversityinternational.org/fileadmin/bioversity/publications/Web_version/174/ch18.htm with minor modifications

**Table 5** International Agricultural Research Centers (IARCs) associated with CGIAR

| Acronym | Centre | Year est. | Research programmes | Location |
|---|---|---|---|---|
| IRRI | International Rice Research Institute | 1960 | Rice | Philippines |
| CIMMYT | International Maize and Wheat Improvement Center | 1964 | Maize, wheat, triticale, barley | Mexico |
| IITA | International Institute of Tropical Agriculture | 1965 | Maize, rice, cowpea, sweet potato, yams, cassava | Nigeria |
| CIAT | International Center for Tropical Agriculture | 1968 | Cassava, beans, rice, pastures | Colombia |
| WARDA | West Africa Rice Development Association | 1971 | Rice | Ivory Coast |
| CIP | International Potato Center | 1972 | Potato | Peru |
| ICRISAT | International Crops Research Institute for the Semi-Arid Tropics | 1972 | Chickpea, pigeonpea, pearl millet, sorghum, groundnut | India |
| ILRAD | International Laboratory for Research on Animal Diseases | 1974 | Trypanosomiasis, theileriosis | Kenya |
| IBPGR | International Board for Plant Genetic Resources | 1974 | Plant genetic resources | Italy |
| ILCA | International Livestock Centre for Africa | 1974 | Livestock production systems | Ethiopia |
| IFPRI | International Food Policy Research Institute | 1975 | Food policy | USA |
| ICARDA | International Centre for Agricultural Research in the Dry Areas | 1976 | Wheat, barley, triticale, faba bean, lentil, chickpea, forages | Syria |
| ISNAR | International Service for National Agricultural Research | 1980 | National agricultural research | Netherlands |

Source: https://www.bioversityinternational.org/fileadmin/bioversity/publications/Web_version/174/ch19.htm with minor modifications

*sativus* L.) and 2050 linseed (*Linum usitatissimum* L.) germplasm accessions (Table 6). Chhattisgarh is popularly known as the 'Rice Bowl of India' due to its rich heritage of rice biodiversity and maximum rice cropping area. Apart from research on the crops, IGKV, Raipur, has given prime importance to the collection, conservation and evaluation of the germplasm of various crops. It has very well understood that countries having rice as a staple food are risk-prone which means that apart from a high yield, tolerance to biotic and abiotic stresses like insects, diseases, weeds, drought, water logging and salinity is one of the issues that would dominate research agenda in the future. Old cultivars, landraces and wild relatives of crop plants are the repository of those essential genes.

**Table 6** Status of germplasm of various crops conserved at IGKV, Raipur, India

| S. no. | Crops | No. of accessions | Place of maintenance |
|---|---|---|---|
| 1. | Rice (*Oryza sativa* L.) | 23,250 | Raipur, India |
| 2. | Lathyrus (*Lathyrus sativus* L.) | 1964 | Raipur, India |
| 3. | Linseed (*Linum usitatissimum*) | 2025 | Raipur, India |
| 4. | Pigeonpea (*Cajanus cajan* L.) | 210 | Raipur, India |
| 5. | Chickpea (*Cicer arietinum* L.) | 189 | Raipur, India |
| 6. | Medicinal plants | 504 | Raipur, India |
| | Total | 28,142 | |

## *7.1 Status of Rice Germplasm at IGKV, Raipur*

After the formation of the new state of Chhattisgarh, renewed emphasis has been laid on enhancing the pace of agricultural research in various fields. Chhattisgarh is predominantly a rice-growing state. The rice research work at Raipur has a long history which started on 1903 with the establishment of the seed production farm at Labhandi, Raipur, by the British Government. Since then, rice research work continues as the main focus of this station. However, the collection of rice germplasm was initiated by late Dr. R.H. Richariya during 1970–1980 who has collected about 18000 rice accessions from Chhattisgarh and nearby places and maintained at IGKV, Raipur, India.

Later on, few more scientists have collected about 5000 rice accessions and total collections were reached up to 23,250 accessions. Presently, the germplasm is being maintained at the Centre for Biodiversity Research Development (CBRD), IGKV, Raipur. The resource persons from the Department of Genetics and Plant Breeding, College of Agriculture, IGKV, Raipur, are working together to maintain the germplasm collections of various crops. The present status of rice germplasm being maintained by the unit is presented in Table 7.

## *7.2 Status of Grass Pea Germplasm at IGKV, Raipur, India*

Chhattisgarh state is a tribal state rich in grass pea biodiversity. IGKV, Raipur (CG), has maintained a total of 1963 grass pea germplasm accessions. Detailed screening of genetic resources in relation to low BOAA (*β-N*-oxalyl-amino-L-alanine) and also with multiple traits related to biotic and abiotic stresses is in progress. The flour of grass pea is richly used with the flour of chickpea to make crispy food products. Apart from this, grass pea is also consumed as green leafy vegetables and green pods and for dal. Henceforth, under crop improvement, focus on this crop is mandatory to screen and identify the potential donors on a need basis and area-specific.

**Table 7** Status of indigenous rice germplasm conserved at IGKV, Raipur, India

| S. no. | Groups | Duration | Number of accession |
|---|---|---|---|
| A. | Indigenous lines | | |
| | (a) *Old collections* | | |
| | 1. Extra early | Up to 95 days | 480 |
| | 2. Very early | 96–110 days | 1197 |
| | 3. Early | 111–125 days | 3880 |
| | 4. Medium | 126–140 days | 5069 |
| | 5. Late | Above 140 days | 7915 |
| | (b) *New collections* | | |
| | 1. On-Farm Conservation Project (IRRI-IGKV-NBPGR) | – | 705 |
| | 2. NATP on Plant Biodiversity Project | – | 1025 |
| | | Subtotal | 20,298 |
| B. | Selected lines (from indigenous) | | 938 |
| C. | Breeding lines (designated as Labhandi numbers and DT numbers) | | 849 |
| D. | Wild rice (number of species – 3): *Oryza nivara, O. officinalis, O. latifolia* and *O. sativa f. spontanea* | | 210 |
| E. | Upland rice, special rice and others | | 955 |
| | | Subtotal | 2952 |
| | | Grand total | 23,250 |

## *7.3 Status of Linseed Germplasm at IGKV, Raipur, India*

IGKV, Raipur, has been working for enhanced production and value addition of linseed crop for the past several years. A total of 16 varieties of linseed have been released, out of which 8 are recent (released after 2020). These varieties have different characteristics and have been released for different environmental conditions and biotic stresses. It holds around 2025 linseed germplasm accessions till date. These accessions have variability for all the important characters like seed colour, plant height, yield, resistance to diseases and pest, etc. We have yellow seeded accessions as well which are known for its golden yellow seed colour and buttery taste for edible market. Flax-type germplasm accessions with plant height more than 95 cm are being utilized for fibre extraction and spinning to boost linseed in the textile and handloom market of the state.

## 7.4 Pigeonpea

The top consumed pulses in India are pigeonpea which is rich in protein, and Indira Gandhi Krishi Vishwavidyalaya has 210 accessions of pigeonpea maintained and multiplied every year at Raipur.

## 7.5 Chickpea

In Chhattisgarh, during *rabi* season, the top coverage of pulses areawise is of chickpea crop due to its suitability of cultivation in the rainfed region. Indira Gandhi Krishi Vishwavidyalaya, Raipur, maintains 189 accessions of this crop at Raipur.

## 7.6 Medicinal Plants

The Chhattisgarh state is also known as the medicinal state since the year 2000 from its inception as a new state separating from Madhya Pradesh. In IGKV, Raipur, India, a total of 504 accessions of 12 medicinal crops is being maintained (Table 8).

**Table 8** List of medicinal plants maintained at IGKV, Raipur, India

| S. no. | Crops | No. of accessions | Place of maintenance |
|---|---|---|---|
| 1. | Kalmegh (*Andrographis paniculata*) | 295 | Raipur, India |
| 2. | Ashwagandha (*Withania somnifera*) | 95 | Raipur, India |
| 3. | Aloe vera (*Aloe barbadensis* miller.) | 13 | Raipur, India |
| 4. | Kewanch (*Mucuna* spp.) | 8 | Raipur, India |
| 5. | Lemongrass (*Cymbopogon citratus*) | 4 | Raipur, India |
| 6. | Betel vine (*Piper betle* L.) | 15 | Raipur, India |
| 7. | Butch (*Betula* spp.) | 3 | Raipur, India |
| 8. | Sarpagandha (*Rauvolfia serpentina*) | 5 | Raipur, India |
| 9. | Satawar (*Asparagus racemosus*) | 8 | Raipur, India |
| 10. | Khus (*Chrysopogon zizanioides*) | 1 | Raipur, India |
| 11. | Charota (*Cassia tora*) | 50 | Raipur, India |
| 12. | Tulsi (*Ocimum* spp.) | 07 | Raipur, India |
| | Total | 504 | |

# 8 Strategies for Conservation of Huge Rice Germplasm Collection at IGKV, Raipur, India

## *8.1 On-Farm Conservation of Rice Germplasm at IGKV, Raipur*

The on-farm conservation unit at Indira Gandhi Krishi Vishwavidyalaya, Raipur, is a secured area fenced with iron angles permanently by surrounding the germplasm field. There are two separate fields of about 4 ha each for regeneration and multiplication of early-, medium- and late-duration rice germplasm. The entire 23,250 rice accessions are not multiplied each year. Approximately 8000 rice accessions are multiplied along with previous seasons' non-germinated or less yielded accessions in each year. After three years, again the same accessions are multiplied and stored in a medium-term storage module available at IGKV, Raipur.

## *8.2 Package of Practices Followed for Obtaining a Healthy Crop in On-Farm Conservation*

- Bed size – One-meter-width beds are prepared and two rows of each accession are sown by the direct seeded method.
- Fertilizer application – Nitrogen, phosphorus and potassium at 50 kg, 35 kg and 20 kg, respectively, per hectare are applied. Whole phosphorus and potash and 50% nitrogen are applied as basal dose at the time of sowing. The remaining 25% nitrogen is applied at the time of tiller initiation and the rest of the 25% nitrogen at the panicle initiation stage.
- Irrigation – Usually a water level of 5–10 cm is maintained in the rice field.
- Intercultural operations – Hand thinning is an important intercultural operation usually practiced 20–25 days after germination. The dense population is uprooted which facilitates proper action and proper grafting the plants.
- Weeding – Usually hand weeding is practiced, but these days, chemical herbicides are also being used. Some herbicides used at the IGKV farm are weed Super + Orbix and Ricestar + Sunrise. Herbicides are usually used at 3–5 leaf stages (post-emergence).
- Roughing – Roughing is the practice of uprooting the off types as per visual appearance. This is one of the most important activities in germplasm purity maintenance and needs to be done very carefully.
- Threshing – The harvested plants are left in sunlight for 3–4 days for proper drying to avoid pest and diseases due to higher moisture content. After proper drying, the panicles are harvested and threshed. Wild accessions are harvested after bagging the panicle of the whole plant due to the highly shattering nature of

panicles and lodging of plant. Very carefully the panicles are covered and harvested in a cotton bag.

- Packing and storage of seeds – Two packets are made using brown paper envelopes: a small seed packet for sowing in the next season and another medium packet also known as reserve seed sample. The reserve seed sample is used in case of distribution of the original crop for seed supply to various institutes and scientists for research purposes.

### *8.3 Medium-Term Storage Facilities at IGKV, Raipur*

Medium-term storage facilities are also available at IGKV, Raipur, for conservation of 23,250 rice accessions. The seeds of each entry are kept in brown paper bags/envelopes and stored inside drawers of the medium-term cabinets. Nowadays, aluminium foil bags are also being used in few samples for testing purposes to test the viability of seeds. Usually, 150 samples or envelopes are kept in one drawer. In these chambers, seeds can be stored for a short duration up to 1–2 years. In general, 7–8 °C temperature with relative humidity of 35–40% is maintained inside the chamber which is ideal for the medium-term storage of seeds.

## 9 Characterization, Evaluation, Cataloguing and Documentation of PGRs

In the solar system, only planet earth is blessed with life on it. The flora and fauna of the planet are diverse due to spatial distribution; this diversity is collectively termed biodiversity, and agricultural diversity is called agro-biodiversity. After entering the sixth extinction phase, species are vanishing at a faster pace. So conservation of biodiversity with proper care is the only option to save this treasure of life on earth. PGRs are the base materials for any breeding programme related to yield augmentation; development of climate-resilient, bio-fortified, disease-resistant and insect-pest-resistant varieties; development of cytoplasmic male sterile lines for hybrid breeding; etc. (Dhillon & Agrawal, 2004). Therefore, exploration of PGRs in a quantitative manner is essential to exploit them for societal benefits.

### *9.1 Characterization*

It is the identification of heritable quasi-quantitative characters present and exhibited by a genotype in a particular environment due to the variability present in them. The ideal characterization shall discriminate genotypes within and between species

and genera (Hidalgo, 2003; Jaramillo & Baena, 2000; Ligarreto, 2003). Characterization may be done through agro-morphological traits, biochemical traits and molecular/DNA markers based on the need, type and nature of the plant/material available, and when used together, they increase the efficiency and precision of characterization.

### 9.1.1 Morphological Characterization

The entries under characterization are raised and maintained under the same environment and agronomic practices and characterized using descriptors. The advantages of this method are as follows: it does not consume a sample, and seed multiplication is carried out simultaneously. It is carried out in a representative population, which is expected to contain 95% of the accession's alleles. This is the most commonly used method of PGR characterization; however, they are influenced with environmental factors, which is a major drawback. The morphological characterization should be performed just after collecting or getting the germplasm and may be finished in a suitable environment, preferably in the area of their adaptation or under a similar environmental condition considering the breeding behaviour and biological status of the germplasm. The internationally/nationally accepted descriptors and descriptor states should be used to record observation such as those developed by UPOV, USDA, Bioversity International (formerly known as IPGRI/IBPGR) or NBPGR Minimal Descriptors for Characterization and Evaluation of Field Crops (Mahajan et al., 2000). Wherever, appropriate descriptors are not available for any crop species; then it should be developed by germplasm curators in consultation with the crop advisory committee and crop experts (Bioversity International, 2007). The field experiment should be conducted with statistically sound experimental design depending upon the quantity and number of germplasm accessions under trial.

### 9.1.2 Biochemical Characterization

It involves the use of biochemical markers (iso-enzymes) and total seed storage proteins for characterization of PGRs. These traits have advantages being that these markers have natural occurrence and no effect on epistasis and the environment (Simpson & Withers, 1986). However, it is quite difficult to perform such process for huge collections of PGRs.

### 9.1.3 Molecular Characterization

The use of molecular markers complements the above two systems and completes the characterization by revealing the polymorphism among variable genotypes, which could not be worked out through morphological characterization. The

molecular markers are not influenced by the environment and do not depend on the developmental stage of the plant. They provide precise information about the variability among genotypes. Common examples of markers used for the purpose include SSRs, SNPs, SCoTs, STMS, AFLP, etc.

Data obtained through characterization may be used in establishing the taxonomic identity of each PGR. In case of more number of accessions in the collections, the core set (comprising 10% of the total collection representing the total variability in PGR) is used for characterization to bring them to a manageable level (Frankel, 1984). Furthermore, if the core set still has a large population, then a mini core set (comprising 10% of the core set) is used for characterization (Upadhyay & Ortiz, 2001).

## *9.2 Evaluation*

It is the process of taking observation of various characters which are under strong influence of the environment and are of agronomic importance such as grain quality and nutritional traits and biotic and abiotic stresses. Evaluation of PGRs is essential to identify the appropriate line with desired traits for their further exploitation in crop breeding. It is preferable to perform evaluation of PGRs which are already characterized and there is enough quantity of seeds/planting materials available. It is a multidisciplinary approach involving the plant breeder, seed technologist, physiologist, germplasm curator, pathologist, entomologist, biochemist and other experts (Rao, 2004). Particular entries are evaluated at multiple sites through network/coordinated approaches and again characterized to reduce the genotype × environment interaction for economically important traits (Jaramillo & Baena, 2000). The accessions should be evaluated in a suitable environment. Evaluation experiments should be conducted with proper experimental design, depending upon the number of accessions to be evaluated. Although characterization and evaluation terms are used interchangeably, they mark a difference in terms of traits under study using the same set of descriptors.

## *9.3 Descriptors*

Descriptors are the traits that are scorable, heritable, morphologically expressed at every stage of the crop and least influenced by the environment. Hence, they are used to differentiate germplasm accessions, as they are the common link between the process of characterization and evaluation and score qualitative and quantitative traits, respectively. Descriptors can be scored easily by the naked eye, with the help of instruments like scales and vernier callipers, RHS colour charts, balances, chemical reagents, etc. They help to identify the hidden potential of the germplasm and its usefulness for cultivar improvement and variety/hybrid development (Hidalgo,

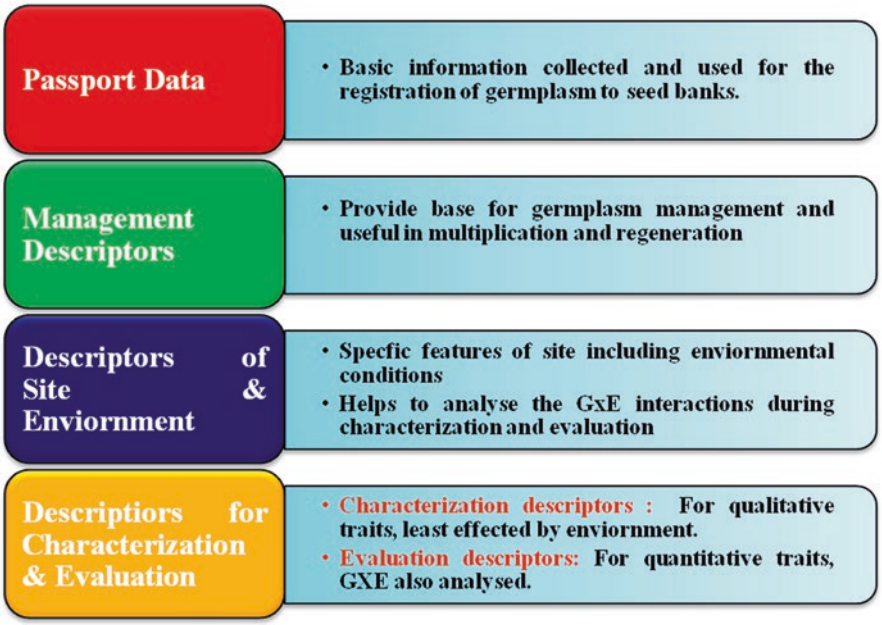



**Fig. 1** Different types of descriptors used for PGR conservation and management

2003). Descriptors are species-specific and are a standardized characterization system which provides an international format for plant genetic resources, descriptors are internationally developed by Bioversity International in collaboration with many other institutes, and they are available online. There are four categories of descriptors that are used in cataloguing the germplasm which are discussed below and summarized in Fig. 1.

### 9.3.1 Characterization Descriptors

These morphological traits differentiate between phenotypes by visual observations only and find expression in all the environments. They are characterized into two categories, viz., botanical taxonomic and morpho-agronomic descriptors.

### 9.3.2 Botanical Taxonomic Descriptors

These are the morphological characters which identify between species but not within species, such as the shape of leaves, seeds, fruits, etc. They have high heritability and little variation.

### 9.3.3 Morpho-Agronomic Descriptors

These are the morphological characters which differentiate the genotypes of the same species into various groups on the basis of qualitative characters like presence or absence of pigmentation on stem or leaves; presence or absence of pubescence on leaves, etc.; stem thickness; presence or absence of aroma, etc. They are measured by colour charts, vernier callipers, etc. They are heritable and less affected by the environment.

### 9.3.4 Evaluation Descriptors

The agronomic characters like plant height, number of tillers, yield, response to biotic and abiotic stresses are evaluation descriptors, as they are governed by polygenes and show a range of variation and hence are usually evaluated on a scale of 0–9 (IPGRI, 1996). Most of these descriptors depend on the environment for expression; therefore, sometimes, evaluation is clubbed with molecular characterization.

## *9.4 Documentation and Cataloguing of PGRs*

Documentation and cataloguing are integral and inseparable parts of germplasm banks and helps humankind to come up with better crop varieties and ensure food security. The role of documentation and cataloguing in PGR management can be understood like multi-cuisine restaurants having so many dishes to serve, but with the help of a menu card, one gets help to decide what to order. The dishes are documented germplasm, and the menu card is a catalogue of documented germplasm which helps breeders to select as per the objective of the breeding programme (Weise et al., 2017). The germplasm banks follow the step-by-step activities summarized in Fig. 2; among them, the sample registration and collection data activities along with descriptors are needful for documentation (Pineda & Hidalgo, 2007).

Accessions are registered to germplasm banks by assigning a unique identification number. The preliminary data including accession number, other code numbers for collector and donors, scientific name (genus, species, sub-taxa), common name of cultivated species, cultivar name/pedigree, date of submission of sample and date of last regeneration of sample is recorded for registering the accessions.

The data generated at the time of collection of any accession also refers to *passport data.* For the uniform coding system of various crops, FAO and Bioversity International together prepared a detailed passport data format known as MCPD (Multi-Crop Passport Descriptors) (Alercia et al., 2001), while a basic passport data documented on registered accessions contains the date of collection; collector's name; number and institute; country and state of collection; locality; longitude, latitude and altitude of collection site; origin of sample; state of sample (wild, landrace, advance cultivar); and number of sampled plants.

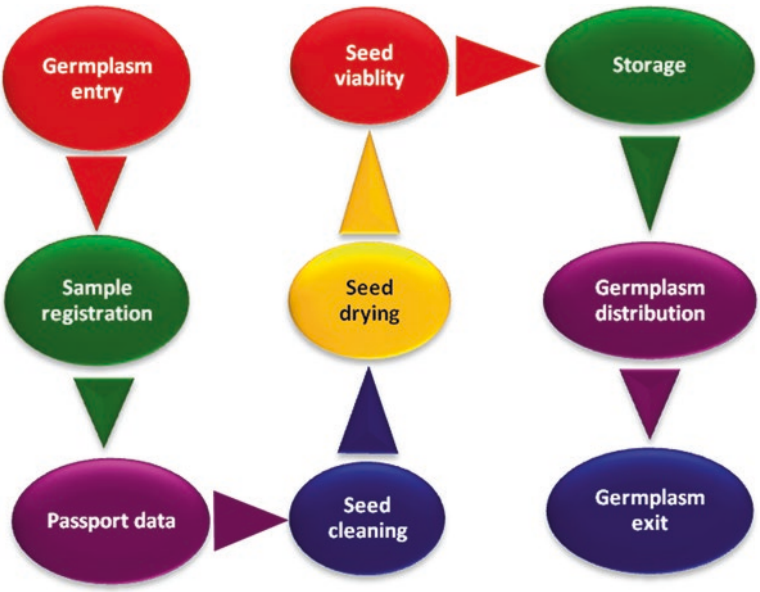



**Fig. 2** Systematic representation of activities performed by germplasm banks to conserve PGRs

The seed saving and exchange between botanical gardens are being practiced since the sixteenth century. The belief that 'sharing is keeping' as if the plant is dead in one garden can be recollected from other gardens. In the eighteenth century, the seed catalogue *Index Seminum* system on the basis of taxonomic classification was established to integrate the botanical gardens and germplasm conservation originations for seed sharing (Havinga et al., 2016). The limited availability and use of electronic systems and non-uniformity among the descriptor for characterization limit the exploitation of the catalogue. So to overcome the non-uniformity of data and less integration between the conservation bodies, the Convention on Biological Diversity 1993 (CBD, https://www.cbd.int/) and Nagoya Protocol of 2014 aimed at fair, equitable access and benefit-sharing through the utilization of plant genetic resources.

In the present era, various PGR conserving institutes made the documentation of accessions and their catalogues available online. All the institutes have a common goal of conservation and utilization by sharing the PGRs, but they operate differently (Bettencourt, 2011). Genis (GENetic Resource Information Management System by Center for Genetic Resources) in the Netherlands (www.cgn.wur.nl/UK/CGN+Plant+Genetic+Resources); The Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Germany (www.ipk-gatersleben.de/Internet); N.I. Vavilov All-Russian Scientific Research Institute of Plant Industry (VIR), Russian Federation (http://www.vir.nw.ru/); Kew's Millennium Seed Bank, United Kingdom (http://data.kew.org/sid/about.html); and many more institutes work at the gene bank level, by establishing their own online information systems which help users to browse, search, view and sometimes download the information of their germplasm collection. Through proper material transfer agreements, they provide material for

research too. While some regional/sub-regional banks like EURISCO, EAPGREN, GRIN Global, etc. collect, conserve, document and catalogue germplasm accessions to make them available to national holdings, ECPGR maintains a list of PGR regional networks, which can be consulted and accessed through the following link: www.ecpgr.cgiar.org/networks/inter_regional_coop/pgr_regional_nw_coordinators.html. Some of them are highlighted below.

### 9.4.1 EURISCO (European Search Catalogue for Plant Genetic Resources)

It was developed between 2001 and 2003 by collaboration between the European Plant Genetic Resources Information Infrastructure (EPGRIS) and the Centre for Genetic Resources, the Netherland CGN, with the participation of the Czech Republic, France, Germany, Portugal, Bioversity International and the Nordic Gene Bank (NGB, now NordGen). Initially, online hosting of EURISCO was done by Bioversity International; later on (15 April 2014), the responsibilities were taken over by Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany. The database provides data of characterization and primary evaluation data of various crops. The accessions registered with EURISCO are maintained by its approximately 400 institutes within the member countries. These institutes provide data to National Focal Points who comply with National Inventories of their respective countries and upload to EURISCO. Data is exchanged as per the Multi-Crop Passport Descriptors for passport data and EURISCO-specific format for phenotypic data (Weise et al., 2017).

### 9.4.2 EAPGREN (The Eastern Africa Plant Genetic Resources Network)

It is a regional project of the national agricultural research systems of Burundi, Eritrea, Ethiopia, Kenya, Madagascar, Rwanda, Sudan and Uganda, which mainly focuses on publishing passport data of all the documented accessions of EAPGREN countries as per the list of Multi-Crop Passport Descriptors (MCPD). EAPGREN is strengthening collaboration and networking between the conservation and utilization of plant genetic resources at both the national and sub-regional levels (www.nordgen.org/portal/index.php?scope=eapgrenPHPSESSID=4pa906im0gh15ll2il8u80l5d1).

### 9.4.3 GRIN Global (Germplasm Resource Information Network Global)

It is a public domain, freely available software developed by the USDA (United States Department of Agriculture) for Agricultural Research Services with the purpose of open access to germplasm information required for research. Since 2011 with joint efforts of the Global Crop Diversity Trust, Bioversity International and

USDA, GRIN Global became freely accessible and benefiting the small organizations with small collections of various crops (Barata et al., 2016). GRIN Global stores, availability of accessions, germplasm health, regeneration, distribution, accession acquisition, phenotypic data, and genotypic data of the stored germplasm (https://www.grin-global.org/).

## *9.5 Cataloguing of PGRs at Various National and International Organizations*

### 9.5.1 National Organizations

Around the world, many countries have their own organizations working on conservation, evaluation, documentation and cataloguing of PGRs which are available online. Their objective is mainly to bring together all region-specific germplasm collections under one roof and act as a gateway to the PGR exchange of a particular country. Some of the leading national organizations are as follows:

(i) ICAR-National Bureau of Plant Genetic Resources (NBPGR) (www.nbpgr.ernet.in)
(ii) Centre for Genetic Resources, Plant Genetic Resources (CGN-PGR), the Netherlands (www.cgn.wur.nl/UK/CGN+Plant+Genetic+Resources)
(iii) National Inventory of Plant Genetic Resources for Food and Agriculture, Austria, (www.genbank.at)
(iv) Nordic Countries (Denmark, Finland, Iceland, Norway, Sweden) (www.nordgen.org/ngb)
(v) N. I. Vavilov All-Russian Scientific Research Institute of Plant Industry (VIR) (www.vir.nw.ru/data/dbf.html)

### 9.5.2 International Collaborations

Article 17 of the International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA) states, 'Contracting Parties shall cooperate in developing and strengthening a global information system to facilitate the exchange of information, based on existing information systems, on scientific, technical and environmental matters related to plant genetic resources for food and agriculture'. In the sixth session in 2015, the governing body emphasises inter alia (Fig. 3): strengthening of existing systems, developing new systems and encouraging the interconnectivity among the systems. This integration is called Global Information System (GLIS), with seven objectives and work plan from 2016 to 2022.

In 2017, the governing body of ITPGRFA welcomed the Digital Object Identifiers (DOI) for PGRFA, which signifies as the most appropriate, web-resolvable,


**Fig. 3** Inter alia of institutes and Global Information System (GLIS) to facilitate the exchange of information related to plant genetic resources

permanent unique identifier. It provides detailed information related to a particular DOI either in publication or in documented form; it also tracks the gene bank in which it is maintained to provide easy and efficient standard material transfer agreement (SMTA) for material exchange and identify duplicates and interoperability between gene banks (Alercia et al., 2018).

The collaborations of the Global Information System started in 2017 with the World Information and Early Warning System (WIEWS), Genesys, GRIN-Global and the European Search Catalogue for Plant Genetic Resources (EURISCO). Meanwhile in 2020–2021, the Secretariat is looking for a partnership with the SPGRC Documentation and Information System (Web-SDIS), as well as the Convention on Biological Diversity's Clearing House Mechanism, the DivSeek International Network, the Global Open Data for Agriculture and Nutrition (GODAN), the CGIAR Platform and the Global Biodiversity Information Facility (GBIF) (Manzella, 2016). GLIS is being established to link the existing systems without replacing them. It is a central platform, facilitating the registration of DOIs for all PGRFA and their identification and tracking with precision and permanence (Alercia et al., 2018).

## 10 Utilization of PGRs in Crop Improvement

Immense efforts have been made for collection; conservation and evaluation of plant genetic resources pay off only with their efficient utilization for enhanced productivity and profitability. Utilization of plant genetic resources varies from direct release of potential germplasm, development of new improved varieties, improving the breeding materials through pre-breeding, development of new novel crops (climate-resilient, biotic and abiotic stress- tolerant, biofortified crops), new gene discovery and allele mining studies. These will be described individually in subsequent paragraphs.

### *10.1 Development of Core Set, Mini-core and Reference Sets*

The characterization, evaluation, utilization and maintenance of huge collections of ex situ conservation of PGRs may be very difficult for the breeders/scientists involved in these processes (Rao & Hodgkin, 2002). Therefore, the concept of core collections has been adapted for easy and efficient characterization, evaluation and utilization of conserved PGRs, by maintaining the genetic diversity of the entire collection (Frankel, 1984; Brown, 1989). According to Frankel (1984), the core collections should have at least 10% of the collection or 2000–3000 accessions (whichever is smaller), which showed the genetic diversity of entire collections. These core collections may be used as base materials for crop improvement programmes. Till now, more than 60 core collections have been recorded in different crops and wild relatives (Rao & Hodgkin, 2002). Furthermore, if the core set still have a large population, then a mini-core set (comprising 10% of the core set) is used for characterization (Upadhyay & Ortiz, 2001). It is worth to note that ICAR-NBPGR has the core sets of brinjal, chickpea, wheat, mung bean, sesame, okra, wild lens sp., etc. for further breeding programmes (Upadhyay & Ortiz, 2001).

### *10.2 Development of Improved Varieties*

A great majority of exotic or indigenous plant genetic resources cannot be directly used for cultivation but they do possess some desirable traits which can be useful to improve the overall production, quality and adaptation of the high-yielding superior genotypes. Nowadays, plant breeders are using molecular marker technology and advanced genomic approaches for the development of improved cultivars in various crops by exploiting the potential of PGRs (Fig. 4). In addition, three possible ways in which available plant genetic resources can be utilized in plant breeding programmes are (a) introgression, (b) incorporation and (c) pre-breeding (Simmonds, 1993).

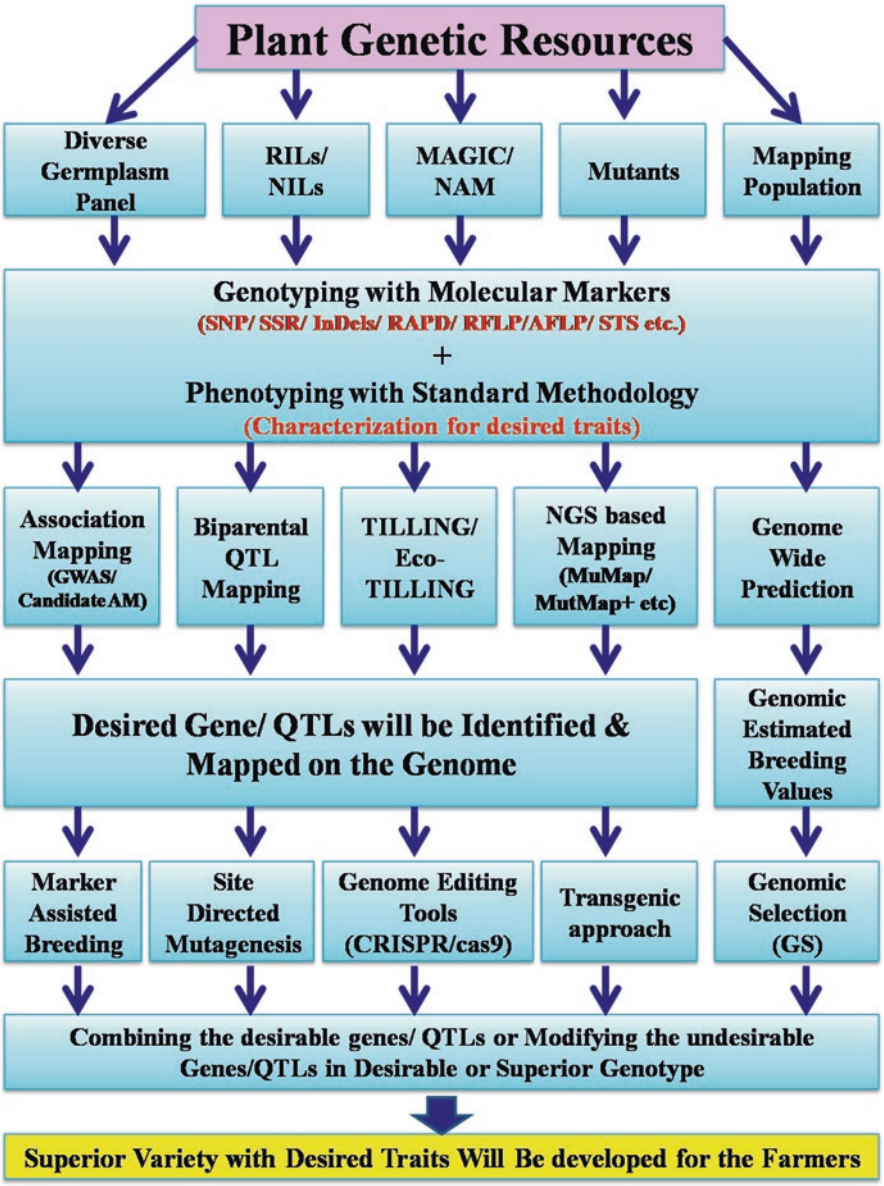



**Fig. 4** Exploitation of plant genetic resources for the development of improved cultivars through advanced genomic approaches

### 10.2.1 Introgression

Introgression basically aims at transferring superior qualitative traits with high heritability from germplasm stocks to advanced breeding materials or popular released varieties. Usually, the backcross method is employed for introgression which suffers from certain issues, for example, linkage drag and longer breeding cycle (Brown & Caligari, 2008). But with the information about the associated markers, it is possible to transfer the trait of interest with the minimum recipient genomic region in a much smaller period of time using marker-assisted backcross (MABC).

There are several occasions where specific genes from genetic resources have created a major impact on varietal development. For instance, a landrace from Japan 'Shiro Daruma' contributed *Rht1* and *Rht2*, which were transferred by Norman E Borlaug to develop high-yielding, lodging-resistant, dwarf wheat which led to the 'Green Revolution' (Sakamoto, 1983). *Sr2* and *Lr34* genes from the genetic resources in combination have been a durable source of resistance against rust disease of wheat (Hoisington et al., 1999). Introgression of accessions from *Cicer reticulatum* and *Cicer echinospermum* in the cultivated varieties was successful in generating high-yielding recombinant lines (Singh & Ocampo, 1997). Valkoun (2001) was able to transfer genes for spike productivity and yellow rust resistance from *Triticum baeoticum, T. urartu* and *Aegilops tauschii* to locally adapted landrace 'Haurani' and improved variety 'Cham 5' of wheat within 4–5 years with limited backcrossing.

### 10.2.2 Incorporation

Another way to exploit potential germplasm is their incorporation for genetic enhancement and increasing the overall genetic variation for various traits in the breeding materials. Different population improvement methods can be employed for this depending on the mode of reproduction (cross-pollinated and self-pollinated). Selection during the initial phase of incorporation is avoided so as to accumulate sufficient diversity by providing ample scope for recombination. The end product may differ according to the breeding goal. A short-term objective can be simply an immediate enhancement of the potential of the breeding materials; a medium-term goal can be to genetic enhancement focusing on the accumulation of various favourable genes to reduce the historical bottleneck effects during the life cycle of the variety, while in the long term, synthetics and composites must be developed (Cooper et al., 2001).

### 10.2.3 Pre-breeding

The success of any breeding programme majorly depends on the choice of potential parents with a desirable trait from various sources which includes landraces, wild species or relatives, obsolete varieties, cultivated varieties and advanced breeding

materials. Hybridization barriers and negative genetic load with the wild relatives, species and landraces sometimes make their direct use in breeding programmes very difficult. Therefore, desirable genes from all such germplasms are first transferred to easy-to-breed advanced breeding materials through a germplasm enhancement programme called pre-breeding.

Pre-breeding, although a tiresome and time-consuming approach, has become an initial phase of every breeding programme involving genetic resources wherein the germplasms are selectively evaluated for both qualitative and quantitative traits followed by the transfer of desirable genes to bridging germplasm with a long-term aim of varietal development using these new populations (Haussmann et al., 2004; Gorjanc et al., 2016; Dempewolf et al., 2017a, b). Bridging germplasm can be developed through three approaches, i.e. selected landraces, doubled haploids of the selected landraces and testcross progenies of landraces and elite germplasm (cultivated variety, obsolete varieties, inbred lines and advanced breeding materials) (Gorjanc et al., 2016). Singh et al. (2018) made three-way crosses of exotic lines (exotic × elite1) × elite2 directly with the elite germplasm without any initial evaluation to obtain pre-breeding lines with exotic and elite genome of 25% and 75%, respectively. This method enabled them to attain greater genetic variation in lesser time. Direct introgression to the elite germplasm from the bridging germplasm is only successful for traits governed by oligogenes. While in the case of polygenetically controlled traits, the genomic selection must be performed to increase the frequency of the favourable genes in the bridging germplasm (Gorjanc et al., 2016).

CIMMYT under its germplasm infusion project 'Seeds of Discovery' has exploited about 1000 exotic accessions from its gene bank to generate nearly 400,000 segregating pre-breeding lines. Singh et al. (2018) were able to identify pre-breeding lines with a high level of Zn content in grain. IPC71 is the pre-breeding line derived from the crosses between *Cicer arietinum* × *Cicer judaicum* which had a high number of primary branches and pods per plant (Chaturvedi & Nadarajan, 2010). Advance generation form of the one-way cross between *Cajanus acutifolius* (an accession from Australia) and *C. cajan* has shown significant resistance to the pod borer (Mallikarjuna & Saxena, 2002). ICRISAT under its groundnut improvement programme has been using several diploid wild relatives to develop amphiploid and autotetraploid pre-breeding materials for introducing several useful traits (Mallikarjuna et al., 2011).

## *10.3 Development of Novel Crop*

The estimated number of species of higher plants present worldwide is 270,000 of which only 0.04% has been domesticated by humans to fulfil various needs. But as time passes, the need of humans changes so that what seems to be once a non-important plant may achieve greater value in future. Genetic diversity stored in plant genetic resources not only offers scope for improving crop varieties but also to develop an altogether new crop. Numerous potential woody plant species are still

being domesticated by the indigenous people all around the world to fulfil their basic needs. A successful story of recent domestication is of macaw palm (*Acrocomia aculeata*) with wider adaptation to various ecological niches that has a tremendous oil yield potential of approximately 2.5–10.9 tonnes/hectare. The vast genetic resource of macaw palm in Brazilian cerrados is said to exhibit high variability for biomass and oil production (Ciconini et al., 2013). The ability to produce biodiesel from the seed oil of *Jatropha* (*Jatropha curcas* L.) has popularized its cultivation not only as an alternate source of biofuel but also for the rehabilitation of the degraded land (Montes & Melchinger, 2016).

## *10.4 Climate Resilience*

In recent years, climate change has emerged as a major challenge for sustainable agriculture and food security. It has increased the onset and intensity of abiotic stress during the various growth stages of the crop. Development of climate-resilient crop varieties will help cope with the limited resource scenario during stress with a faster recovery once stress is relieved for high production potential. Such resilience is multigenic as biochemicals from several physiological processes may be required to achieve an acceptable resilience in the plant. Breeding for climate-resilient varieties begins with the search for genes for various abiotic stresses. The genetic resource of the crops especially landraces, wild relatives and related wild species has been evolving and survived under various environmental stresses. These germplasms may not be higher yielding but do possess novel genes required for surviving such extreme environmental conditions. These novel genes can be used in the breeding programmes to integrate into the modern varieties.

## *10.5 Resistance to Biotic Stress*

Stress to crops caused by various living organisms like fungi, bacteria, viruses, insects, etc. is called biotic stress. These agents restrict the normal growth and development of their host causing loss in plant vigour and yield and in extreme cases can cause death. Use of varieties with inherent ability to resist major disease and insect pests is economical and eco-friendly as it saves the expenses of chemical controls. But these biotic agents, especially microbial pathogens due to their high rate of reproduction and recombination, are therefore able to break this resistance. Therefore, a plant breeder needs to continuously seek for novel genes for resistance and replace older genes for the new ones. Plant genetic resources being the ultimate source of all genetic variation have always been a potential source for novel resistance genes. Resistance genes of southern corn leaf blight have been successfully transferred from *Tripsacum dactyloides* into the cultivated varieties of corn.

## *10.6 Apomixis*

Apomixis is an alternative path of plant reproduction wherein seeds are produced from maternal embryo without any sexual reproduction. The individual derived from such seeds are genetically identical to the mother plant. Apomixis is a revolutionary tool which can increase crop productivity significantly by allowing any breeder to propagate their best genotype indefinitely regardless of their genetic constitution while keeping a low cost of seed production. If it is incorporated into the hybrids, it would be possible to fix the heterosis without any need of replacing the seeds year after year. Several attempts have been made to produce apomictic lines for various cereals but a commercial variety is still far from reality. Among cereals, especially pearl millet and maize are the likely candidates for apomixis breeding since apomictic wild species has already been reported for these crops. Apomixis genes from *Paspalum squamulatum* ($2n = 6x = 54$) have been successfully transferred into a tetraploid pearl millet using *P. purpureum* ($2n = 4x = 28$) as a bridge species (Dujardin & Hanna, 1989). From an attempt to transfer obligate apomixis from *Paspalum squamulatum* ($2n = 6x = 54$) into the cultivated pearl millet variety, several backcross lines with a low number of wild species have been recovered (Ozias-Akins et al., 1993). This shows possibility for transmission of apomixis genes by a single chromosome.

## *10.7 Biofortification*

Crop improvement so far has been focused on improving grain yield and crop productivity neglecting the overall nutrient status of the crops. The food grains hence produced are micronutrient. It creates a state of 'hidden hunger' or micronutrient malnutrition especially in the poor population of developing countries who depend on few staple foods to fill their stomach (Garg et al., 2018). Therefore, biofortified varieties of different crops may help us to win the fight against malnutrition. Breeding for biofortified varieties can be accelerated by harnessing all available genetic variation for micronutrient content among landraces, traditional varieties, related wild species and wild relatives. Screening germplasm is the initial step for such breeding programme of any crop. Accessions with a high level of Zn and Fe have been reported from the *Triticum spelta* and *T. dicoccum* (Velu et al., 2020). A larger variation in the rice germplasms for Fe and Zn content in grain ranging from 9.6 to 44.0 and 9.9 to 39.4 mg/kg, respectively, has been reported by Anuradha et al. (2012). Utilization of wild-related species as a source can increase the possibility of developing nutrient-dense varieties.

## *10.8 Pharmaceutical Industries*

Humans have been using a wide range of organisms in the treatment of many diseases. Among them plants are the easy-to-access and harmless source for improving human health. Active compounds isolated from all such plants with medicinal

properties can be used to make drugs for specific illnesses. Alternatively, chemical compounds isolated from plants can be modified to produce bioactive molecules. Plant-derived pharmaceuticals offer a low-cost drug and vaccine source for developing countries (Ma et al., 2005). Various indigenous traditional knowledge for the medicinal use of plant genetic resources have been documented by several researches. The testing and validation of all such genetic resources can lead to the discovery of bioactive compounds and other potential raw materials for manufacturing herbal medicines (da Silva et al., 2021).

## 11 Major Problems Associated with the Conservation, Evaluation and Enhancement of PGRs

Recent reports indicate that the pressure imposed by both biotic and abiotic agents has caused us massive loss of genetic diversity present in the form of landraces, weedy and wild relatives of crops (Gupta, 2014). All breeding programmes are driven by genetic variability for improving yield, disease or pest resistance and product quality of the crop, forage or ornamental plants. Conservation of plant genetic resources involves active efforts towards retaining the intrinsic diversity present in the gene pool of any plant species of potential value for future use. A brief account on the major problems associated during the conservation, evaluation and utilization of plant genetic resources are discussed in this section.

### *11.1 Population Growth and Deforestation*

With the increasing population of the world, the food grain demands by the year 2030 and 2050 are anticipated to be around 10,094 million and 14,886 million tonnes, respectively, which will impose an extra pressure to increase the food grain production to meet the future demand (Islam & Karim, 2019). This pressure could lead to overexploitation of plant genetic resources. Increasing human population without development will increase the pressure on forests to meet their basic needs. Most of the valuable plant genetic resources in the form of wild relatives and wild forms of several crop plants reside in the forest. Reports suggest that the high rate of population growth followed by the low human development index resulted in increased deforestation, while a high population rate along with a high human development index led to a comparatively lower rate of deforestation.

## *11.2 Varietal Modernization*

Landraces are a dynamic population with a broad genetic base and local adaptation. Most often they are poor yielding and susceptible to lodging, making them unfit for commercial cultivation. The current breeding programmes are focused on developing high-yielding varieties for commercial agriculture. The extensive use of the high-yielding varieties has made landraces go out of cultivation. This has resulted in loss of potential landraces of various crop plants to a magnitude which is irreversible.

## *11.3 Climate Change*

Change in climate has a significant negative effect on plant genetic resources often leading to perturbations such as drought, flood and disease. The significant change in the environment and/or climate may severely affect the vigour and viability of PGRs conserved at the on-farm site. Furthermore, natural calamities or disaster may destroy all the on-farm conservation PGRs within a short period of time. These activities may lead to the 'extinction' of PGRs in those agro-ecosystems (Ogwu et al., 2014). However, at the same time the available diversity and variability in the PGR may give opportunities to withstand adverse environmental conditions by adapting those situations that can provide options to farmers and society to adapt to climate change.

## *11.4 Genetic Erosion*

Genetic erosion, the decrease in population variation due to random genetic drift and inbreeding, is both a symptom and a cause of endangerment of small isolated populations (Woodruff, 2001). The hybrids, HYVs and modern varieties have significantly greater yield over the landraces and farmer varieties which restricted the cultivation of those traditional varieties. Gradually, those landraces are lost unless they are conserved (Wilkes, 1992). In this way, several traditional varieties of various crops have been restricted from the farmers' field.

## *11.5 Genetic Vulnerability*

Genetic vulnerability refers to the inherited characteristics passed on from parents to progenies that make it more likely that a person will develop an addiction. When the whole scientific community may start working on a similar aspect to develop a

similar type of varieties, i.e. resistance to any specific race of pathogen and pest, then the genetic structure of the genotypes may be narrowed towards the specific gene. These activities may enhance the chances of the potential damage from unsuspected pathogens and pests. Since most of the genotypes have similar traits or genes, then they will not have resistance to novel races of pathogens or pests. These may cause a significant impact on genetic vulnerability to that species (Wilkes, 1992). Prolonged spread of the *Ug99* race of wheat stem rust to most of the existing wheat varieties is the best example of genetic vulnerability (Pretorius et al., 2000).

### *11.6 Lack of Technical Knowledge*

During the collection of genetic resources, a highly technical person is required who has basic knowledge about collected materials like their nature, biology and other characteristic features which provide us a useful information regarding collected genetic resources. However, sometimes the person who collects the genetic resources does not have basic knowledge and continues the collection which causes repetition of the same type of genetic resources at a time. Moreover, the PGRs are generally collected from the farmers' field, and if farmers do not have precise information about the PGRs, then the person involved in the collection may not compile enough information. These basic information are very important for further breeding activities and their utilization in crop improvement.

## 12 Way Ahead

To maintain national food security, we need to maintain and conserve the available plant genetic resources. Effective and sustainable utilization of the plant genetic resources will ensure future developments in the crop improvement programmes and industrialization as well. A collaborative approach involving the countries, institutes and organizations must be a matter of primary commitment to discover, collect and conserve the potentially valuable genetic resources. To make the rich genetic diversity easily accessible to breeders, farmers and local communities, it is important to provide a better and more accessible documentation system. The threat of climate change resulting in biodiversity habitat loss has pressed the alarming need of re-examining the strategies of collection and conservation. Efficient survey, monitoring and inventory field studies involving the Geographical Information System (GIS) and remote sensing need to be deployed to supplement the proper understanding/identification of the extent and distribution of agro-biodiversity particularly in inaccessible areas. Also, there is a need to take up harmonizing conservation strategies involving both in situ and ex situ approaches. For in situ conservation, more natural reserved areas should be maintained and due attention is required to be given to genetically rich hotspots including tribal belts and an effective enforcement of laws guarding them. Most of the ex situ collection is

endangered so securing and providing a financial support system should be a priority. Promotion towards identification and increase and share fairly and equitably the benefits derived from the conservation of plant genetic resources should be done. The genetic diversity held at gene banks contains useful genes and alleles to potentially address all kinds of constraints in crop production, but it is difficult to select the most appropriate set of accessions without sufficient information such as phenotypic and genotypic data. For this, the rich resources, when coupled with multi-omics tools including genomics, proteomics and metabolomics on selected materials, will allow selection of genotypes, novel alleles and haplotypes for any trait. Acceleration of these approaches is expected to enhance the breeding process while using the rich conserved diversity. Pre-breeding is needed to incorporate new kinds of pest resistance, to bring in new levels of productivity and stability of performance and to provide quality traits for food and feed products. Public awareness generation at various levels about the value of PGR wealth through training, seminar and media is essential. Also integration of the conservation priorities into the educational curriculum should be encouraged. New useful PGR management alternatives, scientific innovations and advanced technologies promoting the use of PGR have to be achieved. Access and sharing of genetic resources and technologies worldwide are essential to ensure world food security and it must be facilitated under fair terms with the adequate and effective protection of plant intellectual rights.

## 13 Conclusion

Plant genetic resources have great potential in the context of climate change, organic agriculture, food diversity and stability of agricultural production systems, while the need for their conservation and sustainable use worldwide is growing and represents a huge challenge. The germplasm exploration and collection have resulted in the accumulation of enormous genetic diversity of crop plants in gene banks. Therefore, concerted efforts need to be made for its characterization, evaluation and identification of trait-specific accessions especially from unexplored/exotic germplasm using field phenotyping coupled with modern genomic tools to trace the underlying gene. The advances in genotyping and biotechnology tools in recent years are making genetic resources and their variation more accessible for breeders. New breeding tools may also facilitate applications for efficient crop improvement through the removal of deleterious mutations or enrichment of cultivated sequences with alleles from wild relatives.

## References

Alercia, A., Diulgheroff, S., & Metz, T. (2001). *FAO/IPGRI multi-crop passport descriptors [MCPD]* (p. 4).

Alercia, A., López, F. M., Sackville Hamilton, N. R., & Marsella, M. (2018). *Digital Object Identifiers for food crops – Descriptors and guidelines of the Global Information System*. FAO.

Anuradha, K., Agarwal, S., & Rao, Y. V. (2012). Mapping QTLs and candidate genes for iron and zinc concentrations in unpolished rice of Madhukar × Swarna RILs. *Gene, 508*, 233–240.

Bains, N. S., Singh, S., & Dhillon, B. S. (2012). Enhanced utilization of plant genetic resources in crop improvement programme. *Indian Journal of Plant Genetic Resources, 25*(1), 52–62.

Barata, A. M. R. F., Oliveira, J., Lima, J. M., Nobrega, H., Pinheiro de Carvalho, M. A. A., & Dias, S. (2016). Implementation of a PGR global documentation system in Portugal. In N. Maxted, M. E. Dulloo, & B. V. Ford-Lloyd (Eds.), *Enhancing crop genepool use: Capturing wild relative and landrace diversity for crop improvement* (pp. 441–452). CABI.

Bekheet, S. A., Sota, V., & El-Shabrawi, H. M. (2020). Cryopreservation of shoot apices and callus cultures of globe artichoke using vitrification method. *Journal, Genetic Engineering & Biotechnology, 18*, 2.

Bettencourt, E. (2011). Sources of information on existing germplasm collections. In *Collecting plant genetic diversity: Technical guidelines*. Bioversity International.

Bettoni, J. C., Dalla Costa, M., Gardin, J. P. P., Kretzschmar, A. A., & Pathirana, R. (2016). Cryotherapy: A new technique to obtain grapevine plants free of viruses. *Revista Brasileira de Fruticultura, 28*, 1–13.

Bettoni, J. C., Bonnart, R., & Volk, G. M. (2021). Challenges in implementing plant shoot tip cryopreservation technologies. *Plant Cell, Tissue and Organ Culture, 144*, 21–34.

Bioversity International. (2007). *Developing crop descriptor lists-guidelines for developers* (Bioversity Technical Bulletin No.13) (p. 71). Bioversity International.

Brown, A. H. D. (1989). The case for core collections. In A. H. D. Brown, O. H. Frankel, D. R. Marshall, & J. T. Williams (Eds.), *The use of plant genetic resources* (pp. 136–165). Cambridge University Press.

Brown, J., & Caligari, P. (2008). *An introduction to plant breeding*. Blackwell Publishing Ltd.

Chaturvedi, S. K., & Nadarajan, N. (2010). Genetic enhancement for grain yield in chickpea – Accomplishments and resetting research agenda. *Electronic Journal of Plant Breeding, 1*, 611–615.

Ciconini, G., Favaro, S., Roscoe, R., Miranda, C., Tapeti, C., & Miyahira, M. (2013). Biometry and oil contents of Acrocomia aculeata fruits from the Cerrados and Pantanal biomes in Mato Grosso do Sul, Brazil. *Industrial Crops and Products, 45*, 208–214.

Cooper, H. D., Spillane, C., & Hodgkin, T. (2001). *Broadening the genetic base of crop production* (p. 452). CAB International.

Cruz-Cruz, C. A., Gonzalez-Arnao, M. J., & Engelmann, F. (2013). *Biotechnology and conservation of plant biodiversity*. Accessed 16 Feb 2021.

da Silva, L. E., Confortin, C., & Swamy, M. K. (2021). Antibacterial and antifungal plant metabolites from the tropical medicinal plants. In D. Pal & A. K. Nayak (Eds.), *Bioactive natural products for pharmaceutical applications. Advanced structured materials* (Vol. 140). Springer.

de Vicente, M. C., & Andersson, M. S. (2006). *DNA banks – Providing novel options for genebanks? Topical reviews in agricultural biodiversity*. International Plant Genetic Resources Institute.

Dempewolf, H., Baute, G., Anderson, J., Kilian, B., Smith, C., & Guarino, L. (2017a). Past and future use of wild relatives in crop breeding. *Crop Science, 57*, 1–13.

Dempewolf, H., Baute, G., Anderson, J., Kilian, B., Smith, C., & Guarino, L. (2017b). Past and future use of wild relatives in crop breeding. *Crop Science, 57*, 1070–1082.

Dhillon, B. S., & Agrawal, A. (2004). Plant genetic resources: ownership, access and intellectual property rights. In B. S. Dhillon, R. K. Tyagi, S. Saxena, & A. Agrawal (Eds.), *Plant genetic resources: Oilseed and cash crops* (pp. 1–19). Narosa Publishing House.

Dujardin, M., & Hanna, W. W. (1989). Developing apomictic pearl millet characterization of a BC3 plant. *Journal of Genetics and Plant Breeding, 43*, 145–151.

Dulloo, M. E., Rege, J. E. O., Ramirez, M., Drucker, A. G., Padulosi, S., Maxted, N., Sthapit, B. R., Gauchan, D., Thormann, I., Gaisberger, H., & Roux, N. (2017). *Conserving agricultural biodiversity for use in sustainable food systems*.

Edagbo, D. E., Ighere, D. A., Alowonle, A. A., & Clement, M. (2011). Plant genetic resources: A review on the various conservation strategies to explore and the sustainable utilisation of accessible germplasm. *International Journal of Current Research, 3*(12), 043–051.

Engelmann, F. (2000). Importance of cryopreservation for the conservation of plant genetic resources. In F. Engelmann & H. Takagi (Eds.), *Cryopreservation of tropical plant germplasm. Current research progress and application* (pp. 8–20). IPGRI.

Fahelson, J., Erikson, I., Landgren, M., Stymne, S., & Glimelius, K. (1994). Intertribal somatic hybrids between *Brassica napus* and *Thlaspi perfoliatum* with high content of the T. perfoliatum-specific nervonic acid. *Theoretical and Applied Genetics, 87*, 795–804.

Feng, C., Yin, Z., Ma, Y., Zhang, Z., Chen, L., Wang, B., Li, B., Huang, Y., & Wang, Q. (2011). Cryopreservation of sweet potato (*Ipomoea batatas*) and its pathogen eradication by cryotherapy. *Biotechnology Advances, 29*, 84–93.

Food and Agriculture Organization of the United Nations. (2013). *Genebank standards: For plant genetic resources for food and agriculture*. Food and Agriculture Organization of the United Nations.

Frankel, O. H. (1984). Genetic perspectives of germplasm conservation. In W. K. Arber, K. Llimensee, W. J. Peacock, & P. Star Linger (Eds.), *Genetic manipulation: Impact on man and society* (pp. 161–170). Cambridge University Press.

Garg, M., Sharma, N., Sharma, S., Kapoor, P., Kumar, A., & Chunduri, V. (2018). Biofortified crops generated by breeding, agronomy, and transgenic approaches are improving lives of millions of people around the world. *Frontiers in Nutrition, 5*, 12.

Gichuki, S. T., Berenyi, M., DaPeng, Z., Hermann, M., Schmidt, J., Glossl, J., & Berg, K. (2003). Genetic diversity in sweet potato (*Ipomoea batatas* (L.) Lam) in relationship to geographic sources as assessed with RAPD markers. *Genetic Resources and Crop Evolution, 50*, 429–437.

Gollin, D. (2020). Conserving genetic resources for agriculture: Economic implications of emerging science. *Journal of Food Security, 12*, 919–927.

Gorjanc, G., Jenko, J., Hearne, S. J., & Hickey, J. M. (2016). Initiating maize pre-breeding programs using genomic selection to harness polygenic variation from landrace populations. *BMC Genomics, 17*(30), 1–30.

Goyal, S., Wani, M. R., Raina, A., Laskar, R. A., & Khan, S. (2021a). Phenotypic diversity in mutagenized population of urdbean (*Vigna mungo* (L.) Hepper). *Heliyon, 7*(5), e06356.

Goyal, S., Wani, M. R., Raina, A., Laskar, R. A., & Khan, S. (2021b). Quantitative assessments on induced high yielding mutant lines in urdbean [*Vigna mungo* (L.) Hepper]. *Legume Science*, e125.

Gupta. (2014). Plant genetic resources and their utilization in crop improvement. *Biotech Artist, 1*(24), 33.

Hajjar, R., & Hodgkin, T. (2007). The use of wild relatives in crop improvement: A survey of developments over the last 20 years. *Euphytica, 156*, 1–13.

Halewood, M., Chiurugwi, T., Hamilton, R. S., Kurtz, B., Marde, E., Welch, E., Michiels, F., Mozafari, J., Sabran, M., Patron, N., Kersey, P., Bastow, R., Dorius, S., & Dias, S. (2018). Plant genetic resources for food and agriculture: Opportunities and challenges emerging from the science and information technology revolution. *The New Phytologist, 217*(4), 1407–1419.

Haussmann, B. I. G., Parzies, H. K., Prester, T., Susic, Z., & Miedaner, T. (2004). Plant genetic resources in crop improvement. *Plant Genetic Resources, 2*(1), 3–21.

Havinga, R., Kool, A., Achille, F., Bavcon, J., Berg, C., Bonomi, C., & Vange, V. (2016). The Index Seminum: Seeds of change for seed exchange. *Taxon, 65*(2), 333–336.

Hawkes, J. G., Maxted, N., & Ford-Lloyd, B. V. (2012). *The ex situ conservation of plant genetic resources*. Springer.

Heywood, V. H. (2005). *In situ conservation of wild plant species: a critical global review of good practices* (IPGRI Technical Bulletin No. 11).

Hidalgo, R. (2003). Variabilidadgenética y caracterización de especiesvegetales. In T. L. Franco & R. Hidalgo (Eds.), *Análisisestadístico de datos de caracterización morfológica de recursosfitogenéticos* (Boletín Técnico No. 8) (pp. 2–26). IPGRI.

Hoisington, D., Khairallah, M., Reeves, T., Ribaut, J., Skovmand, B., Taba, S., & Warburton, M. (1999). Plant genetic resources: What can they contribute toward increased crop productivity? *PNAS, 96*(11), 5937–5943.

IPGRI. (1996). *Report of the internally commissioned external review of the CGIAR Genebank operations*. International Plant Genetic Resources Institute.

Islam, S. M. F., & Karim, Z. (2019). World's demand for food and water: The consequences of climate change. In *Desalination-challenges and opportunities* (p. 425). Intech Open.

Jaramillo, S., & Baena, M. (2000). *Material de apoyo a la capacitaciónenconservación ex situ de*. www.ipgri.cgiar.org/training/exsitu/web/arr_ppal_modulo.html. recursosfitogenéticos. IPGRI, 209 p.

Julsing, K. M., Quax, J. W., & Kayser, O. (2007). *The engineering of medicinal plants: Prospects and limitations of medicinal plant biotechnology*. Medicinal Plant Biotechnology.

Kaviani, B. (2011). Conservation of plant genetic resources by cryopreservation. *Australian Journal of Crop Science, 5*(6), 778–800.

Kaya, E. (2021). Comparison of three different techniques for eradication of Apple mosaic virus (ApMV) from hazelnut (*Corylus avellana* L.). *Journal of Plant Protection Research, 61*(1), 11–19.

Khursheed, S., Raina, A., Amin, R., Wani, M. R., & Khan, S. (2018a). Quantitative analysis of genetic parameters in the mutagenized population of faba bean (Vicia faba L.). Res. *Crops, 19*(2), 276–284.

Khursheed, S., Raina, A., Laskar, R. A., & Khan, S. (2018b). Effect of gamma radiation and EMS on mutation rate: Their effectiveness and efficiency in faba bean (*Vicia faba* L.). *Caryologia, 71*(4), 397–404.

Khursheed, S., Raina, A., & Khan, S. (2018c). Physiological response of two cultivars of faba bean using physical and chemical mutagenesis. *International Journal of Advance Research in Science and Engineering, 7*(4), 897–905.

Kumar, V., Gajbhiye, N. A., & Manivel, P. (2018). Assessment of the genetic diversity of Ashwagandha (*Withania somnifera* L. Dunal) using agro-morphological, phytochemical and molecular markers. *Research Journal of Biotechnology, 17*(7), 59–68.

Kuraparthy, V. P., Chhuneja, H. S., Dhaliwal, S., Bowden, K. R. L., & Gill, B. S. (2007a). Characterization and mapping of cryptic alien introgression from Aegilops geniculata with novel leaf rust and stripe rust resistance genes *Lr57* and *Yr40* in wheat. *Theoretical and Applied Genetics, 114*, 1379–1389.

Kuraparthy, V., Sood, S., Chhuneja, P., Dhaliwal, H. S., Kaur, S., Bowden, R. L., & Gill, B. S. (2007b). A cryptic wheat-Aegilops triuncialis translocation with leaf rust resistance gene *Lr*58. *Crop Science, 47*, 1995–2003.

Laskar, R. A., Laskar, A. A., Raina, A., & Amin, R. (2018a). Induced mutation analysis with biochemical and molecular characterization of high yielding lentil mutant lines. *International Journal of Biological Macromolecules, 109*, 167–179.

Laskar, R. A., Wani, M. R., Raina, A., Amin, R., & Khan, S. (2018b). Morphological characterization of gamma rays induced multipodding mutant (mp) in lentil cultivar pant L 406. *International Journal of Radiation Biology, 94*(11), 1049–1053.

Lee, J. S., Chebotarov, D., Platten, J. D., McNally, K., & Kohli, A. (2020). Advanced strategic research to promote the use of rice genetic resources. *Agronomy, 10*, 1629.

Ligarreto, G. (2003). Caracterización de germoplasma. In T. L. Franco & R. Hidalgo (Eds.), *Análisisestadístico de datos de caracterizaciónmorfológica de recursosfitogenéticos* (Boletín Técnico No. 8) (pp. 77–79). IPGRI.

Ma, J. K. C., Chikwamba, R., Penny Sparrow, P., Fischer, R., Mahoney, R., & Twyman, R. M. (2005). Plant-derived pharmaceuticals – The road forward. *Trends in Plant Science, 10*(12), 580–585.

Mahajan, R. K., Sapra, R. L., Srivastava, U., Singh, M., & Sharma, G. D. (2000). *Minimal descriptors for characterization and evaluation of agri-horticultural crops (Part I)* (p. 230). National Bureau of Plant Genetic Resources.

Mallikarjuna, N., & Saxena, K. B. (2002). Production of hybrids between *Cajanus acutifolius* and *C. cajan*. *Euphytica, 124*(1), 107–110.

Mallikarjuna, N., Senthilvel, S., & Hoisington, D. (2011). Development of new sources of tetraploid Arachis to broaden the genetic base of cultivated groundnut (*Arachis hypogaea* L.). *Genetic Resources and Crop Evolution, 58*, 889–907.

Mancilla-Álvarez, E., Ramírez-Mosqueda, M. A., Arano-Avalos, S., Núnez-Pastrana, R., & Bello-Bello, J. J. (2019). In vitro techniques to the conservation and plant regeneration of Malanga (Colocasia esculenta L. Schott). *Horticultural Science, 54*(3), 514–518.

Manzella, D. (2016). *The global information system and genomic information: Transparency of rights and obligations*. FAO.

Maxted, N., Hawkes, J. G., Guarino, L., & Sawkins, M. (1997). Towards the selection of taxa for plant genetic conservation. *Genetic Resources and Crop Evolution, 44*(4), 337–348.

Montes, J. M., & Melchinger, A. E. (2016). Domestication and breeding of Jatropha curcas L. *Trends in Plant Science, 21*(12), 1045–1057.

Muthoni, J., Shimelis, H., & Melis, R. (2019). Long-term conservation of potato genetic resources: Methods and status of conservation. *Australian Journal of Crop Science, 13*(5), 717–725.

Nalawade, S. M., Sagare, A. P., Lee, C. Y., Kao, C. L., & Tsay, H. S. (2003). Studies on tissue culture of Chinese medicinal plant resources in Taiwan and their sustainable utilization. *Botanical Bulletin- Academia Sinica, 44*(2), 79–98.

Niino, T., & Sakai, A. (1992). Cryopreservation of alginate coated in-vitro grown shoot tips of apple, pear and mulberry. *Plant Science, 87*(2), 199–206.

Niino, T., Koyaman, A., Shirata, K., Ohuchi, S., Suguli, M., & Sakai, A. (1993). Long term storage of mulberry winter buds by cryopreservation. *Journal of Sericultural Science of Japan, 62*(5), 431–434.

Ogbu, J. U., Essien, B. A., Essien, J. B., & Anaele, M. U. (2010). Conservation and management of genetic resources of horticultural crops in Nigeria: Issues and biotechnological strategies. *Journal of Horticulture and Forestry, 2*(9), 214–222.

Ogwu, M. C., Osawaru, M. E., & Ahana, C. M. (2014). Challenges in conserving and utilizing plant genetic resources (PGR). *International Journal of Genetics and Molecular Biology, 6*(2), 16–22.

Ozias-Akins, P., Lubbers, E. L., Hanna, W. W., & McNay, J. W. (1993). Transmission of the apomictic mode of reproduction in Pennisetum: Co-inheritance of the trait and molecular markers. *Theoretical and Applied Genetics, 85*, 632–638.

Pal, S., Chhuneja, P., Sharma, A., Singh, R. P., & Bains, N. S. (2010). Introgression of quality traits from Triticum dicoccoides to bread wheat variety PBW343. *Crop Improvement, 37*, 183–188.

Pillay, M., Ogundiwin, E., Nwakanma, D. C., Ude, G., & Tenkouano, A. (2001). Analysis of genetic diversity and relationships in East African banana germplasm. *Theoretical and Applied Genetics, 102*, 965–970.

Pineda, B., & Hidalgo, R. (2007). *Multi-institutional distance learning course on the ex situ conservation of plant genetic resources* (Vol. 360, p. 283). CIAT Publication.

Pretorius, Z. A., Singh, R. P., Wagoire, W. W., & Payne, T. S. (2000). Detection of virulence to wheat stem rust resistance gene *Sr31* in *Puccinia graminis*. f. sp. *tritici* in Uganda. *Plant Diseases, 84*, 203–208.

Raina, A., Laskar, R. A., Tantray, Y. R., Khursheed, S., Wani, M. R., & Khan, S. (2020a). Characterization of induced high yielding cowpea mutant lines using physiological, biochemical and molecular markers. *Scientific Reports, 10*(1), 1–22.

Raina, A., Khan, S., Sahu, P., & Roa, R. (2020b). Increasing rice grain yield under abiotic stresses: Mutagenesis, transgenics and genomics approaches. In C. Aryadeep (Ed.), *Rice research for quality improvement: Genomics and genetic engineering* (pp. 753–777). Springer. https://doi.org/10.1007/978-981-15-4120-9_31

Raina, A., Laskar, R. A., Wani, M. R., Jan, B. L., Ali, S., & Khan, S. (2022a). Gamma rays and sodium azide induced genetic variability in high yielding and biofortified mutant lines in

cowpea [*Vigna unguiculata* (L.) Walp.]. *Frontiers in Plant Science, 13*, 911049. https://doi.org/10.3389/fpls.2022.911049

Raina, A., Laskar, R. A., Wani, M. R., Jan, B. L., Ali, S., & Khan, S. (2022b). Comparative mutagenic effectiveness and efficiency of gamma rays and sodium azide in inducing chlorophyll and morphological mutants of cowpea. *Plants, 11*, 1322. https://doi.org/10.3390/plants11101322

Raina, A., Laskar, R. A., Wani, M. R., & Khan, S. (2022c). Chemical mutagenesis: Role in breeding and biofortification of lentil (*Lens culinaris* Medik) mutant lines. *Molecular Biology Reports, 49*(12), 11313–11325. https://doi.org/10.1007/s11033-022-07678-6

Raina, A., Laskar, R. A., Wani, M. R., & Khan, S. (2022d). Plant breeding strategies for abiotic stress tolerance in cereals. In *Omics approach to manage abiotic stress in cereals* (pp. 151–177). Springer. https://doi.org/10.1007/978-981-19-0140-9_8

Ramya, A. R., Rajesh, V., Jyothi, P., & Swathi, G. (2014). A review on in situ and ex situ conservation strategies for crop germplasm. *International Journal of Applied Biology and Pharmaceutical Technology, 5*(1), 267–273.

Rao, N. K. (2004). Plant genetic resources: Advancing conservation and use through biotechnology. *African Journal of Biotechnology, 3*(2), 136–145.

Rao, V. R., & Hodgkin, T. (2002). Genetic diversity and conservation and utilization of plant genetic resources. *Plant Cell, Tissue and Organ Culture, 68*(1), 1–19.

Rao, N. K., Hanson, J., Dulloo, M. E., Ghosh, K., Nowell, D., & Larinde, M. (2006). *Handbooks for Genebanks No. 8. Manual of seed handling in Genebanks* (p. 163).

Rasik, S., Raina, A., Laskar, R. A., Wani, M. R., Reshi, Z., & Khan, S. (2022). Lower doses of Sodium azide and Methyl methanesulphonate improved yield and pigment contents in vegetable cowpea [*Vigna unguiculata* (L.) Walp.]. *South African Journal of Botany, 148*, 727–736. https://doi.org/10.1016/j.sajb.2022.04.034

Rawat, N., Tiwari, V. K., Neelam, K., Randhawa, G. S., Chhuneja, P., Singh, K., & Dhaliwal, H. S. (2009). Development and characterization of Triticum aestivum–Aegilops kotschyi amphiploids with high grain iron and zinc contents. *Plant Genetic Resources: Characterisation and Utilisation, 7*, 271–280.

Reed, B. M. (2004). *Technical guidelines for the management of field and in vitro germplasm collections (No. 7)*. Bioversity International.

Sakamoto, S. (Ed.). (1983). *Proceedings of the 6th international wheat genetic symposium*. Plant Germplasm Institute, University of Kyoto.

Sellapillai, L., Dhanarajan, A., Raina, A., & Ganesan, A. (2022). Gamma ray induced positive alterations in morphogenetic and yield attributing traits of finger millet (*Eleusine coracana* (L.) Gaertn.) in M2 generation. *Plant Science Today, 9*(4), 939–949.

Sharma, S. K. (2007). *Indian Plant Genetic Resources (PGR) system: Role of NBPGR. Training manual on in vitro and cryopreservation techniques for conservation of PGR*. NBPGR and Bioversity Int..

Sharma, D. R., Kaur, R., & Kumar, K. (1996). Embryo rescue in plants – A review. *Euphytica, 89*, 325–337.

Simmonds, N. W. (1993). Introgression and incorporation. Strategies for the use of crop genetic resources. *Biological Reviews, 68*, 539–562.

Simpson, M. J. A. & Withers, L. A. (1986). Characterization of plant genetic resources using isozyme electrophoresis: a guide to the literature. IBPGR, Rome.

Singh, C. (2017). Recent advances in conservation of plant genetic resources. *Agricultural Research & Technology, 7*(4), 110–111.

Singh, K. B., & Ocampo, B. (1997). Exploitation of wild Cicer species for yield improvement in chickpea. *Theoretical and Applied Genetics, 95*, 418–423.

Singh, S., Vikram, P., Sehgal, D., Burgueño, J., Sharma, A., Singh, S. K., Sansaloni, C. P., Joynson, R., Brabbs, T., Ortiz, C., Solis-Moya, E., Govindan, V., Gupta, N., Sidhu, H. S., Basandrai, A. K., Basandrai, D., Ledesma-Ramires, L., Suaste-Franco, M. P., Fuentes-Dávila, G., Moreno, J. I., & Pixley, K. V. (2018). Harnessing genetic potential of wheat germplasm banks through

impact-oriented-prebreeding for future food and nutritional security. *Scientific Reports, 8*(1), 12527.

Singh, K., Gupta, K., Tyagi, V., & Rajkumar, S. (2020). Plant genetic resources in India: Management and utilization. *Journal of Genetics and Plant Breeding, 24*(3), 306–314.

Spoor, W., & Simmonds, N. W. (2001). Base-broadening: Introgression and incorporation. In H. D. Cooper, C. Spillane, & T. Hodgkin (Eds.), *Broadening the genetic base of crop production* (pp. 71–80). CAB International.

Ulukan, H. (2011). Plant genetic resources and breeding: Current scenario and future prospects. *International Journal of Agriculture and Biology, 13*, 447–454.

Upadhyay, H. D., & Ortiz, R. (2001). A mini core sub set for capturing diversity and promoting utilization of chickpea genetic resources in crop improvement. *Theoretical and Applied Genetics, 102*, 1292–1298.

Valkoun, J. J. (2001). Wheat pre-breeding using wild progenitors. *Euphytica, 119*, 17–23.

Velu, G., Singh, R. P., & Joshi, A. K. (2020). A decade of progress on genetic enhancement of grain zinc and iron in CIMMYT wheat germplasm. *Wheat and Barley Grain Biofortification*, 129–138. https://doi.org/10.1016/B978-0-12-818444-8.00005-5.

Vijayan, K. (2020). *Role of biotechnology in genetic conservation of mulberry plants for crop improvement*, *9*, S4 003.

Wambugu, W. P., Ndjiondpp, M. N., & Henry, R. J. (2018). Role of genomics in promoting the utilization of plant genetic resources in genebanks. Briefings in functional genom. Accessed 15 Feb 2021.

Weise, S., Oppermann, M., Maggioni, L., van Hintum, T., & Knüpffer, H. (2017). EURISCO: The European search catalogue for plant genetic resources. *Nucleic Acids Research, 45*, 1003–1008.

Wilkes, G. (1992). Strategies for sustaining crop germplasm preservation, enhancement, and use. *Consultative Group on International Agricultural Research*, 12–12.

Withers, L. A., & Engelmann, F. (1997). *In vitro conservation of plant genetic resources. Biotechnology in Agriculture* (pp. 57–88). Marcel Dekker Inc..

Woodruff, D. S. (2001). Declines of biomes and biotas and the future of evolution. *Proceedings of the National Academy of Sciences of the United States of America, 98*, 5471–5476.

# SINE Markers as a Powerful Tool for Assessing Genetic Diversity to Improve Potato


**Nasya Tomlekova, Sibel Aziz, Emiliya Nacheva, Beatrice Weber, Aamir Raina, and Kathrin M. Seibt**

**Abstract** Potato is an important global food source. The tetraploid potato is a genetically complex and heterozygous crop, which makes it difficult to introgress desirable traits from wild relatives. Therefore, in order to increase the genetic variability of the breeding material, mutagenesis is effectively used, for instance, by chemical agents.

This chapter summarizes the results obtained at the Maritsa Vegetable Crops Research Institute in Plovdiv, Bulgaria, with regard to the evaluation of the genetic diversity of local potato varieties and 16 advanced mutant lines ($M_1V_8$) and the corresponding parental components which were divided into four groups according the origin on the initial genotypes, using the retrotransposon-based molecular technique of inter-SINE amplified polymorphism (ISAP).

ISAP reactions with the two SINE families (SolS-IIIa and SolS-IV) proved to be the most efficient for the molecular identification. Of all the three reactions (SolS-IIIa-F/R, SolS-IV-F/R, SolS-IIIa-F/SolS-IV-R) performed on the 16 mutant lines,

N. Tomlekova (✉)
Molecular Biology Laboratory, Maritsa Vegetable Crops Research Institute, Agricultural Academy, Plovdiv, Bulgaria

S. Aziz
Department of Breeding, Agricultural Academy, Maritsa Vegetable Crops Research Institute, Plovdiv, Bulgaria

Department of Developmental Biology, Faculty of Biology, University of Plovdiv "Paisii Hilendarski", Plovdiv, Bulgaria

E. Nacheva
Department of Breeding, Agricultural Academy, Maritsa Vegetable Crops Research Institute, Plovdiv, Bulgaria

B. Weber · K. M. Seibt
Technische Universität Dresden, Institute of Botany, Dresden, Germany

A. Raina
Mutation Breeding Laboratory, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

Botany Section, Women's College, Aligarh Muslim University, Aligarh, Uttar Pradesh, India



A. Raina et al. (eds.), *Advanced Crop Improvement, Volume 2*,
https://doi.org/10.1007/978-3-031-26669-0_2

including parents and control lines, three mutant lines (M-I-8, M-IV-17, M-VII-27), two control lines (K-IV-3, K-VII-4), and all parent lines (PC428, PC490, PC707, PC538, PC757) were characterized by unique profiles.

The present study can be used as a basis for future research in potato breeding. The selection of mutant lines with high-productivity morphological and molecular data and their combination are of great importance for breeding new valuable mutant varieties.

**Keywords** ISAP · Induced mutagenesis · *Solanum tuberosum* L. · Transposable elements

Abbreviations

| | |
|---|---|
| BSA | Bovine Serum Albumin |
| CTAB | Hexadecyltrimethylammonium Bromide [(C16H33)N(CH3)3]Br |
| EDTA | Ethylenediaminetetraacetic Acid $[CH_2N(CH_2CO_2H)_2]_2$ |
| ISAP | Inter-SINE Amplified Polymorphism |
| M | Multiplex reaction |
| SINE | Short Interspersed Nuclear Element |
| Sol | Solanaceae |
| TE | Transposable Elements |

## 1 Introduction

Potato belongs to the Solanaceae, a large plant family with more than 3000 species, which also includes several other economically important species such as tomatoes, eggplants, petunias, tobacco, and peppers. Potato (*Solanum tuberosum* L.) is a valuable global food source and the fourth most important crop in the world after wheat, rice, and maize (Zhang et al., 2017). The most commonly cultivated potatoes are tetraploid (2n = 4x = 48), whereas wild species vary in ploidy from diploid to hexaploid (Lara-Cabrera and Spooner, 2004). This complicates both breeding improvement of cultivated potato and elucidation of genetic traits (De Boer et al., 2011). The utility and economic importance of potato derives from the tuber, of which approximately 82% of its dry matter is starchy carbohydrates. The quality of the tuber is determined by a combination of structural, physiological, biochemical, and chemical properties, which are affected both genetically and by developmental and postharvest conditions (Flinn et al., 2005).

Today's potato breeders are facing the challenge of producing varieties that are widely adaptive to increasing biotic and abiotic stress factors while at the same time exhibiting higher productivity and quality. The tetraploid potato is a genetically complex and heterozygous crop, which makes the introgression of desirable traits from related wild species difficult. Therefore, in order to enlarge the genetic variability of the breeding material, mutagenesis by chemical agents is frequently used (Tomlekova, 2010). Ethyl methanesulfonate (EMS) treatment is very effective in this case, as it induces high-frequency point mutations that can lead to a large

variation in numerous traits in EMS-treated plants compared to their untreated counterparts of the initial genotypes (Tomlekova et al., 2014a, b).

Genotyping DNA sequence variants in highly heterozygous polyploid species, such as potato (*Solanum tuberosum* L.), is more challenging than in diploid species, because a given gene may be represented by more than two different alleles per locus per genotype (Uitdewilligen et al., 2013). Molecular genetic markers have therefore become useful tools for estimating genetic diversity and phylogeny in plants in the most unbiased manner possible (Barone, 2004; Clegg, 1989; El-Fiki et al., 2018).

The application of molecular techniques using DNA sequence homologies and DNA polymorphisms has become a reliable approach toward the identification and establishment of genetic relatedness (Khadhair et al., 1997). This has made the main objective of crop breeding more feasible: obtaining new varieties with improved yields, quality traits, and resistance to biotic and abiotic stresses. For most domesticated crop species, various genetic resources are available, such as related species and crop wild relatives (Callow et al., 1997).

> No two plants are exactly alike. They're all different, and as a consequence, you have to know that difference.

This short quote from Barbara McClintock, a famous plant geneticist, impressively illustrates how important the individual level is when considering genetic diversity within a species or a population. To ensure their adaptation to certain environmental factors and thus preserve their ability to evolve, broad biological variety is essential. It offers plant breeders the opportunity to develop new and improved varieties with desirable characteristics. However, the ongoing genetic erosion caused by the large-scale replacement of local varieties by improved varieties increases the susceptibility of crops to diseases and thus risks global food security. Therefore, plant breeders today face the challenge of improving varieties while preserving the diversity of different traits within a species.

To address this important issue, molecular markers have become standard tools in a wide variety of plant genetic applications. The detection of polymorphisms between individuals not only allows them to be clearly distinguished, but also provides information on the degree of genetic variation within a population, which is essential for the maintenance and monitoring of genetic diversity as well. In some cases, molecular markers can also be linked to desirable traits, allowing the targeted selection of suitable individuals in breeding programs (Andersen and Lubberstedt, 2003; Philips and Vasil, 2013; Dou et al., 2021). This marker-assisted selection (MAS) increases the effectiveness in breeding and have the potential to significantly speed up the breeding process.

The development of massively parallel sequencing technologies has accelerated genome sequencing while decreasing sequencing costs. Thus, virtually any organism can be studied on a genomic scale. In the course of this, recent research has shown that it is advantageous to analyze multiple genome assemblies for one species (Della Coletta et al., 2021). Such pan-genome projects help to capture the entire diversity of a species, to link phenotypic traits to genomic positions, and to identify gene variants. Moreover, high-throughput single nucleotide polymorphism (SNP)

discovery and simultaneous genotyping in multiple DNA samples, known as genotyping by sequencing (GBS), is possible for an increasing number of organisms. Nevertheless, such innovative techniques, while important for crop improvement, are still far from practical application. Breeders primarily need simple, reliable, and robust marker systems for the unambiguous identification of varieties for cultivation, the selection of most genetically diverse crossing parents possible, and the protection of plant varieties.

Consequently, marker methods developed earlier still have their relevance. While the first molecular marker system was based on the detection of restriction fragment length polymorphisms (RFLPs) by hybridization (Botstein et al., 1980), the use of the polymerase chain reaction (PCR) quickly expanded the technological spectrum. DNA fingerprints could thus be generated by either random amplification of polymorphic DNA (RAPD; Williams et al., 1990), selective amplification of restriction fragments (amplified fragment length polymorphisms, AFLPs; Vos et al., 1995), or amplification of microsatellite loci (simple sequence repeats, SSRs; Chung and Staub, 2003). Besides the improvements that these applications bring to breeding, they also have individual disadvantages, such as the fact that some of them are unable to distinguish between homozygosity and heterozygosity, that they incur high development costs, and that their application is often time-consuming and laborious. Therefore, it is obvious to also consider genomic sequences, which are conserved and accumulated in almost all eukaryotic organisms.

## 2 Retrotransposon-Based Marker Systems Are Well Suited to Assess Genetic Diversity

With the increase in sequence data, it is becoming clear that transposable elements (TEs) are an integral part of eukaryotic genomes, sometimes constituting the majority of plant genomes (SanMiguel et al., 1996; Bennetzen et al., 2005). Originally considered as junk DNA (Ohno, 1972) that is passively accumulated and does not code for any proteins, it is now accepted that TEs make a significant contribution to genetic diversity (Feschotte et al., 2002; Biémont and Vieira, 2006).

Their ability to move within the genome generates mutations and genetic polymorphisms (McClintock, 1956; Bourque et al. 2018). They drive the evolution of genomes by facilitating the translocation of genomic sequences, the shuffling of exons, and the repair of double-strand breaks (Stapley et al., 2015). Insertions and transposition can also alter gene regulatory regions and their inactivation by host silencing mechanisms contributes to different layers of epigenetic genome regulation (Slotkin and Martienssen, 2007). Therefore, it is not surprising that rapid TE turnover and waves of TE amplification during plant evolution massively altered genomic regions through insertion and deletion of TEs (Wicker et al., 2018).

The direct link between TEs and genetic polymorphisms makes these mobile DNAs an ideal source for molecular markers. The integration of retrotransposons is

assumed to be irreversible because, unlike DNA transposons, they cannot excise themselves from their insertion sites (Finnegan, 1989). This makes them well suited as cladistic markers for phylogenetic studies based on their presence or absence, since the empty site represents the known ancestral state (Batzer et al., 1994; Nikaido et al., 1999).

Retrotransposons are the most abundant and widespread class of TEs, and due to their copy-and-paste replication mode, they are widely distributed across the plant genome. This contributes to insertion polymorphisms both within and between species (Waugh et al., 1997). The relatively high copy number of retrotransposons combined with their high mobility results in a considerable number of polymorphisms, which are a common source of genomic diversity and consequently an excellent basis for genotyping. With the sequence-specific amplification polymorphism (S-SAP), the inter-retrotransposon amplified polymorphism (IRAP), and the retrotransposon-microsatellite amplified polymorphism (REMAP), new DNA fingerprinting techniques have been developed that allow the discrimination of individuals without prior treatment of genomic DNA (Waugh et al., 1997; Kalendar et al., 1999). These marker systems rely on PCR amplification between retrotransposons or a component of flanking genomic DNA, such as microsatellites, to generate marker bands. Considering that these jumping genes are ubiquitously found in almost all eukaryotic genomes (Feschotte et al., 2002; Huang et al., 2012), retrotransposon-based marker systems are universally applicable.

## 3 SINE Retrotransposons Are Informative Molecular Markers for Plant Breeding

Due to their genome-specific distribution, short interspersed nuclear elements (SINEs) can also be used as appropriate genomic components for the detection of polymorphisms. These retrotransposons are ubiquitous in plants, where their random insertion behavior and different activity levels have led to considerable variation in amplified interspaces between individuals (Shedlock & Okada, 2000; Seibt et al., 2016; Meng et al., 2020). Several studies revealed that they are frequently observed in regions adjacent to genes (Medstrand et al., 2002; Ben-David et al., 2013; Seibt et al., 2016). SINEs are short and noncoding retrotransposons with a length of about 80–700 base pairs. They are a heterogeneous group of elements derived from noncoding RNAs, such as tRNA, 7SL RNA, and 5S RNA (Kapitonov and Jurka, 2003); while primates contain mostly 7SL RNA-like SINEs, other eukaryotes, especially plants, primarily harbor tRNA-like SINEs (Kramerov and Vassetzky, 2011).

As nonautonomous retrotransposons, their transposition is dependent on proteins encoded by an autonomous partner long interspersed nuclear elements (LINEs) and/or host components (Boeke, 1997; Okada et al., 1997; Ogiwara et al., 1999; Kajikawa & Okada, 2002; Dewannieux et al., 2003). For this reason, they are

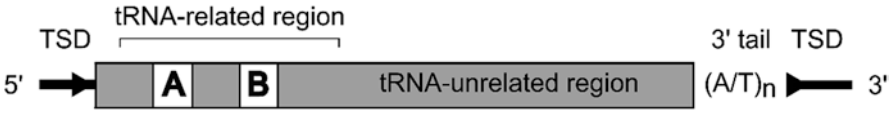



**Fig. 1** A SINE and two TSDs

usually terminated by a poly(A) stretch, a poly(T) stretch, or a simple sequence repeat at the 3′ end, as is the case for LINEs. Their descent from tRNA genes is reflected by the presence of an internal RNA polymerase III promoter within the 5′-terminus, which ensures transcriptional activity and thus their transpositional activity. The central body is tRNA-unrelated and exhibits high family specificity. In some cases, a short region within the body can be conserved in a wide range of SINEs, with otherwise unrelated tRNA- and LINE-derived parts (Luchetti and Mantovani, 2016; Seibt et al., 2020). A mechanism called target primed reverse transcription (TPRT) is responsible for the synthesis and proper integration of a new SINE copy, which is then flanked by target site duplications (TSDs) (Fig. 1).

SINEs are typically characterized by a tRNA-derived 5′-region containing the RNA polymerase III promoter motif (box A and box B), a non-tRNA-related region of mostly unknown origin, and an A/T-rich 3′-tail or simple sequence repeat. The flanking target site duplication (TSD) results from the integration of new copy.

## 4 SINEs Can Be Identified from Available Sequence Data

Through this small quantity of characteristic features, which are in most cases genome specific, SINEs were discovered rather by chance in the past. However, their systematic and targeted identification in genome data became possible through the development of a sequence-based algorithm. In the SINE-Finder program, common characteristics of known eukaryotic tRNA-derived SINEs are used to identify SINE candidates from sequence data in FASTA format (Wenke et al., 2011).

The central search pattern consists of two degenerate consensus motifs for the polymerase III promoter boxes (box A, RVTGG, and box B, GTTCRA) separated by a variable distance between 25 and 50 nucleotides. In addition, the termination site is accounted for by a poly(A) or poly(T) stretch 2 up to 500 nucleotides downstream from the box B motif as well as a target site duplication (TSD) of variable length and sequence flanking the SINE copy (reviewed in Kramenov and Vassetzky, 2005). The search algorithm of the SINE-Finder is based on a Python script that uses the following expression: pattern = ((‘TSD_region_1’, “{,40}”), (‘a_box’, “[GA][CGA]TGG”), (‘spacer_1’, “{25,50}”), (‘b_box’, “GTTC[AG] A”), (‘spacer_2’, “{20,500}”), (‘polyA’, “A{6,}|T{6,}”), (‘TSD_region_2’, “{,40}”)). Depending on the analyzed organism, a manual modification of the algorithm might be appropriate.

After running the SINE-Finder on the target sequence data, the retrieved hits must be filtered to identify high-confidence SINE candidates. The first step is the pairwise comparison of all SINE candidate hits with BLAST (Altschul et al., 1990). Subsequently, families of homologous sequences can be defined, e.g., using SiLiX-based clustering (Miele et al., 2011) experimenting with different thresholds for sequence identity and length. Clusters with less than three genomic hits are usually excluded from further analysis, as no repetitive character is recognizable. After aligning all sequences belonging to a cluster, the corresponding alignment has to be evaluated with respect to further SINE-typical features in order to exclude false-positive repetitive but SINE-unrelated sequences. This can be achieved by taking advantage of the fact that the integration of a SINE copy is accompanied by the formation of a unique TSD. While members of a SINE family exhibit high sequence conservation in the SINE body, their flanking regions are characterized by high variability (Fig. 2). In addition, the lengths of tail and TSD also vary between individual copies.

The sequence alignment of multiple SINE copies clearly shows the high identity of SINE members, while the flanking regions differ significantly with variation in tail length and individual TSDs (Fig. 2). Point mutations and indels highlight the variability within SINE sequences.

The SINE-Finder only detects those genomic sequences that match the search pattern, while others with mutations in the promoter motifs, the tail, or TSDs are not retrieved. Thus, it is essential in a further step to identify further family members that could not be detected so far due to deviations. For this purpose, after manual refinement of the alignment, a consensus sequence should be derived as query to perform a genomic search for all family members, including diversified SINE copies. Candidates are then filtered based on a minimum identity of 60% (Wenke et al.,

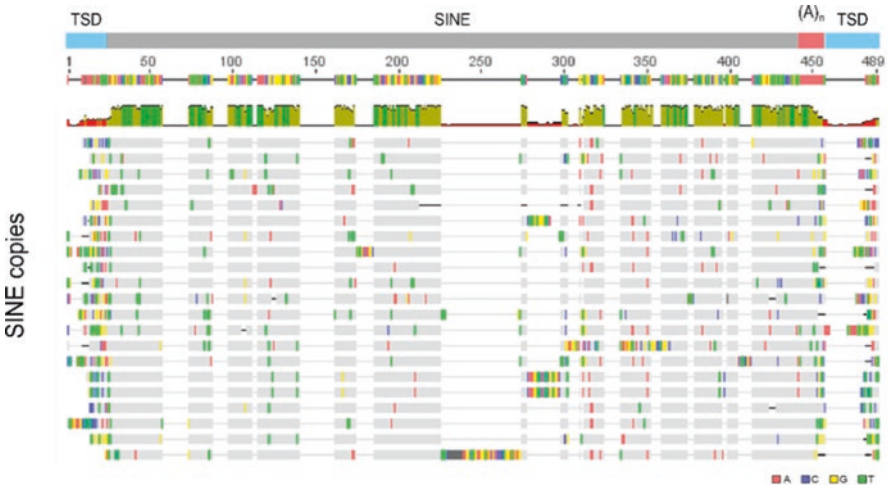



**Fig. 2** Sequence alignment of multiple SINE copies belonging to the same family

2011). For further characterization of the SINE family, only full-length copies should be considered to ensure unambiguous assignment to a SINE family and to allow comparability with other species.

## 5 Genetic Fingerprinting Applying the Inter-SINE Amplified Polymorphism (ISAP) Marker System

The principle of the ISAP method is the amplification of genomic regions between SINE copies that are close to each other. The genotype-specific SINE distribution can thus lead to fingerprint patterns that allow differentiation at the individual level. Outward-facing primers have to derive from suitable SINE families to amplify inter-SINE regions (Fig. 3). Here, the most conserved regions are suitable, and promoter box motifs should be excluded to prevent cross-hybridization between different families.

For the development of the ISAP marker system, the comprehensive characterization of the SINE landscape is indispensable. Both the number of full-length members and their conservation to the in silico consensus sequence provide information about the extent and history of transposition. On this basis, a decision can be made to select those SINE families for PCR primer derivation, which (i) have at least a moderate number of copies for good genome coverage and (ii) show sequence regions with high identity values to ensure primer annealing. In addition, the distribution pattern of the particular SINE family and the homologies within and between SINE families are crucial parameters that should be considered when designing primers.

In addition to the use of family-specific primer sets, the combination of primers from different SINE families as well as single primer reactions can enable the generation of numerous polymorphic ISAP patterns. The resulting PCR products can be separated according to their size by standard agarose gel electrophoresis or capillary electrophoresis on an automated sequencer.

The principle of the inter-SINE amplified polymorphism (ISAP) method is based on the amplification of genomic DNA between neighboring SINEs by PCR with outward-facing SINE reverse (R) and forward primers (F). PCR amplicons are separated by electrophoresis according to their size (Seibt et al., 2012).

To determine the marker information content (number of polymorphic bands), the primers have to be applied to a diverse set of genotypes. Moreover, the extension of the primer at the 5′ end by a 20mer of an arbitrary GC-rich sequence (e.g., CTG

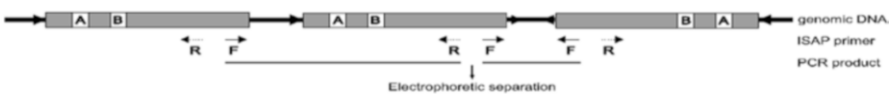



**Fig. 3** Schematic representation of ISAP reaction

ACG GGC CTA ACG GAG CG; Seibt et al., 2012) allows the application of high annealing temperature to develop more robust markers.

To analyze the resulting polymorphisms, either gel images of the ISAP patterns or electrophoregrams of capillary electrophoresis have to be processed by software that enables the handling of fingerprints. Using BioNumerics (Applied Maths NV, Belgium), a large number of samples from several ISAP experiments can be processed and stored in a database. For this purpose, the images have to be normalized in order to detect and classify the bands according to their size and count them. This enables the comparison and combination of ISAP runs and hence the calculation of pairwise similarities based on band presence and absence. Furthermore, cluster analysis could be performed for calculating dendrograms from pairwise similarity values.

The ISAP reaction has been applied to potatoes to distinguish closely related representatives, such as varieties. We assume that it can serve to distinguish the molecular profiles of EMS mutants, which were obtained from the treatment of $F_1$ hybrids obtained by crossing genotypes belonging to the crop species *Solanum tuberosum* L.

## 6 Phenotyping Bulgarian Potato Variety and Mutant Lines from the MVCRI Collection

The morphological characteristics of the analyzed varieties possessed a diverse wide range of phenotypic variation shown in Table 1 and Fig. 4.

The study reported by Tomlekova et al. (2017) included eight Bulgarian potato varieties; six of them (“Perun”, “Rozhen”, “Iverce”, “Nadezhda”, “Orfei”, “Pavelsko”) were bred in the Maritsa Vegetable Crops Research Institute (MVCRI) and two of them in the Experimental Potato Station, Samokov (“Kalina”, “Bor”).

## 7 Morphological Characteristics of Potato Mutant Lines in the Maritsa VCRI Collection

Induced mutations are a proven tool for creating desirable genetic variability in plants that leads to increased accumulation of essential minerals, synthesis of precursors of vitamins, and modified quantities and qualities of starch, proteins, and oils as well as secondary plant metabolites that play critical roles in improving human health and nutrition (Tomlekova, 2014).

In their study, Nacheva et al. (2012) reported an EMS treatment for potato genotypes performed in 2010 on a total of 534 seeds, with 100 seeds set aside as controls. The number of control seeds was 10% of the total number of treated seeds.

**Table 1** Morphological description of the Bulgarian potato variety collection

| Variety name | Bred in | Maturity | Consumer quality | Tuber characteristics | Dry matter content | Resistances | References |
|---|---|---|---|---|---|---|---|
| "Perun" | VCRI | Mid–early | For fresh consumption and French fries, very good taste, non-discolored flesh | Mid–large, round-oval tubers, yellow netlike skin, shallow eyes, light yellow flesh | High | *Globodera rostochiensis*, tuber blight, common scab | Nacheva (2004a, b) |
| "Rozhen" | VCRI | Mid–late | For fresh consumption, French fries and chips, free from discoloration after cooking | Large, long ova, red skin, shallow eyes, yellow flesh | Moderate to high | *Rhizoctoniose,* early blight, leaf blight, tuber blight, common scab | Nacheva (2004a, b) |
| "Iverce" | VCRI | Very early | For fresh consumption, very good taste, free from discoloration after cooking | Large oval tubers, yellow skin, shallow eyes, yellow flesh | Moderate to fairly low | Tuber blight, common scab | Nacheva et al. (2010) |
| "Nadezhda" | VCRI | Early | For fresh consumption, very good taste, free from discoloration after cooking | Large, long oval tubers, yellow skin, shallow eyes, light yellow flesh | Moderate | Tuber blight, common scab, field resistance to virus diseases | Nacheva et al. (2010 |
| "Orfei" | VCRI | Early | For fresh consumption, very good taste, non-discolored flesh | Mid–large, round-oval tubers, yellow netlike skin, shallow eyes, light yellow flesh | Moderate | *Globodera rostochiensis, Globodera pallid,* field resistance to tuber blight | Nacheva et al. (2011) |
| "Pavelsko" | VCRI | Mid–early | For fresh consumption and chips, free from discoloration after cooking | Mid–large, round-oval tubers, yellow netlike skin, shallow eyes, yellow flesh | Very high | *Globodera rostochiensis,* tuber blight, field resistance to virus diseases | Nacheva (2014) |
| "Kalina" | EPS | Mid–late | For fresh consumption and chips, free from discoloration after cooking | Large, round-oval tubers, yellow, sometimes netlike skin, moderately deep eyes, yellow flesh | High | Leaf blight, tuber blight, field resistance to virus diseases | Nacheva et al. (2010) |
| "Bor" | EPS | Late | For fresh consumption, very good taste, non-discolored flesh | Large, oval tubers, dark pink skin, shallow eyes, creamy white flesh | Moderate to high | Wart disease, leaf blight, tuber blight | Nacheva et al. (2010) |

Maritsa Vegetable Crops Research Institute (VCRI), Plovdiv, Experimental Potato Station (EPS), Samokov

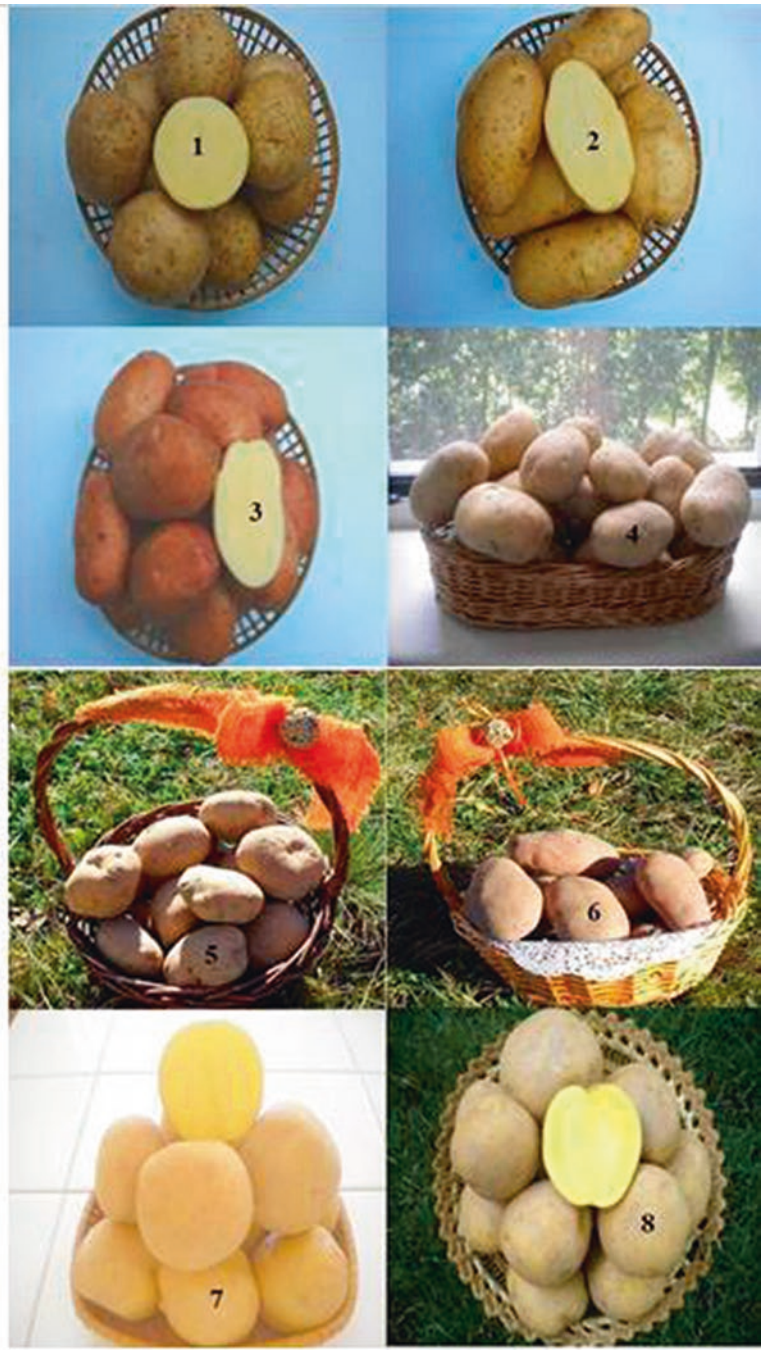



**Fig. 4** Bulgarian potato variety of Maritsa VCRI, Plovdiv, and Experimental Potato Station, Samokov. 1, "Perun"; 2, "Nadezhda"; 3, "Rozhen"; 4, "Ivertse"; 5, "Kalina"; 6, "Bor"; 7, "Orfei"; 8, "Pavelsko"

The mutant lines, controls, and parental components included in the study to describe the mutant population developed differed significantly in their morphological and economic characters (Nacheva, 2004a, b; Nacheva et al., 2012). The four groups are formed according to the origin of the genotypes.

The plant height of mutants ranged from 8 cm (M-III-1) to 72 cm (M-III-17, M-III-50, M-IV-6) and from 15 to 81 cm in the untreated controls. As compared to the parental forms, an average of 40% of the resulting mutants exhibited a positive heterosis effect for the plant height character. The average number of stems in the parental lines was 3. In mutant forms, the amplitude variation was from 1 to 7 stems.

The data showing maximum values for this character were recorded in hybrid combination I (PC 428 × PC 490) for mutants M-I-6 and M-I-9. The majority of mutant lines were late ripening, compared to the parental forms. Of the four hybrids, hybrid combination III (PC 692 × PC 490) was the earliest ripening, with an average duration of the vegetation period of 80 days, followed by hybrid combination VII (PC 757 × PC 538) at 81 days. The shortest vegetation period was recorded in mutant lines M-VII-22 (64 days), M-III-29, and M-III-32 with 65 days. The number of standard-size tubers per plant in the mutant lines of hybrid combination I ranged from 1 (M-I-20) to 14 (M-I-22), with an average number of 8.5 for the parental forms. A positive heterosis effect for this character is reported for M-I-2, M-I-9, M-I-14, M-I-16, M-I-18, M-I-21, and M-I-22, in which the number of standard tubers per plant exceeded 10. In mutants of hybrid combination III, the highest value for the character of the number of standard-size tubers per plant was recorded in M-III-17 (24 tubers), followed by M-III-25 (20 tubers). Mutant lines M-IV-6 of hybrid combination IV (PC 707 × PC 428) and M-VII-20 of hybrid combination VII (PC 757 × PC 538) were characterized by 24 standard tubers per plant. Of all mutants obtained, the highest number of standard-size tubers per plant was recorded in M-VII-9–29 tubers. The amplitude of variation of this character studied in the untreated controls in the four hybrid combinations was from 0 to 13. The number of nonstandard tubers ranged from 6 to 8 in the parental forms, from 1 to 18 in the controls, and from 0 to 30 in the mutants. The smallest number of nonstandard tubers was formed in the mutants of hybrid combination IV (six tubers on average).

The total number of tubers per plant was a minimum of 2 and a maximum of 56. In five of the mutant lines of hybrid combination I (M-I-6, M-I-14, M-I-18, M-I-21, and M-I-2, M-I-22), a greater number of tubers were recorded (over 17) in comparison with the parent with a higher value of this character (PC 428). The mutants in the other hybrid combinations were characterized by even greater variability: in hybrid combination III, from 2 (M-III-1) to 41 (M-III-1, M-III-17); in hybrid combination IV, from 3 (M-IV-2) to 27 (M-IV-10); and in hybrid combination VII, from 4 (M-VII-13) to 56 tubers (M-VII-9). The variation in the controls had a minimum value of 2 (K-I-1) and a maximum of 28 (K-III-2). The weight of standard-size tubers in a single plant in the mutant lines of hybrid combination I was 447 g on average, compared to 332 g in the parental forms, and 72 g in the control. In 60% of the mutants in this hybrid combination, there was a positive heterosis effect for the studied character. M-I-2 was characterized by a maximum value (930 g). Three of the mutant lines in hybrid combination III formed standard-size tubers of over

1000 g per plant – M-III-1, M-III-17, M-III-25, and M-III-50. A maximum value for this character was recorded in mutant M-IV-14 in hybrid combination IV (1000 g). The highest average productivity characterized the mutant lines in hybrid combination VII (596 g). In this hybrid combination, 31.3% of the mutants formed standard-size tubers of over 1000 g per plant – M-VII-7, M-VII-9, M-VII-16, M-VII-20, and M-VII-26.

As a whole, the total weight of tubers per plant followed the trend outlined in the analysis of tuber standard weight in the sense that the mutant lines with the highest standard weight of tubers are characterized by the highest value of the character total tuber weight in a single plant. The mutant lines with a total yield of more than 1000 g per plant were M-III-17, M-III-25, M-III-50, M-IV-14, M-VII-7, M-VII-9, M-VII-16, M-VII-16, and M-VII-26.

The weight of a standard tuber in mutant lines had an amplitude of variation from 20 g (M-IV-7) to 125 g (M-III-50) and in the untreated controls from 42 g (K-III-2) to 102 g (K-IV-1). The average weight of 80 g of a standard-size tuber was formed by the mutants M-I-2, M-I-27, M-III-50, M-VII-7, and M-VII-10 (Nacheva et al., 2012).

## 8 Genotyping of Bulgarian Potato Variety by Using ISAP Molecular Reactions

A study by Tomlekova et al. (2017) reported ISAP banding patterns identified with SINE reactions at Bulgarian potato varieties showing exhibited genotype variation as expected. The results of ISAP reactions with different primer combinations enable their genotyping using primer pairs SolS-IIIa-F/SolS-IIIa-R, SolS-IIIa-F/SolS-IV-F, and SolS-IIIa-F/SolS-IV-R, as revealed by distinct polymorphic profiles.

The summarized results for the number of monomorphic and pol-ymorphic fragments, as well as generated polymorphic profiles be-tween the studied Bulgarian varieties with each SINE reaction are presented in Table 2, and the electrophoretic separation results are shown in Fig. 5 with the permission of the author's team.

The profiles of the varieties were generated by the following primer pairs for the ISAP reaction: lanes 1–8 by SolS-IIIa-F/SolS-IIIa-R, lanes 10–17 by SolS-IIIa-F/SolS-IV-F, lanes 19–26 by SolS-IIIa-F/SolS-IV-R, and lanes 9, 18, and 27, DNA ladder.

**Table 2** Profiles of eight Bulgarian potato varieties by three ISAP reactions

| Primers | G | TM | PA | Fragment size of template | MB | PB |
|---|---|---|---|---|---|---|
| SolS-IIIa-F/SolS-IIIa-R | 8 | 13 | 6 | 2000/200 | 3 | 10 |
| SolS-IIIa-F/SolS-IV-F | 8 | 10 | 5 | 3000/500 | 4 | 6 |
| SolS-IIIa-F/SolS-IV-R | 8 | 15 | 6 | 2000/200 | 2 | 13 |

Tomlekova et al. (2017)

**G**, number of analyzed genotypes; **TM**, total number of amplified fragments; **PA**, polymorphic profiles; **MB**, number of monomorphic bands; **PB**, number of polymorphic bands

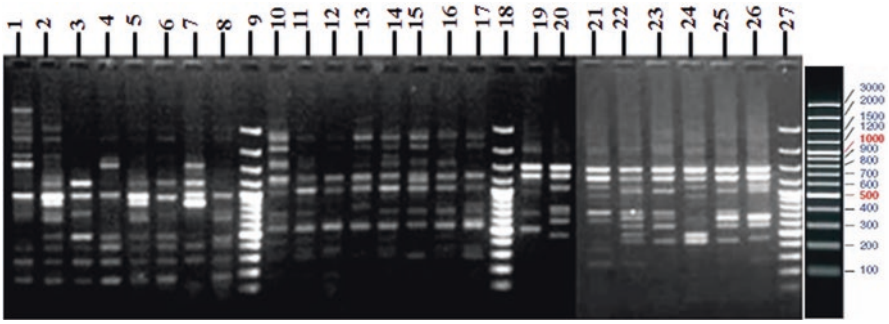



**Fig. 5** ISAP profiles of potato varieties obtained by the three primer pairs SolS-IIIa-F/SolS-IIIa-R (1–8), SolS-IIIa-F/SolS-IV-F (10–17), and SolS-IIIa-F/SolS-IV-R (19–26). Lanes on the gel: "Iverce" (1, 10, 19), "Orfei" (2, 11, 20), "Nadezhda" (3, 12, 21), "Perun" (4, 13, 22), "Pavelsko" (5, 14, 23), "Rozhen" (6, 15, 24), "Kalina" (7, 16, 25), "Bor" (8, 17, 26), ladders (9, 18, 27) (modified by Tomlekova et al., 2017)

**Table 3** Profiles of Bulgarian potato varieties generated by ISAP reactions with primer pairs which showed the best results

| Primer combinations | ISAP profiles | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Iverce | Orfei | Nadezhda | Perun | Pavelsko | Rozhen | Kalina | Bor |
| SolS-IIIa-F/ SolS-IIIa-R | 1 | 2 | 3 | 4 | 2 | 3 | 5 | 6 |
| SolS-IIIa-F/ SolS-IV-F | 1 | 1 | 2 | 3 | 1 | 4 | 1 | 5 |
| SolS-IIIa-F/ SolS-IV-R | 1 | 2 | 3 | 4 | 2 | 5 | 2 | 6 |

Modified by Tomlekova et al. (2017)
Different numbers in this table from 1 to 6 correspond to the six different profiles generated by the three reactions with the most efficient primers

Three ISAP reactions presenting the best results have generated a total of 38 fragments, of which 9 were monomorphic and 29 polymorphic (Table 2) (Tomlekova et al., 2017).

Table 3 presents a distribution of the generated amplification profiles in the eight Bulgarian potato varieties, with the three most informative reactions, as the authors reported six identified varieties with unique profiles, but two of them have monomorphic profiles and were studied with other SINE reactions (SolS-II-F/ SolS-II-R), while also amplified monomorphic profiles in others, but polymorphic with other varieties (Tomlekova et al., 2017). The variety "Orfei" was bred by individual clone selection in the hybrid progeny of the line ML 83.508/4 and the variety "Koretta". The variety "Pavelsko" was bred by individual clone selection in the hybrid progeny of "Britta" and line ML 75.25/18yN. The two varieties did not have the same origin.

The reactions carried out by the ISAP reaction with the primer pair SolS-IV-F/SolS-IV-R amplified monomorphic profiles of the eight studied Bulgarian potato varieties.

These results were presented by part of the author's team (Tomlekova et al., 2017) and served as a basis for the study of $M_1V_8$ mutant potato lines.

# 9 Genotyping Bulgarian Mutant Lines Using ISAP Molecular Technique: Case Study

The objective of this chapter is to demonstrate the discriminatory power of ISAP markers by genotyping EMS advanced ($M_1V_8$) mutant potato lines developed at the Maritsa Vegetable Crops Research Institute in Bulgaria.

## *9.1 Material and Methods*

Based on the above-described morphological indicators, productivity of mutant lines (Nacheva et al., 2012), and biochemical analysis (Tomlekova, unpublished data), a selection of the most promising mutants was made. The most reliable genotypes were only preserved and grown, which is the subject of the genotyping by the ISAP technique. The genotypes' origin of the parents, controls, and $M_1V_8$ mutant lines included in this study are demonstrated in Table 4.

**Table 4** Parental genotypes, mutant lines, and controls ($F_1$ hybrids): origin, groups, and number of mutants

| Mutant group (family no) | Hybrid combination | Origin | $M_1V_8$ mutant lines no) | $M_1V_8$ mutant lines | Controls |
|---|---|---|---|---|---|
| M-I | PC 428 × PC 490 | "Nadezhda" × I 75.127 N | 3 | M-I-3, M-I-8, M-I-17 | |
| M-III | PC 692 × PC 490 | "Orlik" × I 75.127 N | 6 | M-III-8, M-III-9, M-III-25, M-III-30, M-III-48, M-III-50 | K-III-2 |
| M-IV | PC 707 × PC 428 | "Olza" × "Nadezhda" | 3 | M-IV-14, M-IV-15, M-IV-17 | K-IV-3 |
| M-VII | PC 757 × PC 538 | E 402 × "Karlena" | 4 | M-VII-7, M-VII-9, M-VII-19, M-VII-27 | K-VII-4 |

Modified by Nacheva et al. (2012)

## 9.2 Molecular Method

Genomic DNA was isolated from young leaves by using the CTAB protocol, including RNaseA treatment. DNA quality was determined by Lambda DNA (Thermo Scientific Life Sciences, cat. no. SD0011, Lithuania), on a 1% LE agarose gels (Lonza, cat. no. 50004, USA) with ethidium bromide (VWR International, cat. no. 4007–07, Austria). Buffers (TAE and TBE) were prepared by Tris base (Sigma-Aldrich, cat. no. RDD008, USA), acetic acid (VWR International, cat. no. 20104.298, Germany), and boric acid (Sigma Aldrich, cat. no. B0252, Germany), respectively, and EDTA (Sigma Aldrich, cat. no. E-5134, Germany). The 20 μL reaction mixture consisted of 10 ng DNA template, 1× Green PCR buffer (10× Green buffer, Thermo Scientific, cat. no. B71, Lithuania), 0.2 mM dNTPs (Thermo Scientific, cat. no. R0192, Lithuania), 0.15 μL each primer (Table 2), 0.1 mg/mL BSA (Sigma-Aldrich, cat. no. A6003, USA), and 0.5 U DreamTaq DNA polymerase (Thermo Scientific, cat. no. EP0702, Lithuania) (Seibt et al., 2012; Tomlekova et al., 2017).

ISAP amplification reactions with primers from SolS-IIIa and SolS-IV families were performed using the following conditions: initial denaturation for 5 min at 93 °C, followed by 30 cycles with 20 s at 93 °C, 30 s at 52 °C, and 120 s at 72 °C, and a final elongation of 5 min at 72 °C. The primers and PCR program were according to Seibt et al. (2012). The resulting amplicons were separated on 2% LE agarose gels, prestained with ethidium bromide, in standard 1× TAE buffer. The comparison of the length of the fragments was done with DNA Ladder Gene Ruler 100 bp Plus DNA (Thermo Scientific, cat. no. SM0321, Lithuania).

The visualization of the ISAP banding patterns was performed using the gel documentation system AZURE Biosystem C600.

To compile matrices (the total number of amplified fragments in all the studied genotypes), only reproducible fragments with defined lengths were taken into account, e.g., for primer combinations SolS-IIIa-F/SolS-IIIa-R of 3200 to 300 bp, SolS-IV-F/SolS-IV-R of 2500 bp to 280 bp, and multiplex reactions with lengths, SolS-IIIa-F/SolS-IV-R, of 3000 to 480 bp.

The profiles amplified by each of the reactions included the lengths of the amplified fragments in all analyzed genotypes compared to the DNA marker used. The different intensity of the fragments was not taken into account. The intensity was involved only in the selection of fragments and assessment of their reproducibility. Only reproducible fragments of certain lengths for each reaction were taken into account for the compilation of the profiles.

## 9.3 Statistical Method

The amplified fragments were scored as present (1) or absent (0) for the three most informative reactions to generate a binary data matrix, which was analyzed using SPSS Statistics software (IBM Corp. Released, 2019). This is a graphical method for visualizing the proximity and difference between the units.

The graphics show the distribution of the mutant lines in each group, including their parental genotypes and controls based on the three SINE reactions. These data complement the information obtained from the molecular profiles and confirm the clear differentiation between the groups.

## *9.4 Results and Discussion*

The results from the analyses of mutant lines from the collection of the MVCRI with the two most informative families (SolS-IIIa and SolS-IV) conducted as single and multiplex reactions demonstrated a high level of polymorphism in the analyzed mutant potato lines. Three representative combinations of primers proposed by Seibt et al. (2012) were selected in the analyzed mutant potato lines, due to their high proportion of polymorphic bands: SolS-IIIa-F/SolS IIIa-R, SolS-IV-F/SolS-IV-R, and SolS-IIIa-F/SolS-IV-R (Table 5). Reaction with SolS-IIIa-F/SolS-IIIa-R resulted in the generation of 20 fragments representing a matrix of all templates, of which 17 were polymorphic. The reaction SolS-IV-F/SolS-IV-R generated a total of 12 fragments – 7 polymorphic and 5 monomorphic. The reaction SolS-IIIa-F/SolS-IV-R generated a total of 18 fragments. These results are shown in Table 5.

The three reactions presenting the best results of the applied ISAP method in the mutant potato collection amplified a total of 50 fragments, of which 11 were monomorphic and 39 were polymorphic. The monomorphic and polymorphic ISAP profiles composed by the amplified fragments were assessed. All the parent components of each group available for the present study were distinguished between them accordingly. Some of the mutant genotypes and controls were also distinguished in the groups (Table 6).

The ISAP reaction with the primer pair SolS-IV-F/SolS-IV-R provided the best resolution and generated 12 profiles. Genotypes PC428, PC490, PC538, PC757, M-I-8, K-III-2, and K-VII-4 amplified unique profiles, different from all the others. The rest of the profiles included the studied genotypes grouped by two and three representatives per profile.

Single-family ISAP assays SolS-IIIa and SolS-IV in the studied potato collection amplified different patterns with a different number of bands. Primer pairs from SolS-SINE families IIIa and IV led to amplification of a number of fragments with

**Table 5** ISAP amplifications of potato mutant lines

| ISAP primers | G | TM | PA | Fragment size of template | MB | PB |
|---|---|---|---|---|---|---|
| SolS-IIIa-F/SolS-IIIa-R | 24 | 20 | 15 | 300/3200 | 3 | 17 |
| SolS-IV-F/SolS-IV-R | 24 | 12 | 12 | 280/2500 | 5 | 7 |
| SolS-IIIa-F/SolS-IV-R | 24 | 18 | 9 | 480/3000 | 3 | 15 |

**G**, number of analyzed genotypes; **TM**, total number of matrix bands; **PA**, polymorphic profiles; **MB**, number of monomorphic bands; **PB**, number of polymorphic bands

**Table 6** Profiles of mutant lines of potato generated by ISAP reactions with primer pairs which showed the best results

| ISAP reactions with primer combinations | PC 428 | PC 490 | M-I-3 | M-I-8 | M-I-17 | M-III-8 | M-III-9 | M-III-25 | M-III-30 | M-III-48 | M-III-50 | K-III-2 | M-IV-14 | M-IV-15 | M-IV-17 | K-IV-3 | PC707 | PC538 | PC757 | M-VII-7 | M-VII-9 | M-VII-19 | M-VII-27 | K-VII-4 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SolS-IIIa-F/ SolS-IIIa-R | 1 | 2 | 3 | 3 | 3 | 4 | 5 | 5 | 4 | 6 | 6 | 6 | 7 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 13 | 13 | 14 | 15 |
| SolS-IV-F/ SolS-IV-R | 1 | 2 | 3 | 4 | 5 | 3 | 5 | 5 | 3 | 6 | 6 | 7 | 8 | 8 | 8 | 8 | 8 | 9 | 10 | 11 | 11 | 11 | 11 | 12 |
| SolS-IIIa-F/ SolS-IV-R | 1 | 2 | 3 | 4 | 4 | 3 | 5 | 5 | 5 | 6 | 6 | 6 | 4 | 3 | 3 | 3 | 6 | 7 | 8 | 9 | 9 | 9 | 9 | 9 |

Different numbers in this table from 1 to 15 correspond to the different profiles generated

suitable length and were included in multiplex reaction combining forward and reverse primers from these families.

With all the three selected ISAP reactions, the parental genotypes, available for this study, generated polymorphic profiles when compared to each other within the groups as well as between groups (Table 6).

The amplified fragments by ISAP reaction with primer pair SolS-IIIa-F/SolS-IIIa-R led to the construction of a matrix including 20 fragments of the matrix with lengths from 300 bp to 3200 bp. The ISAP with the SolS-IIIa family conducted in the parent genotypes of the first mutant group, PC 428 and PC 490, led to distinction of their amplification profiles by seven polymorphic fragments. The same PC 428, one of the parent components also of the fourth group, compared to PC 707, the other parent genotype of this group, differed by eight polymorphic fragments. The parental genotypes of the seventh mutant group, PC 538 and PC 757, also generated different profiles between them, amplifying five different polymorphic fragments (Fig. 6).

The matrix of ISAP with SolS-IV was composed of 12 amplified fragments with length from 280 bp to 2500 bp. With the SolS-IV-F/SolS-IV-R reaction, the profiles of PC 428 and PC 490 differed by three fragments. PC 538 and PC 757 were composed of the same number of amplified fragments, and two of them differed by fragment lengths. The parental genotype of the fourth mutant group, PC 707, differed from the amplification profiles of the other parental genotypes by generating a smaller number of fragments that were amplified at different lengths in the template (Fig. 6).

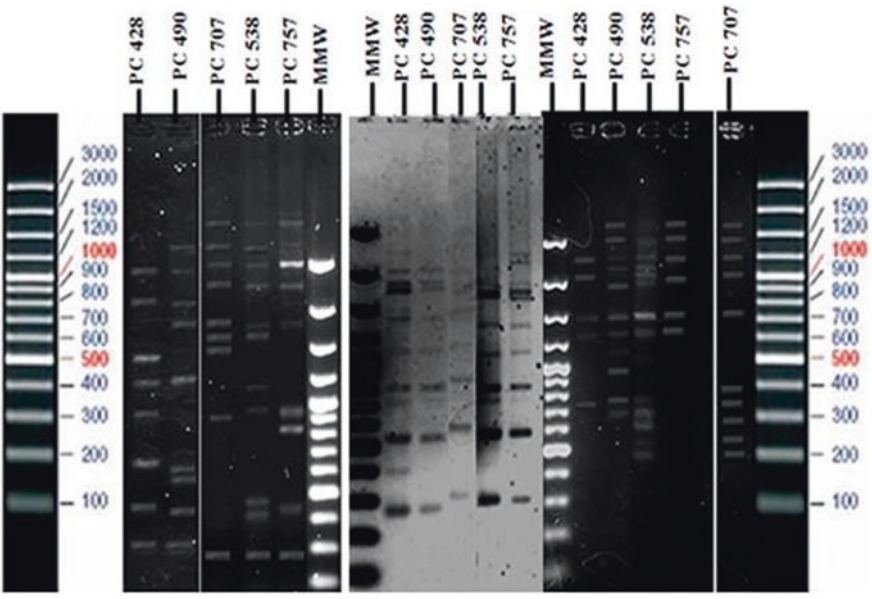



**Fig. 6** Amplified profiles of parental genotypes by ISAP reactions
**Lanes 1–5 (SolS-IIIa-F/SolS-IIIa-R); lanes 8–12 (SolS-IV-F/SolS-IV-R), lanes 14–18 (SolS-IIIa-F/SolS-IV-R), lanes 6, 7, and 13, DNA ladder (100 bp Gene Ruler)**

The matrix of ISAP with representatives of two SolS-IIIa and SolS-IV was composed of 18 amplified fragments with lengths from 480 bp to 3000 bp. The profile of the parent variety PC 428 was polymorphic with PC 490 and also with PC 707. They differed by six amplified fragments. Parental components PC 757 and PC 538 differed by five fragments with length in the range of 500–800 bp (Fig. 6).

The amplified ISAP profiles of the other studied genotypes, mutants, and controls are shown in Fig. 7.

The SolS-IIIa-F/SolS-IIIa-R reaction generated ten polymorphic profiles in the 19 analyzed mutant lines and controls. According to the number of amplified fragments and their lengths with this reaction, the five parental genotypes were also amplified with polymorphic profiles that differed from the mutants and controls.

The profiles described below correspond to the number of lanes in Fig. 7. The results obtained through generating the amplification profiles of the mutant lines with the three reactions performed are shown in Table 6.

The profile in Fig. 7, lane 1, included three mutant lines from the first group (M-I-3, M-I-8, M-I-17), the lane 2 profile included two mutant lines (M-III-8, M-III-30) (Fig. 7), and the profile on lane 3 included three mutants (M-III-48, M-III-50, K-III-2) (Fig. 7, lane 3). The profile in Fig. 7, lane 4, included two genotypes (M-III-9, M-III-25) from the third group. The profile in Fig. 7, lane 5, included one genotype (M-IV-17) that was polymorphic with all the others studied with this reaction. The profile of three mutant lines of the seventh group (M-VII-7, M-VII-9, M-VII-19), which was monomorphic to each other, was polymorphic with the other mutant and parental lines (Fig. 6, lane 6). Two genotypes generated unique profiles (M-VII-27) (K-VII-4) (Fig. 7, lanes 7 and 8). The profile included the control

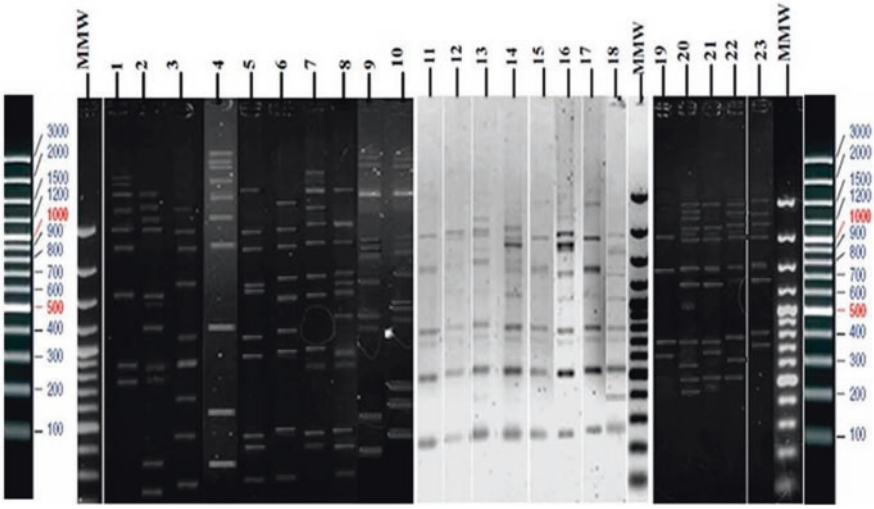



**Fig. 7** Amplified polymorphic profiles in mutant potato lines by ISAP reactions **SolS-IIIa-F/SolS-IIIa-R (lanes 1–10), SolS-IV-F/SolS-IV-R (lanes 11–18), SolS-IIIa-F/SolS-IV-R (lanes 19–23)**

(K-IV-3) of the fourth group with a SolS-IIIa-F/SolS-IIIa-R reaction (Fig. 7, lane 9). This reaction identified the controls of the fourth and seventh mutant groups with different polymorphic profiles (Fig. 7, lanes 8 and 9), and the control of the third mutant group amplified an identical profile with two of the mutant lines from the same group (M-III-48, M-III-50) (Fig. 7, lane 3). Among the mutant lines, a unique profile was amplified in genotype M-VII-27, polymorphic with the rest of the mutants, and a control (Fig. 7, lane 10). The parental genotypes in this group also amplified unique profiles (Fig. 6). Another mutant line M-IV-17 generated a unique profile, and the ISAP amplification profile of the other genotypes of this group (M-IV-14, M-IV-15) was represented by lane 10 in Fig. 7, and their profiles differed from the two parental genotypes of this group (Fig. 6).

The profiles in Fig. 7, lanes 11 to 18, showed the characteristic polymorphic profiles of mutant lines amplified with the SolS-IV-F/SolS-IV-R ISAP reaction. A total of seven profiles were generated among the mutant lines, and similar to the first reaction (SolS-IIIa-F/SolS-IIIa-R), the control of the seventh group (K-VII-4) amplified an individual profile (Fig. 7, lane 17), which differed with their parental genotypes (PC 538 and PC 757) (Fig. 6). The other genotypes studied (M-VII-7, M-VII-9, M-VII-19, M-VII-27) from this group have a common monomorphic amplification profile (Fig. 7, lane 16), but polymorphic compared to the others. The profiles of the two parental genotypes also differed (Fig. 6). Profile no. 11 shown in Fig. 7 is typical for M-I-3, M-III-8, and M-III-30. A different profile was generated in the mutant lines M-I-17, M-III-9, M-III-25 (Fig. 7, lane 13). The parental genotypes differed both from each other and compared to mutant lines in the group. A profile included one representative from the first group (M-I-8) (Fig. 7, lane 12) and generated an individual profile. An amplified profile included two mutant lines of the third group (M-III-48, M-III-50) (Fig. 7, lane 14), and the ISAP profile of the control line of the group (K-III-2) was shown (Fig. 7, lane 18). All mutant lines of the fourth group of genotypes amplified a common profile shown in Fig. 7, lane 15. The profile was monomorphic with the control from this group and one parent genotype (PC707) but polymorphic with another parental line (PC428).

With the third informative ISAP reaction SolS-IIIa-F/SolS-IV-R, five polymorphic profiles were amplified among the studied genotypes, and the profile in Fig. 7, lane 19, included mutant lines (M-I-3, M-III-8, M-IV-15, M-IV-17, K-IV-3), all with different origins. Some profiles of mutants from different groups, shown with the same numbers in Table 6, were monomorphic, which we attribute to a random coincidence of amplicons of the same length, constituting profiles of different mutant groups. This is a random resemblance of a profile obtained from a reaction. To avoid such random similarities, we conducted a mathematical analysis with SPSS Statistics, a program that, based on a matrix of three highly polymorphic reactions, comprising the information for monomorphic and polymorphic profiles from all conducted reactions in the study gives the distribution of genotypes within each group (see Fig. 8).

More ISAP reactions performed in this study can differentiate all the five mutants included in profile no. 19.

Two mutant lines of the first group (M-I-8 and M-I-17) were shown in Fig. 7, lane 20. They amplified seven different length fragments compared to parental genotypes. The profile of the parental genotype PC490 was polymorphic and compared to the representatives of the third mutant group, shown in lanes 21 and 22. The grouping is demonstrated in Fig. 8b.

The other parental genotype of third group was the PC692 line. During the reproduction of the plant material, it was lost and is not available in the collection, respectively for the study. The origin of this genotype is "Orlik" – a Poland variety who has valuable economic qualities, including resistances to bacterial, fungal, and viral diseases (https://www.europotato.org/varieties/view/Orlik%20%281989%29-E).

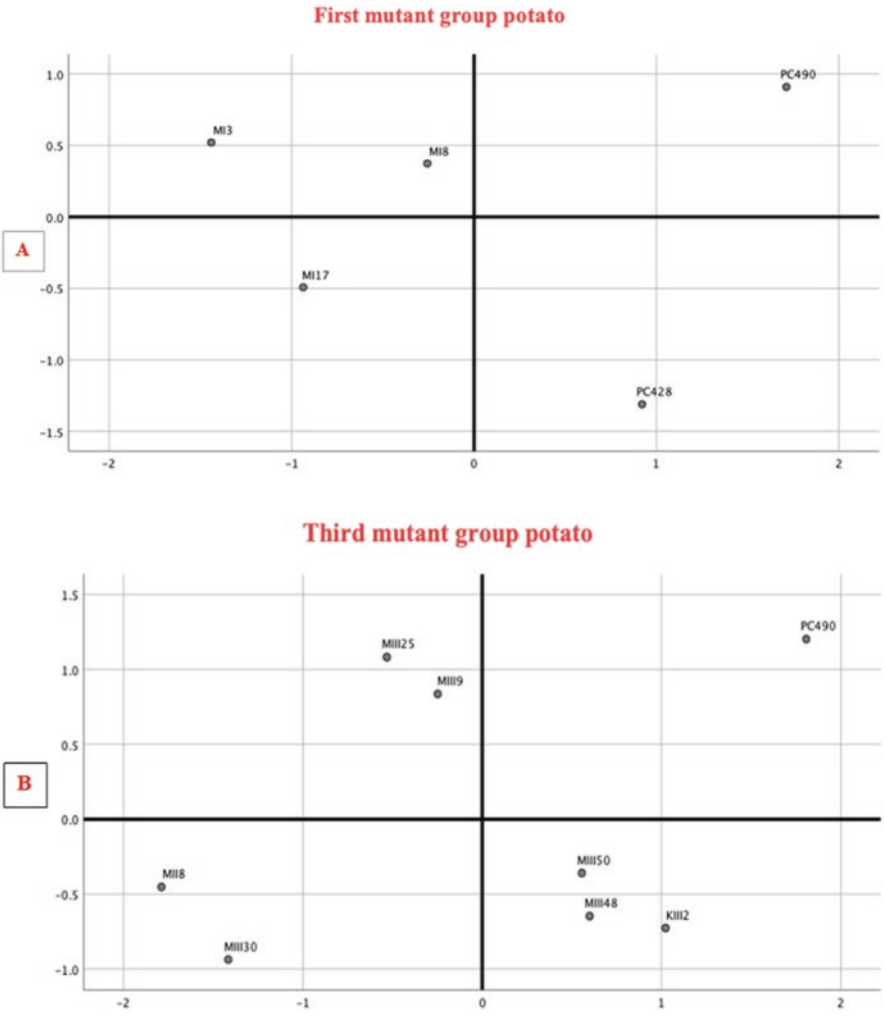



**Fig. 8** Result of multidimensional scaling of mutant potato groups

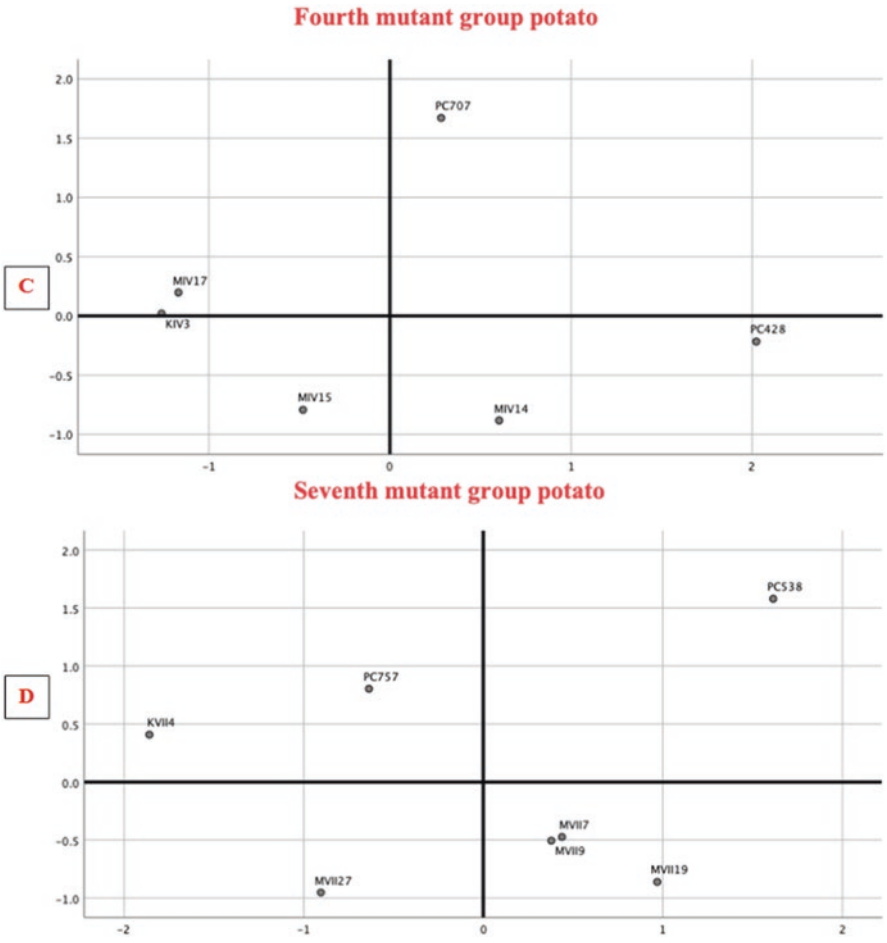



**Fig. 8** (continued)

The origin of the parental genotypes of the fourth mutant group is the Bulgarian variety "Nadezhda" (PC428) and the Poland variety "Olza" (PC707). Morphological characteristics on "Nadezhda" are presented in Table 1, and the Poland genotype "Olza" is characterized by a high yield potential and other important economical qualities, including resistance to fungal, bacterial, viral, and pest diseases (https://www.europotato.org/varieties/view/Olza-E). The amplified profiles of the mutant lines of the seventh group generated mainly long fragments with a length of 1200 bp to 3000 bp. The parental genotypes amplified different profiles. The origin of one of the parents was related to a German variety "Karlena", which characterized early maturity, medium yield potential, high to very high dry matter content, and other economically important traits, including resistance to fungal, bacterial, viral, and pest diseases (https://www.europotato.org/varieties/view/Karlena-E).

The information presented for the varieties “Orlik”, “Olza”, and “Karlena” is taken from the European cultivated potato database (https://www.europotato.org/).

Cluster analysis of the profiles obtained from the four primers of the ISAP reaction confirmed the expected genealogy of these potato genotypes according to the reported origin of the germplasm (Fig. 8). From the graphics the relative position of the genotypes to each other in a group and the number and composition of clusters can be assessed.

Nowadays, classic breeding is proving to be an insufficient model in breeding programs. In order to classify valuable accessions, the use of modern biotechnological methods is inevitable. When identifying varieties and mutant lines, molecular markers prove to be a valuable resource in combination with phenotypic traits. Existing molecular marker systems, especially those affecting highly variable regions – microsatellites and retrotransposons in the genome – can lead to genotyping with specific DNA profiles and further identification.

Their use in the present study of ISAP genotyping is based on the detection of SINE presence/absence at a particular locus (Schmidt et al., 1998).

SINEs are not only components of the eukaryotic genome, but also have an important role in genomic organization and gene evolution. According to Seibt et al. (2012), SINE SolS-IIIa and SolS-IV are the most common in the potato genome, indicating that ISAP reactions with these primers will have the greatest potential for genotyping and identifying potato genotypes. The study by Tomlekova et al. (2017) conducted in the Bulgarian varieties and the mutant potato lines confirms this statement, as only two genotypes could not identify among the analyzed eight varieties. We reported polymorphism for 16 mutant lines compared to their parental genotypes, their controls, and between them – a mutant with mutant. According to Seibt et al. (2012), a large number of amplifying fragments were identified with primer combinations SolS-II, IIIa, and IV, but only a small number with SolS-VI and VII.

Diekmann et al. (2017) analyzed 185 cultivated potato (*S. tuberosum* L.) accessions with the three most informative reactions reported by Seibt et al. (2012) and determined 115 distinguishable ISAP fragments.

In studies by Sormin et al. (2021) in 22 *Cucumis melo* L. accessions, ISAP primers described by Seibt et al. (2012) from potatoes were used in the study and showed a high level of polymorphism. SolS-IIIa-F generated five fragments and showed 60% polymorphism. SolS-IV-R resulted in the generation of eight fragments and showed a 100% polymorphism, which did not coincide with this polymorphism that reported 100% in the constructed melon primers.

In a study using the ISAP technique performed by Tomlekova et al. (2017) (unpublished result) on mutant pepper lines, the most successful profiles were amplified with SolS-II-F/SolS-II-R and SolS-V-F/SolS-V-R primer pairs.

Aziz et al. (2020) reported three pairs of primers – SolS-Ib-F/R, SolS-IIIb-F/R, and SolS-IIIa-F/R – used in tomato, as single-family and multiplex combinations among them and with other informative reactions such as SolS-Ia-F/R and SolS-II--F/R. These families showed the best results from the ISAP reactions in tomato varieties. The primer pairs SolS-IIIa-F/SolS-IIIa-R, SolS-IV-F/SolS-IV-R, and

SolS-IIIa-F/SolS-IV-R used for ISAP amplifications in our study allowed the generation of profiles with the most polymorphic fragments. The richest profiles were generated with SolS-IIIa-F/SolS-IV-F. The most easily distinguishable were the profiles generated by the pair SolS-IV-F/SolS-IV-R. The results obtained in the present study were consistent with the research studies of German genotypes published by Seibt et al. (2012).

The amplification of specific polymorphic profiles and the genotyping of potato accessions to be applied at an early stage of plant development allow accelerating the selection process. Induced mutagenesis leads to the activation of mobile genetic elements in plants, which makes them a suitable tool for the study of newly generated mutant forms. The DNA sequence of a gene could include a transposable element, in particular a SINE(s) (Schmidt et al., 1998). With this property, they are a suitable tool for the study of induced mutants.

## 10 Conclusions

The study underlines the potential and accuracy of the ISAP technique to perform precise genotyping of representatives of *Solanum tuberosum* L. and identify closely related potato genotypes.

The best resolution was obtained by the ISAP method with the SINE family SolS-IV.

Of all the reactions performed, five mutant/control lines (M-I-8, M-IV-17, M-VII-27, K-IV-3, K-VII-4) were molecularly identified by unique profiles. Polymorphism between parental genotypes was proven with the three SINE reactions performed.

The present study can serve as a basis for further research on potato breeding. The combination of morphological with molecular studies data, high productivity of genotypes, is of great importance in order to register new mutant variety/varieties.

**Acknowledgments** This research was funded by the International Atomic Energy Agency, grant numbers BUL/5/016 and RER/5/024.

**Conflicts of Interest** The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

**Author Contributions** Conceptualization, N.T.; methodology, K.S., N.T., and B.W.; growing plant material, E.N.; breeding accessions for analysis, E.N. and N.T.; molecular analysis, S.A. and N.T.; interpretation of results, N.T. and S.A.; writing–original draft preparation, S.A., B.W., N.T., K.S., and E.N.; and writing–review and editing, N.T. All authors have read and agreed to the published version of the manuscript.

## References


Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology, 215*(3), 403–410.

Andersen, J. R., & Lübberstedt, T. (2003). Functional markers in plants. *Trends in Plant Science, 8*, 554–560.

Aziz, S., Tomlekova, N., & Ganeva, D. (2020). Adaptation of ISAP technique for genotyping tomato. Scientific research of the Union of Scientists in Bulgaria – Plovdiv, series B. *Natural Sciences and Humanities, XX*, 20–26.

Barone, A. (2004). Molecular marker-assisted selection for potato breeding. *American Journal of Potato Research, 81*, 111–117.

Batzer, M. A., Stoneking, M., Alegria-Hartman, M., Bazan, H., Kass, D. H., Shaikh, T. H., Novick, G. E., Ioannou, P. A., Scheer, W. D., & Herrera, R. J. (1994). African origin of human-specific polymorphic Alu insertions. *Proceedings of the National Academy of Sciences, 91*(25), 12288–12292.

Ben-David, S., Yaakov, B., & Kashkush, K. (2013). Genome-wide analysis of short interspersed nuclear elements SINEs revealed high sequence conservation, gene association and retrotranspositional activity in wheat. *Plant Journal, 76*(2), 201–210.

Bennetzen, J.L., Ma, J., .Devos K.M. (2005). Mechanisms of recent genome size variation in flowering plants. Annals of Botany, 95(1): 127–132.

Biémont, C., & Vieira, C. (2006). Junk DNA as an evolutionary force. *Nature, 443*(7111), 521–524.

Boeke, J. D. (1997). LINEs and Alus - the polyA connection. *Nature Genetics, 16*(1), 6–7.

Botstein, D., White, R. L., Skolnick, M., & Davis, R. W. (1980). Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *American Journal of Human Genetics, 32*(3), 314–331.

Bourque, G., Burns, K. H., Gehring, M., Gorbunova, V., Seluanov, A., Hammell, M., Imbeault, M., Izsvák, Z., Levin, H. L., Macfarlan, T. S., Mager, D., & Feschotte, C. (2018). Ten things you should know about transposable elements. *Genome Biology, 19*(1), 1–12.

Callow, J. A., Ford-Lloyd, B. V., & Newbury, H. J. (1997). *Biotechnology and plant genetic resources: Conservation and use/edited by JA Callow, BV Ford-Lloyd and HJ Newbury* (Biotechnology in agriculture series; 19) (308 p). CAB.

Chung, S. M., & Staub, J. E. (2003). The development and evaluation of consensus chloroplast primer pairs that possess highly variable sequence regions in a diverse array of plant taxa. *Theoretical and Applied Genetics, 107*(4), 757–767.

Clegg, M. T. (1989). Molecular diversity in plant populations, in plant population genetics, breeding and genetic resources. In A. H. D. Brown, M. T. Clegg, A. L. Kahler, & B. S. Weir (Eds.), *Plant population genetics, breeding, and genetic resources* (pp. 98–115). Sinauer Association Inc.

De Boer, J. M., Borm, T. J., Jesse, T., Brugmans, B., Tang, X., Bryan, G. J., Bakker, H. J., van Eck, H. J., & Visser, R. G. (2011). A hybrid BAC physical map of potato: A framework for sequencing a heterozygous genome. *BMC Genomics, 12*(1), 1–23.

Della Coletta, R., Qiu, Y., Ou, S., Hufford, M. B., & Hirsch, C. N. (2021). How the pan-genome is changing crop genomics and improvement. *Genome Biology, 22*(1), 1–19.

Dewannieux, M., Esnault, C., & Heidmann, T. (2003). LINE-mediated retrotransposition of marked Alu sequences. *Nature Genetics, 35*(1), 41–48.

Diekmann, K., Seibt, K. M., Muders, K., Wenke, T., Junghans, H., Schmidt, T., & Dehmer, K. J. (2017). Diversity studies in genetic resources of *solanum spp*. (section *Petota*) by comparative application of ISAP markers. *Genetic Resources and Crop Evolution, 64*(8), 1937–1953.

Dou, Y., Xia, W., Mason, A. S., Huang, D., Sun, X., Fan, H., & Xiao, Y. (2021). Developing functional markers for vitamin E biosynthesis in oil palm. *PLoS One, 16*(11), e0259684.

El-Fiki, A., Adam, Z., Mohamed, T., Sobieh, S., & Salah, A. (2018). Molecular diversity analysis of two *in vitro* and irradiated potato varieties expressed by random amplified polymorphic DNA. *Notulae Scientia Biologicae, 10*(1), 45–51.

Feschotte, C., Jiang, N., & Wessler, S. R. (2002). Plant transposable elements: Where genetics meets genomics. *Nature Reviews Genetics, 3*(5), 329–341.

Finnegan, D. J. (1989). Eukaryotic transposable elements and genome evolution. *Trends in Genetics, 5*, 103–107.

Flinn, B., Rothwell, C., Griffiths, R., Lague, M., DeKoeyer, D., Sardana, R., Audy, P., Goyer, C., Li, X. O., Wang-Pruski, G., & Regan, S. (2005). Potato expressed sequence tag generation and analysis using standard and unique cDNA libraries. *Plant Molecular Biology, 59*, 407–433.

Huang, C. R. L., Burns, K. H., & Boeke, J. D. (2012). Active transposition in genomes. *Annual Review of Genetics, 46*, 651–675.

IBM Corp. Released (2019). IBM SPSS statistics for windows, version 26.0. Armonk, NY: IBM Corp.

Kajikawa, M., & Okada, N. (2002). LINEs mobilize SINEs in the eel through a shared 3′ sequence. *Cell, 111*(3), 433–444.

Kalendar, R., Grob, T., Regina, M., Suoniemi, A., & Schulman, A. (1999). IRAP and REMAP: Two new retrotransposon-based DNA fingerprinting techniques. *Theoretical and Applied Genetics, 98*(5), 704–711.

Kapitonov, V. V., & Jurka, J. (2003). A novel class of SINE elements derived from 5S rRNA. *Molecular Biology and Evolution, 20*(5), 694–702.

Khadhair, A. H., Hiruki, C., Hwang, S. F., & Wang, K. (1997). Molecular identification and relatedness of potato witches - broom phytoplasma isolates from four potato cultivars. *Microbiological Research, 152*, 281–286.

Kramerov, D. A., & Vassetzky, N. S. (2005). Short retroposons in eukaryotic genomes. *International Review of Cytology, 247*, 165–221.

Kramerov, D. A., & Vassetzky, N. S. (2011). Origin and evolution of SINEs in eukaryotic genomes. *Heredity, 107*(6), 487–495.

Lara-Cabrera, S., & Spooner, D. (2004). Taxonomy of north and central American diploid wild potato (*solanum sect. Petota*) species: AFLP data. *Plant Systematics and Evolution, 248*, 129–142.

Luchetti, A., & Mantovani, B. (2016). Rare horizontal transmission does not hide long-term inheritance of SINE highly conserved domains in the metazoan evolution. *Current zoology, 62*(6), 667–674.

McClintock, B. (1956). Controlling elements and the gene. In *In cold Spring Harbor symposia on quantitative biology, 21: 197–216* (Vol. 21, p. 197). Press.

Medstrand, P., Van De Lagemaat, L. N., & Mager, D. L. (2002). Retroelement distributions in the human genome: Variations associated with age and proximity to genes. *Genome Research, 12*(10), 1483–1495.

Meng, H., Feng, J., Bai, T., Jian, Z., Chen, Y., & Wu, G. (2020). Genome-wide analysis of short interspersed nuclear elements provides insight into gene and genome evolution in citrus. *DNA Research, 27*(1), dsaa004.

Miele, V., Penel, S., & Duret, L. (2011). Ultra-fast sequence clustering from similarity networks with SiLiX. *BMC Bioinformatics, 12*(1), 1–9.

Nacheva, E. (2004a). *Peroun – A new Bulgarian potato variety suitable for fresh consumption and processing. Stara Zagora union of scientists. Scientific conference with international participation. Vol. II, agrarian sciences – Plant growing. Genetics, selection, weeds* (pp. 178–183). Diseases and Wreckers, Part 2. /in Bulgarian/.

Nacheva, E. (2004b). *"Rozhen" – A new Bulgarian potato variety suitable for fresh consumption and processing. Lucrari stiintifice. Anale. Institutul de Cercetare – Dezvoltare Pentru Cartof si Sfecla de Zahar Vol. XXXI* (pp. 57–65). Proceedings of EAPR Agronomy Section Meeting. Mamaia, Romania, June 23th – 27th 2004 Brasov.

Nacheva, E., & Blagoeva, V. (2010). Bulgarian potato varieties. *Journal of Agriculture Plus, 4*, 10–11. /in Bulgarian/.

Nacheva, E., Petkova, V., Pevicharova, G., & Samaliev, H. (2011). *Orfei – A new Bulgarian potato variety resistant to potato cyst nematodes Globodera rostochiensis and Globodera pallida* (pp. 128–131). Proceedings of the XIV International scientific and practical conference "Agricultural Science – Agricultural production in Siberia, Mongolia, Kazakhstan and Bulgaria", part 1.

Nacheva, E., Tomlekova, N., & Petkova, V. (2012). Induced mutagenesis in $F_1$ hybrid seeds of potato. *Ecology and Future, 11*(4), 30–35.

Nacheva, E., Petkova, V., Pevicharova, G., & Samaliev, H. (2014). "Pavelsko" – A new Bulgarian potato variety suitable for chips. *Turkish Journal of Agricultural and Natural Science, 1*(2), 1554–1558.

Nikaido, M., Rooney, A. P., & Okada, N. (1999). Phylogenetic relationships among cetartiodactyls based on insertions of short and long interspersed elements: Hippopotamuses are the closest extant relatives of whales. *Proceedings of the National Academy of Sciences, 96*(18), 10261–10266.

Ogiwara, I., Miya, M., Ohshima, K., & Okada, N. (1999). Retropositional parasitism of SINEs on LINEs: Identification of SINEs and LINEs in elasmobranchs. *Molecular Biology and Evolution, 16*(9), 1238–1250.

Ohno, S. (1972). So much 'junk' DNA in our genome. In *Evolution of genetic systems* (pp. 366–370). Brookhaven Symposium Biol.

Okada, N., Hamada, M., Ogiwara, I., & Ohshima, K. (1997). SINEs and LINEs share common 3′ sequences: A review. *Gene, 205*(1–2), 229–243.

Phillips, R. L., & Vasil, I. K. (Eds.). (2013). *DNA-based markers in plants 6*. Springer Science & Business Media.

San Miguel, P., Tikhonov, A., Jin, Y. K., Motchoulskaia, N., Zakharov, D., Melake-Berhan, A., Springer, P. S., Edwards, K. J., Lee, M., Avramova, Z., & Bennetzen, J. L. (1996). Nested retrotransposons in the intergenic regions of the maize genome. *Science, 274*(5288), 765–768.

Schmidt, T., & Heslop-Harrison, J. S. (1998). Genomes, genes and junk: The large-scale organization of plant chromosomes. *Trends in Plant Science, 3*, 195–199.

Seibt, K. M., Wenke, T., Wollrab, C., Junghans, H., Muders, K., Dehmer, K. J., Diekmann, K., & Schmidt, T. (2012). Development and application of SINE-based markers for genotyping of potato varieties. *Theoretical and Applied Genetics, 125*(1), 185–196.

Seibt, K., Wenke, T., Muders, K., Truberg, B., & Schmidt, T. (2016). Short interspersed nuclear elements (SINEs) are abundant in Solanaceae and have a family- specific impact on gene structure and genome organization. *The Plant Journal, 86*(3), 268–285.

Seibt, K. M., Schmidt, T., & Heitkam, T. (2020). The conserved 3′ Angio-domain defines a superfamily of short interspersed nuclear elements (SINEs) in higher plants. *The Plant Journal, 101*(3), 681–699.

Shedlock, A. M., & Okada, N. (2000). SINE insertions: Powerful tools for molecular systematics. *BioEssays, 22*(2), 148–160.

Slotkin, R. K., & Martienssen, R. (2007). Transposable elements and the epigenetic regulation of the genome. *Nature Reviews Genetics, 8*(4), 272–285.

Sormin, S., Purwantoro, A., Setiawan, A., & Teo, C. (2021). Application of inter-SINE amplified polymorphism (ISAP) markers for genotyping of *Cucumis melo* accessions and its transferability in coleus spp. *Biodiversitas, 22*(5), 2918–2929.

Stapley, J., Santure, A. W., & Dennis, S. R. (2015). Transposable elements as agents of rapid adaptation may explain the genetic paradox of invasive species. *Molecular Ecology, 24*(9), 2241–2252.

Tomlekova, N. (2010). Induced mutagenesis for crop improvement in Bulgaria. *Plant Mutation Reports, 2*(2), 4–27.

Tomlekova, N. (2014). Use of mutant genes for exploring and altering carotenoid biosynthetic pathway of sweet pepper: The Bulgarian viewpoint. In N. B. Tomlekova, M. I. Kozgar, & M. R. Wani (Eds.), *Mutagenesis: Exploring novel genes and pathways* (pp. 433–454). Wageningen Academic Publishers.

Tomlekova, N., Kozgrar, M., & Wani, M. (2014a). *Mutagenesis: Exploring novel genes and pathways* (p. 467). Wageningen Academic Publishers.

Tomlekova, N., Kozgrar, M., & Wani, M. (2014b). *Mutagenesis: Exploring genetic diversity of crops* (p. 394). Wageningen Academic Publishers.

Tomlekova, N., Spasova-Apostolova, V., Nacheva, E., Stoyanova, M., Teneva, A., Petrov, N., & Schmidt, T. (2017). Genotyping of Bulgarian potato varieties by SINE–based ISAP markers. *Comptes Rendus de l'Académie Bulgare des Sciences, 70*, 61–70.

Uitdewilligen, J. G., Wolters, A. M., D'hoop, B. B., Borm, T. J., Visser, R. G., & Eck, H. J. (2013). A next-generation sequencing method for genotyping-by-sequencing of highly heterozygous autotetraploid potato. *PLoS One, 8*(5), e62355.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Lee, T. V. D., Hornes, M., Friters, A., Pot, J., Paleman, J., Kuiper, M., & Zabeau, M. (1995). AFLP: A new technique for DNA fingerprinting. *Nucleic Acids Research, 23*(21), 4407–4414.

Waugh, R., McLean, K., Flavell, A. J., Pearce, S. R., Kumar, A., Thomas, B. B. T., & Powell, W. (1997). Genetic distribution of Bare-1-like retrotransposable elements in the barley genome revealed by sequence-specific amplification polymorphisms (S-SAP). *Molecular and General Genetics MGG, 253*(6), 687–694.

Wenke, T., Döbel, T., Sörensen, T. R., Junghans, H., Weisshaar, B., & Schmidt, T. (2011). Targeted identification of short interspersed nuclear element families shows their widespread existence and extreme heterogeneity in plant genomes. *The Plant Cell, 23*(9), 3117–3128.

Wicker, T., Gundlach, H., Spannagl, M., Uauy, C., Borrill, P., Ramírez-González, R. H., De Oliveira, R., International Wheat Genome Consortium, Mayer, K. F. X., Paux, E., & Choulet, F. (2018). Impact of transposable elements on genome structure and evolution in bread wheat. *Genome Biology, 19*(1), 1–18.

Williams, J. G., Kubelik, A. R., Livak, K. J., Rafalski, J. A., & Tingey, S. V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research, 18*(22), 6531–6535.

Zhang, H., Xu, F., Wu, Y., Hu, H.-H., & Dai, X.-F. (2017). Progress of potato staple food research and industry development in China. *Journal of Integrative Agriculture, 16*, 2924–2932.

# Applicability of ISAP and RAPD Techniques for Capsicum Collection Genotyping


**Nasya Tomlekova, Velichka Spasova-Apostolova, Nikolay Panayotov, Ivelin Panchev, Fatma Sarsu, Mohammad Rafiq Wani, and Kathrin M. Seibt**

**Abstract** Bulgaria was the secondary gene pool for many crops, and one of the first was pepper. However, during the political transforming and economic crises, the lands for growing pepper (*Capsicum* spp.) were reduced, and thereafter, the genetic diversity was lost. With pepper, Bulgaria still has priority providing on European scale valuable pepper germplasm, and this priority should be evaluated and preserved. We present our efforts to characterize pepper accessions using RAPD as well as the retroelement-based Inter-SINE Amplified Polymorphism (ISAP) method initially developed for potatoes. Several short interspersed nuclear element (SINE) families were active within the common ancestor of potato and pepper. We studied the degree of polymorphisms in a collection of 73 pepper genotypes, divided into six groups, using ISAP with primers derived from seven Solanaceae SINE families as well as two subfamilies. Two primer pairs from the families SolS-II and SolS-V generated the most fragments and most informative banding patterns. These SINE-based ISAP reactions are best suited for identifying species of the *Capsicum*

N. Tomlekova (✉)
Molecular Biology Laboratory, Maritsa Vegetable Crops Research Institute, Agricultural Academy, Plovdiv, Bulgaria

V. Spasova-Apostolova
Agricultural Academy, Tobacco and Tobacco Products Institute, Markovo, Bulgaria

N. Panayotov
Department of Horticulture, Agricultural University, Plovdiv, Bulgaria

I. Panchev
Department of Biochemistry, Sofia University "St. Kliment Ohridski", Sofia, Bulgaria

F. Sarsu
General Directorate of Agricultural Research and Policies, Ministry of Agriculture and Forestry Universiteler Mah, Ankara, Cankaya, Turkey

M. R. Wani
Department of Botany, Abdul Ahad Azad Memorial Degree College Bemina, Cluster University, Srinagar, Jammu and Kashmir, India

K. M. Seibt
Institute of Botany, Technische Universität Dresden, Dresden, Germany



A. Raina et al. (eds.), *Advanced Crop Improvement, Volume 2*,
https://doi.org/10.1007/978-3-031-26669-0_3

genus. The most polymorphic profiles within all studied were generated by *C. baccatum* accessions. In contrast, intraspecific application of the SINE-based markers yielded a high percentage of conserved ISAP fragments. From a total of 56 *C. annuum* accessions, only three of them with two different profiles were identified. Our results demonstrate that potato-based SolS-SINE primers can be adapted for molecular genotyping in peppers. The low intraspecies polymorphism generated by ISAP forced us to investigate RAPD as an alternative low-cost genotyping approach. RAPD was successfully applied on a group of mutant lines and corresponding source lines, carrying valuable breeding traits. Despite the low polymorphic levels, we have identified four RAPD primers, capable to discriminate among several genotypes.

**Keywords** *Capsicum* spp. · SINE · Transposable elements · ISAP · RAPD

## Abbreviations

| | |
|---|---|
| BSA | Bovin serum albumin |
| CTAB | Hexadecyltrimethylammonium bromide [(C16H33)N(CH3)3]Br |
| EDTA | Ethylenediaminetetraacetic acid $[CH_2N(CH_2CO_2H)_2]_2$ |
| M | Multiplex reaction |
| Sol | Solanaceae |

## 1 Introduction

In Bulgarian agriculture, most of the pepper varieties belong to *Capsicum annuum* L. (Todorova et al., 2014). Bulgaria is a secondary breeding center where sweet pepper varieties dominate the production, complemented with limited genetic diversity available to the breeders (Poryazov et al., 2013).

### *1.1 Molecular Marker Systems*

Detailed genetic characterization in pepper spp. started in mid-twentieth century. Initially, it was based on phenotype assays, gradually supplemented with physiological and biochemical methods. The development in molecular biology led to introduction of powerful DNA-based methods like RFLP, RAPD, AFLP, SSRs, CAPS, SCAR, and SNP (Paran, 2013).

RFLP, while first introduced in the 1980s (Livingstone et al., 1999; Tanksley et al., 1988) and still in use today (Paran, 2013), was quickly replaced by AFLP – a

"hybrid" between RFLP and PCR. AFLP generates a vast amount of data allowing to detect polymorphism at intraspecies level and even within the progeny of a single parent (Vos et al., 1995). AFLP was used in pepper for QTL analyses of different traits (Ogundiwin et al., 2005) not only in *C. annuum* L. but also on other species like *C. chinense* Jacq., *C. baccatum* L., and *C. frutescens* L. Despite the high informativity of the method, low levels of polymorphism were obtained (Ht 0.119) resulting in a low index of genetic diversity (GST 0.331). These results demonstrated that the modern varieties had a very narrow genetic base (Toquica et al., 2003).

## *1.2 RAPD for Pepper Genotyping*

RAPD is an anonymous PCR-based method for the detection of polymorphism. The method employs a single decamer primer to amplify regions where this primer anneals in opposite direction at a distance of 50–2000 bp. The method is simple and inexpensive but had problems with reproducibility. Also, it requires preliminary screening of a large primer set to identify primers that generate informative patterns (usually 5–20 bands) (Welsh & McClelland, 1990; Williams et al., 1990).

Despite the limitations, RAPD was successfully applied in pepper genetic research. Rodriguez et al. (1999) screened 134 accessions with 110 primers and determined that three accessions previously classified as *Capsicum annuum* on morphology base actually belong to other species.

Analysis of 22 accessions of *C. annuum*, *C. baccatum*, *C. chinense*, *C. eximium*, *C. frutescens*, and *C. luteum* with 27 RAPD and eight ISSR primers revealed genetic similarity between 23% and 96% (Thul et al., 2012). Combining RAPD with UPGMA analysis allowed Adetula (2005) to separate 40 accessions of *C. annuum* and *C. frutescens* into four groups. At population level, RAPD allowed for identification of novel genetic variation (Votava et al., 2002).

RAPD was also used for genetic characterization of disease resistance loci. Analysis of 800 primers identified one RAPD marker (UBC191432) related to *Pvr4* gene, conferring resistance to PVY (Arnedo-Andrés et al., 2002). The marker was converted into SCAR and successfully used in a breeding program (Moodley et al., 2014). RAPD can also be applied for characterization of source material for hybrid production (Ilbi, 2003), hybrid identification (Jang et al., 2004), and hybrid quality tests (Ballester & Carmen de Vicente, 1998). Baoxi et al. (2000) and Kumar et al. (2007) successfully applied RAPD for identification of genetic bases of fertility restoration.

Despite its limitations RAPD can be successfully used for the primary screening of genetic material and further serving as a base for SCAR markers for more sustainable analysis.

## 1.3 Application of RAPD for Pepper Characterization

RAPD analyzes were performed using 40 decamer primers (Fig. 1a, b) (Tomlekova et al., 2016). Sixteen from all 40 analyzed primers have been found to generate profiles suitable for genotype identification. Selected primers generated 162 bands in total with size ranging from 300 bp to 3000 bp (Fig. 1 and Table 1).

RAPD reactions with 12 of the selected primers generated monomorphic profiles in all investigated pepper genotypes within all 113 scored bands (Fig. 2). Details of these primers are shown in Table 1.

Four primers generated polymorphic patterns in some of the analyzed lines. Three of them (A13, A18, and B01) produced polymorphism in line 1928, while primer B10 generated polymorphism in genotypes 1928 and 1966 (Fig. 3 and Table 2).

RAPD reactions with primers A13, A18, and B01 generate monomorphic patterns in genotypes 1966, 1917, 1931, 1933, and 1935. The primer A13 amplifies ten

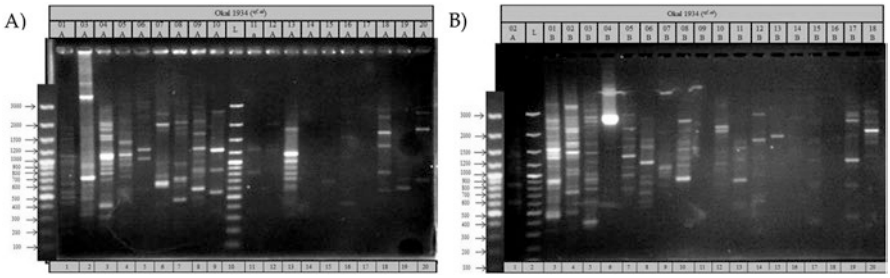



**Fig. 1** RAPD amplification patterns from pepper genotype 1934*(of. al)*

**Table 1** Primer sequences and band score for the monomorphic RAPD reactions in pepper initial and mutant genotypes

| Random primer | Sequence of primers (5′ → 3′) | A | T | Fragment size (bp) |
|---|---|---|---|---|
| A01 | CAG GCC CTT C | 7 | 19 | 250–2200 |
| A02 | TGC CGA GCT G | 12 | 15 | 250–2900 |
| A04 | AAT CGG GCT G | 12 | 12 | 450–3000 |
| A05 | AGG GGT CTT G | 12 | 8 | 450–1800 |
| A08 | GTG ACG TAG G | 7 | 7 | 550–2200 |
| A09 | GGG TAA CGC C | 7 | 17 | 390–3000 |
| B02 | TGA TCC CTG G | 7 | 13 | 700–2900 |
| B03 | CAT CCC CCT G | 7 | 15 | 310–3000 |
| B05 | TGC GCC CTT C | 7 | 12 | 500–2800 |
| B06 | TGC TCT GCC C | 7 | 9 | 550–2800 |
| B08 | GTC CAC ACG G | 7 | 6 | 850–2700 |
| B17 | AGG GAA CGA G | 7 | 12 | 350–2800 |

A = number of analyzed genotypes, T = total number of bands

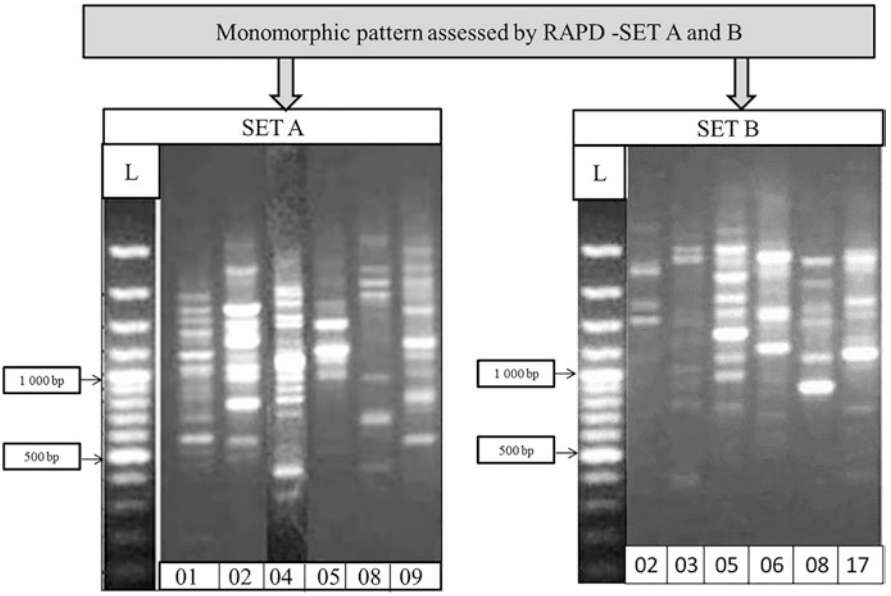



**Fig. 2** Monomorphic pattern assessed by RAPD amplification in all analyzed pepper genotypes. Lane 7 (L) – 100 bp Plus DNA Ladder

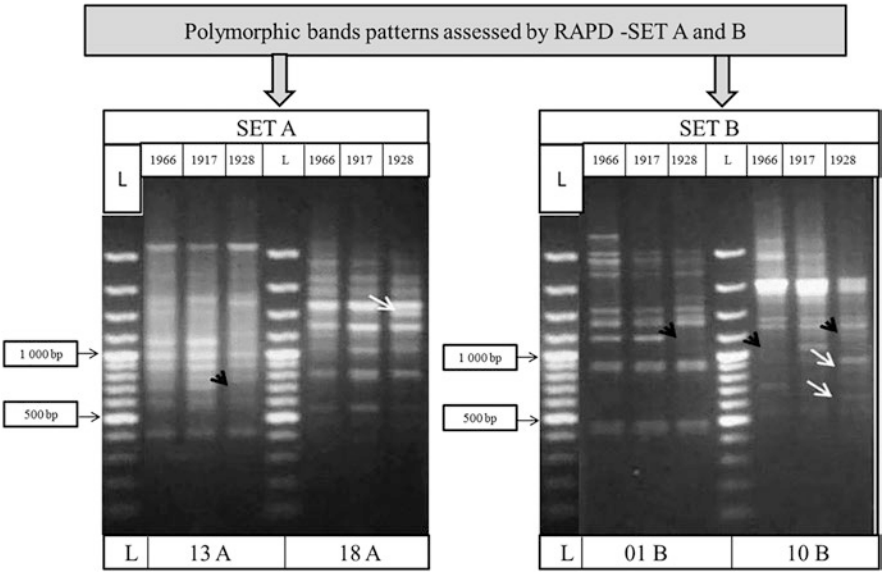



**Fig. 3** Polymorphic band patterns (or RAPD amplification profile) found in three pepper genotypes (1966, 1917, and 1928). The white arrow shows additional polymorphic bands and the black arrow shows polymorphic bands absent in the patterns

**Table 2** Primer sequences and band score for polymorphic RAPD reactions in pepper genotypes

| Random primers | Sequence of primers (5′ → 3′) | A | PA | % PA | T | Fragment size (bp) | P | % P |
|---|---|---|---|---|---|---|---|---|
| A13 | CAG CAC CCA C | 7 | 1 | 14.29 | 10 | 300–1900 | 1 | 10 |
| A18 | AGG TGA CCG T | 7 | 1 | 14.29 | 13 | 400–2800 | 1 | 7.69 |
| B01 | GTT TCG CTC C | 7 | 1 | 14.29 | 12 | 410–3000 | 1 | 8.33 |
| B10 | CTG CTG GGA C | 7 | 2 | 28.57 | 14 | 550–2900 | 3 | 21.43 |

A = number of analyzed genotypes, PA = polymorphic genotypes, % PA = percent polymorphic genotypes, T = total number of bands, P = polymorphic bands, % P = percent polymorphic bands

fragments in genotypes 1930, 1966, 1917, 1931, 1933, and 1935 (length of 1900 bp, 1600 bp, 1190 bp, 1000 bp, 900 bp, 800 bp, 700 bp, 600 bp, 450 bp, and 300 bp) and nine fragments in line 1928, whereas the fragment with length 700 bp is not amplified (Fig. 3 and Table 2).

The primer A18 amplifies patterns consisting of 12 fragments in accessions 1966, 1917, 1931, 1933, and 1935 and 13 fragments in mutant line 1928, where an additional fragment with length 1500 bp was amplified.

The amplification with primer B01 results in lack of amplification of a fragment with length 1200 bp in line 1928 (11 fragments) as compared to the pattern generated in genotypes 1966, 1917, 1931, 1933, and 1935 where 12 fragments are amplified.

The primer B10 amplifies monomorphic profiles containing 12 fragments in mutant genotypes 1930, 1917, 1931, 1933, and 1935, while it generates polymorphic patterns in variety 1966 and line 1928. The pattern of line 1928 (13 amplified fragments) contains two additional fragments with lengths 850 bp and 600 bp, and it does not contain fragment with length 1050 bp. The pattern of variety 1966 (11 amplified fragments) lacks the fragment of length 1050 bp.

The RAPD reaction with primers A13, A18, B01, and B10 generates a total of 6 polymorphic bands from all 49 amplified fragments, thus resulting in average polymorphic content of 12.24% and polymorphic band size ranging from 300 bp to 2900 bp (Table 2).

Varieties 1966 (initial) and 1917 (mutant) can be distinguished by an amplification using primer B10. Eleven fragments of genotype 1966 and 12 fragments of genotype 1917 were amplified by RAPD with primer B10. The additional fragment of 1917 amplified by the RAPD reaction with primer B10 was with 1050 bp length. Mutant lines 1928 and 1930 can be distinguished with all four selected primers. Primer A13 amplifies 10 fragments of the genome of line 1930 and 9 fragments of line 1928 where a 700-bp fragment is not amplified. The primer A18 amplifies patterns consisting of 13 fragments of genome of line 1928 and 12 fragments of line 1930. Line 1930 is characterized with an additional amplified fragment with length 1500 bp.

The amplification with primer B01 results in lack of amplification of a fragment with length 1200 bp of line 1928 (11 fragments) as compared to the pattern generated from lines 1930 (12 fragments). The profile of line 1928 consists of 13 fragments amplified by primer B10 and of line 1930 – of 12 fragments. The differences

are more complicated since primer B10 generates two additional fragments with lengths 850 bp and 600 bp of line 1928 and does not amplify the fragment with length 1050 bp in the RAPD-B10 profile of line 1930. Polymorphism is not detected in the comparison of mutant lines 1931, 1933, and 1935 by the RAPD reactions with primers used in this study.

RAPD is a well-known technique for genetic analyses in pepper (Bahurupe et al., 2013; Pradeepkumar et al., 2001). In the present work, we have identified four RAPD primers capable of differentiating between the analyzed pepper genotypes. Three of these primers generate polymorphic fragments in line 1928, while the fourth one – B10 – generates polymorphism also in variety 1966. The established level of polymorphism is 12.2% calculated only for these primers. A recalculation including all tested primers will result in polymorphic levels below 1–2%. These RAPD primers (A13, A18, B01, and B10) successfully discriminate between 'Zlaten medal *ms* 8' from its mutant derivative 1929$^{(of,\ al,\ ms8)}$. Moreover, primer B10 discriminates between initial variety 'Pazardzhishka kapia 794' and its mutant progeny 'Oranzheva kapia' (Tomlekova et al., 2016). These results are interesting since we were not able to discriminate these genotypes using the potato-based ISAP. Another important outcome is the demonstration of the applicability of RAPD as a useful and affordable tool in pepper molecular breeding. Despite several known drawbacks of this method, it allows quick and cheap generation of polymorphic bands suitable for further conversion into more reproducible markers.

## *1.4 Microsatellite Analysis in Pepper*

Microsatellites are short DNA sequences containing tandemly repeated motifs of 2–15 bp, i.e., (CA)n or (GATA)n (Lijun & Xuexiao, 2012; Min et al., 2008). Each motif is represented several hundred times in eukaryotic genome, and for some of them, a high level of polymorphism is observed. The polymorphism is due to changes in the number of repeated motifs and the mechanisms of such change are largely unknown. It is also unknown why in some loci no polymorphism is observed, while for others change might occur even after a single cell division. The use of microsatellites as markers is possible in two ways. The first one is similar to RAPD when the motif sequence is used as single primer. In this case, primers amplify the region between two adjacent loci of oppositely oriented sequences divided by less than 2000 bp. This technique is known as ISSR and has similar requirements like RAPD and generates similar amplification patterns.

The second approach needs preliminary isolation of individual locus, its sequencing and design of locus-specific primer pair. Further, each primer pair needs to be checked in order to confirm whether the locus is polymorphic or not. This SSR technique requires extensive time-consuming and expensive work without guarantee that all characterized loci will be useful for genetic research.

Nevertheless, when such work was performed, SSR markers were successfully applied for gene mapping and for QTL analyses in pepper (Minamiyama et al.,

2006; Paran, 2013). Combing SSR with NGS in two pepper varieties identified 2067 and 2494 promising microsatellite loci. Most represented motifs were trinucleotides (49%) followed by di- (39.7%), hexa- (5.3%), tetra- (2.9%), and pentanucleotide (2.8%). Among them, AGC/CAG/GCA (17.7%) are the most frequent, followed by GGT/GTG/TGG (16%), AAG/AGA/GAA (14.3%), ATC/CAT/TCA (11.9%), and AAC/ACA/CAA (7.5%) (Ahn et al., 2014).

ISSR and SSR were applied for variety identification of *C. annuum* and *C. pubescens*. Moreover, primers designed for *C. annuum* were amplifying similar loci in *C. pubescens* (Ibarra-Torresa et al., 2015). ISSR was used for analysis of genetic diversity in *C. chinense* (Gozukirmizi et al., 2015; Hazarika & Neog, 2014). ISSR was also used for revealing genetic variations upon in vitro regeneration of pepper variety 'Habanero' (Bello-Bello et al., 2014).

A variation of SSR targeting ORFs and known as SRAP was developed in 2001 for genetic analysis in *Brassica* (Li & Quiros, 2001). This technique was successfully applied for analysis of loci determining resistance to *Phytophthora capsici* in pepper (Xu et al., 2014). Also, similar application of COSII markers developed for other species can be used in pepper (Wu et al., 2009). Combining available markers allows for better and more complete germplasm characterization worldwide (Geleta et al., 2005).

## *1.5 Mobile Genetic Elements as a Base for Molecular Marker Systems*

Achievements in genetics allowed for the development of novel marker systems based on mobile genetic elements (MGEs). Mobile genetic elements are complex systems allowing a nucleotide sequence to change its position within genome. For the first time, mobile elements were described by Barbara McClintock (1950). Further research had revealed that MGEs are diverse groups forming a substantial part of eukaryotic genomes and their activities supplement the classic genetic paradigm (Ravindran, 2012). The current view of MGE roles suggests that they might participate in the regulation of gene expression as "promoter donors" (Hedges & Batzer, 2005) or providing places for binding of regulatory factors (Lee et al., 2015; Sundaram et al., 2014; Wang et al., 2007). MGEs might serve as donors of novel exons or entire protein-coding genes (Ferguson et al., 2013). MGEs can also affect the chromosome structures and stability (Lippman et al., 2004).

MGEs are widely distributed within chromosomes and their number can change not only during evolution but also during stress conditions (Schulman, 2007). Since the last effect can occur in short periods of time, it might serve as a basis for the search of novel MGE-based molecular markers. MGEs can be separated in two groups depending on their propagation cycle. Type I involves an RNA intermediate along with the DNA form (retrotransposons), while Type II involves only a DNA form (transposons) (Xiong & Eickbush, 1990; Wessler, 2006; Wicker et al., 2007;

Munoz-Lopez et al., 2010). Depending of the replication mechanism, Type II MGEs are divided to replicative and non-replicative transposons (Feschotte et al., 2002).

Type I retrotransposons can be divided into two groups – retroelements with long terminal repeats (LTR) and non-LTR retroelements (Schmidt, 1999; Wessler et al., 2006).

LTR retrotransposons like *Ty1* or *gypsy* can be represented in approximately a million copies in some plant genomes (Kumar et al., 1997). In pepper, MGEs comprise 76%–79.6% of genome in *C. annuum* CM334 and *C. chinense*, respectively, and 70% of them are LTR retrotransposons (Kim et al., 2014).

The main non-LTR retroelements are LINE and SINE (Deragon & Zhang, 2006; Wessler, 2006). LINE comprises a single bicistronic RNA encoding an RNA-binding protein and a protein with endonuclease and reverse transcriptase activities. Thus, LINEs are self-sufficient for propagation (Cost & Boeke, 1998) but their integration depends on cellular DNA repair mechanisms (Moore & Haber, 1996).

LINEs are widely distributed among eukaryotes and can be divided in several main families – R2, L1, RTE, I, and Jockey (Eickbush & Malik, 2002; Xiong & Eickbush, 1990). In plants, most represented are members of the L1 clade and RTE (Biedler & Tu, 2003; Wicker et al., 2007). There is a hypothesis that LINEs are the oldest retroelements and evolutionary predecessors of LTR retrotransposons (Xiong & Eickbush, 1990).

SINEs are a heterologous group derived from different RNA types (i.e., tRNA, 7S RNA, 5S RNA) and do not encode proteins necessary for their own propagation (Park et al., 2011; Wicker et al., 2007). All known SINEs are transcribed by RNA polymerase III but their propagation depends on enzymes produced by active LINEs or retrotransposons (Kajikawa et al., 2005; Schmidt, 1999). SINE organization resembles that of pseudogenes (Weiner et al., 1986; Winkfein et al., 1988).

SINEs are also a widely spread group of MGEs in eukaryotic genomes with several hundred thousand to several million copies per genome. Recently, SINE activity was demonstrated in potato (Seibt et al., 2016). Along with their high copy number, this information pointed on the possibility to use SINEs in a novel marker system. Such a system was developed for potato and is known as ISAP (Alzohairy et al., 2015; Seibt et al., 2012; Wenke et al., 2011).

In potato genome, seven SolS-SINE families and two subfamilies have been identified (Seibt et al., 2016; Wenke et al., 2011). These SolS-SINEs were detected in a number of Solanaceae crops such as potato, tomato, and pepper (Seibt et al., 2016). Interestingly, SINEs were frequently observed in and near genes (Seibt et al., 2016). The Solanaceae SINE families differed in their copy numbers and the estimated age of these copies and were successfully used to ISAP markers for potato genotyping (Seibt et al., 2012, 2016). Although initially developed for potatoes, sequence similarities between SINEs across Solanaceae species indicate that these ISAP markers could be adapted for related species (Seibt et al., 2016).

The robustness and ease of application make ISAP a suitable alternative to more advanced techniques like AFLP or SNP. ISAP may not replace these standard methods in molecular genetics, but can be a powerful tool for quick preliminary characterization of the available germplasm and the identification of markers for particular

traits (Tam et al., 2005). Hence, it could be easily incorporated into breeding programs. Since all SolS-SINE families appeared to be active during evolution of the Solanaceae family, it can be expected that SINE-derived primers can be exploited for genetic analyses within this family (Seibt et al., 2016). Solanaceae family encloses several economically important crops like pepper, tobacco, tomato, and potato to name a few (Wu et al., 2010). According to the C value database, the pepper genome is similar in size compared to tobacco, but twice that of the genomes of potato and tomato. The genome of the tomato is most closely related to the potato (Wu et al., 2010). The accumulation of repeated elements that led to genome size enlargement in pepper during evolution outlines the possibility of successful employment of potato-based ISAP primers on pepper (Kim et al., 2014).

The advantages of the ISAP method are the simplicity of the experimental design, reproducibility, and sufficient levels of polymorphism. ISAP is similar to RAPD in terms of time and labor requirements, but ISAP is more reproducible and generates more complex amplification patterns (Wenke et al., 2011). The three most informative ISAP reactions in potato were based on the SINE families SolS-IIIa and SolS-IV (primer pairs SolS-IIIa-F/SolS-IIIa-R, SolS-IIIa-F/SolS-IV-F, and SolS-IIIa-F/SolS-IV-R). Of all, this is SolS-IIIa-F/SolS-IV-R, which generates the most polymorphic banding patterns in potato (Seibt et al., 2012; Tomlekova et al., 2017b). Retrotransposons can be used as markers because their integration creates new joints between genomic DNA and their conserved ends. Apart from the ISAP technique under consideration to detect polymorphisms for retrotransposon insertion, in 2006 Kalendar and Schulman developed two methods, retrotransposon-microsatellite amplified polymorphism (REMAP) analysis and inter-retrotransposon amplified polymorphism (IRAP) analysis, that require neither restriction enzyme digestion nor ligation to generate the marker bands for PCR amplification with a single primer or with two primers and applied in different crops (Kalendar et al., 2004; Kalendar et al., 2011).

## *1.6 Applicability of Potato-Derived ISAP for Pepper Genome Characterization*

### 1.6.1 Materials and Methods

Plant Material

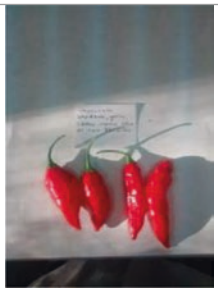
Seventy-three pepper accessions used in genotyping experiments are described in Tables 3, 4, 5, 6, 7 and 8. X-Rays were applied to the initial (parent) ‘Pasardzhishka kapia 794’ pepper variety during the 1980s. Later, the orange-fruit variety ‘Oranzheva kapia’ was developed through the mutation breeding process. The mutant variety had more β-carotene and other beneficial mutations were induced as well (Tomlekova et al., 2021). Evaluating and increasing genetic diversity has always been one of the most important targets in Bulgarian pepper breeding

**Table 3** Group I. Seventeen accessions belonging to *Capsicum fasciculatum* Sturtev., *C. frutescens* L., *C. baccatum* L., and *C. pubescens* Ruiz & Pav. species

| Number of accession | Classification | Origin | Photos |
|---|---|---|---|
| 100 | *C. fasciculatum* Sturtev. | Introduction from Greece |  |
| 98 | *C. frutescens* L. | Local accession from Bulgaria |  |
| 101 | *C. baccatum* L. | Local accession from Bulgaria |  |
| 106 | *C. baccatum* L. | Local accession from Bulgaria |  |
| 110 | *C. frutescens* L. | Introduction from Crete |  |

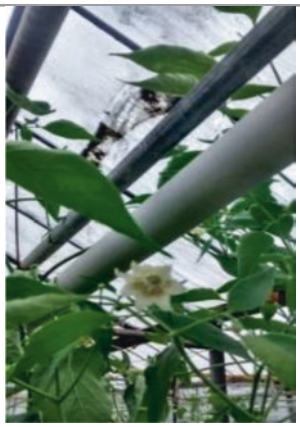
(continued)

**Table 3** (continued)

| Number of accession | Classification | Origin | Photos |
|---|---|---|---|
| CBU | *C. baccatum* L. var. *umbilicatum* | Argentina | |
| CBP | *C. baccatum* L. var. *pendulum* | Argentina | |
| Peru 1 | *C. frutescens* L. | Introduction from Peru |  |
| Peru 2 | *C. frutescens* L. | Introduction from Peru |  |
| Peru 4 | *C. frutescens* L. | Introduction from Peru |  |
| Peru 5 | *C. frutescens* L. | Introduction from Peru |  |

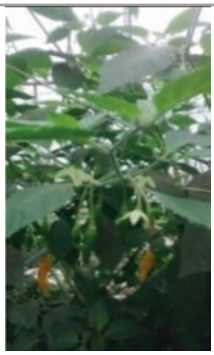
(continued)

**Table 3** (continued)

| Number of accession | Classification | Origin | Photos |
|---|---|---|---|
| Peru 6 | *C. baccatum* L. | Introduction from Peru |  |
| Peru 7 | *C. frutescens* L. | Introduction from Peru |  |
| Peru 8 | *C. frutescens* L. | Introduction from Peru |  |
| Peru 9 | *C. baccatum* L. | Introduction from Peru |  |

(continued)

**Table 3** (continued)

| Number of accession | Classification | Origin | Photos |
|---|---|---|---|
| Peru 10 | *C. frutescens* L. | Introduction from Peru |  |
| Peru 11 | *C. pubescence* Ruiz & Pav. | Introduction from Peru |  |

Introductions and local accessions from different countries belong to the collection of the Maritsa Vegetable Crops Research Institute and Agricultural University, Plovdiv
The photos are copyright and taken in the course of the research of the plant material grown. Kindly provided by Prof. Eduardo Moscone from the collection of the University of Cordoba, Argentina for analyses

**Table 4** Group II. Twelve foreign accessions belonging to *C. annuum*

| Number of accession | Type | Origin |
|---|---|---|
| China 1 | $F_1$ hybrid | China |
| China 2 | $F_1$ hybrid | China |
| China 3 | $F_1$ hybrid | China |
| China 4 | $F_1$ hybrid | China |
| China 5 | $F_1$ hybrid | China |
| England 1 | $F_1$ hybrid | England |
| England 2 | $F_1$ hybrid | England |
| 47 | Local accession | Egypt |
| 108 | $F_1$ hybrid | Poland |
| 114 | $F_1$ hybrid | Poland |
| 23 | Local accession | Egypt |
| 60 | $F_1$ hybrid | Spain |

**Table 5** Group III. Seven local accessions and breeding lines, belonging to *C. annuum*

| Number of accessions and breeding lines | Origin |
|---|---|
| 73 – Breeding line | Selected from breeding line (DMR × 'Kapia 1300') |
| 123 – Local accession | Plovdiv, Bulgaria |
| 43 – Local accession | Svishtov, Bulgaria |
| 85 – Local accession | Svishtov, Bulgaria |
| 86 – Local accession | Svishtov, Bulgaria |
| 90 – Local accession | Svishtov, Bulgaria |
| 18 – Breeding line | Selected from variety 'Osmarsko Kambe' |

DMR – Doux Marconi Rouge

**Table 6** Group IV. Eleven breeding lines belonging to *C. annuum*

| Number of accession | Phenotype and origin |
|---|---|
| 34 | Cone-like, slightly curved at the tip, red color; breeding line from (DMSS × 'Kalinkov' 807/5) |
| 5 | Cone-like, rounded tip, orange color; breeding line from (DMG × 'Zlaten Medal 7') |
| 2 | Cone-like, pointed tip, light beige; breeding line from (DMG × 'Zlaten Medal 7') |
| 13 | Oval, conic tip, orange color; breeding line from local accession from Bulgaria |
| 27 | Cone-like, dual wall, orange color; breeding line from local accession from Greece |
| 29 | Cone-like, dual wall, orange color, breeding line from (DMG × 'Kurtovska Kapia 1619') |
| 32 | Short conical with rounded tip, yellow-orange color; breeding line from (DMG × 'Kurtovska Kapia 1619') |
| 37 | Cone-like, dual-wall, yellow-orange color; breeding line from (DMSS × 'Kalinkov' 807/5) |
| 51 | Cone-like, dual wall, rounded tip intense orange color; breeding line from local accession from Egypt |
| 53 | Cone-like, dual wall, rounded tip, light orange to yellow color; breeding line from local accession from Egypt |
| 91 | Cone-like, dual wall, orange color; breeding line from local accession from Bulgaria |

*DMSS* Doux Marconi San Semences, *DMG* Doux Marconi Geonet the corresponding
*Breeding lines belonging to the collection of the Agricultural University

**Table 7** Group V. Eleven accessions of *C. annuum* – local varieties (initial) and advanced mutant lines

| No | Description | Photos |
|---|---|---|
| 1966 | 'Pazardzhishka Kapia 794'. Traditional local variety from the Pazardzhik region. |  |
| 1917 | 'Oranzheva Kapia'. High β-carotene concentration. Mutation – Orange fruits (*of*). Mutant variety obtained upon X-ray irradiation with 120 Gy of parent variety 'Pazardzhishka Kapia 794'. |  |
| 1928 | Zlaten medal *ms*8 ($M_{ms8}$). Male sterile advanced mutant (*ms*8) line. Obtained from 'Zlaten medal 7' (red fruits) obtained upon X-ray irradiation with 120 Gy. |  |
| 1929 | Advanced mutant line obtained by successive backcrosses [1928 x *Okal*$^{(of.al)}$] and self-pollinations. It contains *of, al,* and *ms8* mutations in a single genotype. | Photos of plant and fruit similar to 1930 |

(continued)

**Table 7** (continued)

| No | Description | Photos |
|---|---|---|
| 1930 | Advanced mutant line obtained by successive backcrosses [1928 x *Okal*$^{(of,al)}$] and self-pollinations. It contains *of, al,* and *ms8* mutations in a single genotype. A new registered variety 'Desislava' with orange fruits obtained from 1930. |   |
| 1931 | 'Albena'$^{(al)}$ free of anthocyanins. Mutant variety obtained from 'Zlaten medal 7' upon X-ray irradiation with 120 Gy. It contains red fruits. |  |
| 1932 | Advanced *Okal*$^{(of,al)}$ mutant line obtained by successive backcrosses between 'Oranzheva Kapia' and 'Albena'. Orange fruit (*of*) in anthocyaninless (*al*) genotype. |  |

(continued)

**Table 7** (continued)

| No | Description | Photos |
|---|---|---|
| 1933 | Advanced *Okal*$^{(of, al)}$ (inbred) mutant line obtained by successive backcrosses between 'Oranzheva Kapia' and 'Albena'. Orange fruit (*of*) in anthocyaninless (*al*) genotype. |  |
| 1934 | Advanced *Okal*$^{(of, al)}$ (inbred) mutant line obtained by successive backcrosses between 'Oranzheva Kapia' and 'Albena'. Orange fruit (*of*) in anthocyaninless (*al*) genotype. | Photos of plant and fruit similar to 1933 |
| 1935 | Advanced *Okal*$^{(of, al)}$ (inbred) mutant line obtained by successive backcrosses between 'Oranzheva Kapia' and 'Albena'. Orange fruit (*of*) in anthocyaninless (*al*) genotype. | Photos of plant and fruit similar to 1933 |

(continued)

**Table 7** (continued)

| No | Description | Photos |
|---|---|---|
| 1936 | Advanced *Okal*$^{(of, al)}$ (inbred) mutant line obtained by successive backcrosses between 'Oranzheva Kapia' and 'Albena'. Orange fruit (*of*) in anthocyaninless (*al*) genotype. | Photos of plant and fruit similar to 1933 |

The photos are copyright and taken in the course of the research

**Table 8** Group VI. Fifteen Bulgarian local varieties of *C. annuum*

| No. | Description | Photos |
|---|---|---|
| 'Bouquet 50' | 'Gorogled 6' × *C. fasciculatum* | |
| 'Bulgarski Ratund' | Traditional local variety type Ratund |  |
| 'Byala Shipka' | Traditional local variety | |
| 'Dzhulunska Shipka 1021' | Obtained from traditional local variety |  |
| 'Zlaten medal 7' | Obtained from traditional local variety 'Bjala Kapia' |  |
| 'IZK Delikates' | Obtained from variety 'Chorbadzhijski' | |
| 'IZK Kalin' | New high-yield variety | |
| 'IZK Rubin' | Obtained from local varieties | |
| 'Kapia UV' | Medium-early variety. Obtained from 'Kurtovska Kapia 1619' | |

(continued)

**Table 8** (continued)

| No. | Description | Photos |
|---|---|---|
| 'Kurtovska Kapia 1' | Obtained from local variety 'Kurtovska Kapia 1619' |  |
| 'Kurtovska Kapia 1619' | Local variety obtained by individual selection | |
| 'Sivria 600' | Local variety obtained by individual selection |  |
| 'Sofiiska Kapia' | Obtained from local variety 'Bjala Kapia' |  |
| 'Stryama' | High-yield variety; ('Sivria 600' × no. 12) × C156 $F_8$ × 'Podarok Moldavii' | |
| 'Hebar' | No. 786 × 'Bjala Kapia 1' | |

The photos are copyright and taken in the course of the research

programs, and germplasms of different *Capsicum* species, local varieties, local accessions, and induced and spontaneous mutations were generated and genotypically characterized (Tomlekova et al., 2017a).

Introductions of group II belong to the collection of the Maritsa Vegetable Crops Research Institute and of the Agricultural University, Plovdiv.

*Local accessions from different Bulgarian regions and breeding lines belong to the collection of the Agricultural University, Plovdiv and to the collection of the Maritsa Vegetable Crops Research Institute.

*Breeding lines and local accessions belong to the collection of the Agricultural University located in Plovdiv.

All the advanced mutant lines are inbred/homogeneous and varieties were developed in the ancient Institute of Genetics, Sofia, and the Maritsa Vegetable Crops Research Institute, Plovdiv, and currently maintained in the Maritsa Institute in Plovdiv.

All the advanced mutant lines are in $M_8$ generation and were induced and developed in the ancient Institute of Genetics, Sofia and currently maintained in the working collection of the Maritsa Vegetable Crops Research Institute, Plovdiv.

### 1.6.2 Molecular Methods

Genomic DNA was prepared from young pepper leaves according to the CTAB protocol (Murray & Thompson, 1980; Saghai-Maroof et al., 1984) or by using Nucleon PhytoPure Kit (Amersham, Cat. RPN 8510, Austria) following company's protocols including an RNase A treatment. Agarose Lambda DNA (Thermo Scientific Life Sciences, Cat. SD0011, Lithuania) was used to determine DNA quantity on 1% LE agarose gels (Lonza, Cat. 50,004, USA) with ethidium bromide (VWR International, Cat. 4007-07, Austria). Buffers (TAE and TBE) were prepared with a Tris base (Sigma-Aldrich, Cat. RDD008, USA), acetic acid (VWR International, Cat. 20104.298, Germany), and boric acid (Sigma Aldrich, Cat. B0252, Germany), respectively, and EDTA (Sigma Aldrich, Cat. E-5134, Germany).

For the ISAP method, the 20 μL PCR mixture consisted of 10 ng template DNA, 1× Green PCR buffer (10× Green buffer, Thermo Scientific, Cat. No. B71, Lithuania), 0.2 mM dNTPs (Thermo Scientific, Cat. No. R0192, Lithuania), 0.15 μL each primer (Seibt et al., 2012) (Table 1), 0.1 mg/mL BSA (Sigma-Aldrich, Cat. No. A6003, USA), and 0.5 U Dream*Taq* DNA polymerase (Thermo Scientific, Cat. No. EP0702, Lithuania) (Seibt et al., 2012). Amplification reactions were performed using the following conditions: initial denaturation for 5 min at 93 °C, followed by 30 cycles with 20 s at 94 °C, 30 s at 52 °C, 120 s at 72 °C, and a final elongation of 5 min at 72 °C.

The result products were separated on 2% LE agarose gels in 1x TAE buffer containing fluorescent dye 0.1 $\mu g.mL^{-1}$ ethidium bromide (Sigma-Aldrich, E1510, Austria). The fragment lengths were estimated by comparing with DNA Ladder Gene Ruler 100 bp Plus DNA (Thermo Scientific, Cat. No.SM0321, Lithuania).

#### Data Analysis

Images were captured by the Gel Doc 2000 Gel Documentation System (GenoMini, VWR, Belgium) and analyzed with GenoSoft Imaging software (VWR Int.). To compile matrices, only reproducible fragments with defined lengths were taken into account. Data analysis was performed using SPSS Statistics for Windows version 17.0 (SPSS Inc., released 2008, Chicago: SPSS Inc.).

The monomorphic and polymorphic profiles were grouped according to the degree of similarity and difference between them in the number of amplified fragments and the distances between the samples were determined. The Statistical Package for the Social Sciences (SPSS) program was applied for grouping and visualization of the different distances of the amplified polymorphic profiles, depending on the number of polymorphic fragments.

### 1.6.3 Results

The first stage was to identify primers capable of amplification on pepper genomic DNA. It included primer pairs arising from a single SINE family (two primers F and R from two different SINE families, as well as primer combinations from the same SolS-SINE family). The selected reactions were performed with only one pepper genotype. This allowed us to assess the effectiveness of the marker system.

#### Results of ISAPInter-SINE Amplified Polymorphism (ISAP) Reactions Performed with a Forward and a Reverse Primer Designed from Different Sol-SINE Families

The effectiveness of the ISAP reactions with individual forward and reverse primers from different Sol-SINE families was assessed with two representative pepper genotypes. Thus, the application of the selected efficient primers was further used to assess polymorphism between closely related genotypes such as the traditional Bulgarian pepper variety ‘Pazardzhishka Kapia 794’ – parent of the mutant one, ‘Oranzheva Kapia’. In the performed test design to adapt ISAP for pepper germplasm, a total of 24 ISAP assays with primer pairs from different families were tested on both genotypes. After no different profiles were found between the parent and mutant genotypes, in the next stage of the analyses, the obtained promising reactions were conducted on a larger number of accessions.

The results from the ISAP reactions with different primer combinations diversed in the number of generated ISAP amplifications and in the fragments’ size (Table 9). In general, the selected SINE families and the resulting primer combination strongly affected the ISAP banding pattern and the primer combinations differed in the degree of informativeness. Five primer pairs (SolS-Ia-R/SolS-IIIb-R, SolS-Ia-R/SolS-IIIb-F, SolS-Ia-R/SolS-Ib-F, SolS-II-F/SolS-IIIb-R, and SolS-Ib-R/SolS-IIIb-R) did not generate reliable amplification products (Table 9).

**Table 9** ISAP profiles in the pepper accessions obtained by reactions carried out with two primers of different Sol-SINE families

| Primers | Number of amplified fragments | Reproducible fragments (bp) |
|---|---|---|
| SolS-Ib-F/ SolS-II-F | 5 | 2500–690 |
| SolS-Ib-F/ SolS-II-R | 6 | 2500–250 |
| SoSolS-Ib-R/ SolS-II-F | 10 | 3000–310 |
| SolS-Ib-F/ SolS-IIIb-F | 5 | 1900–400 |
| SolS-Ib-F/ SolS-IIIb-R | 3 | 1600–1000 |
| SolS-Ib-R/ SolS-II-R | 3 | 750–300 |
| SolS-Ib-R/ SolS-IIIb-F | 5 | 1700–400 |
| SolS-Ib-R/ SolS-IIIb-R | 0 | n.a. |
| SolS-II-F/ SolS-IIIb-F | 7 | 2500–210 |
| SolS-II-R/ SolS-IIIb-F | 4 | 1990–590 |
| SolS-II-R/ SolS-IIIb-R | 3 | 1300–650 |
| SolS-II-F/ SolS-IIIb-R | 0 | n.a. |
| SolS-Ia-F/ SolS-Ib-F | 2 | 800–600 |
| SolS-Ia-F/ SolS-Ib-R | 5 | 2200–550 |
| SolS-Ia-F/ SolS-II-F | 1 | 2000 |
| SolS-Ia-F/ SolS-II-R | 6 | 1600–490 |
| SolS-Ia-F/ SolS-IIIb-F | 3 | 1200–1000 |
| SolS-Ia-F/ SolS-IIIb-R | 5 | 1900–950 |
| SolS-Ia-R/ SolS-Ib-R | 2 | 790–350 |
| SolS-Ia-R/ SolS-II-F | 4 | 1200–320 |
| SolS-Ia-R/ SolS-II-R | 1 | 1000 |
| SolS-Ia-R/ SolS-Ib-F | 0 | n.a. |
| SolS-Ia-R/ SolS-IIIb-F | 0 | n.a. |
| SolS-Ia-R/ SolS-IIIb-R | 0 | n.a. |

### ISAP Reactions Performed with Primers Designed from Three and More Sol-SINE Families

Other primer combinations included primer pairs from three SINE families, for instance, SolS-Ib-R/F, SolS-II-R/F, and SolS-IIIb-R/F, and the ISAP reactions produced 10 amplified fragments tested in 14 accessions of the two different groups – *C. annuum* local varieties (initial lines) and advanced mutant lines and Bulgarian varieties group of *C. annuum* (Table 8) with 1300-100-bp reproducible fragment length, and the estimated product lengths were 1300, 1050, 850, 750, 480, 400, 300, 250, 180, and 100.

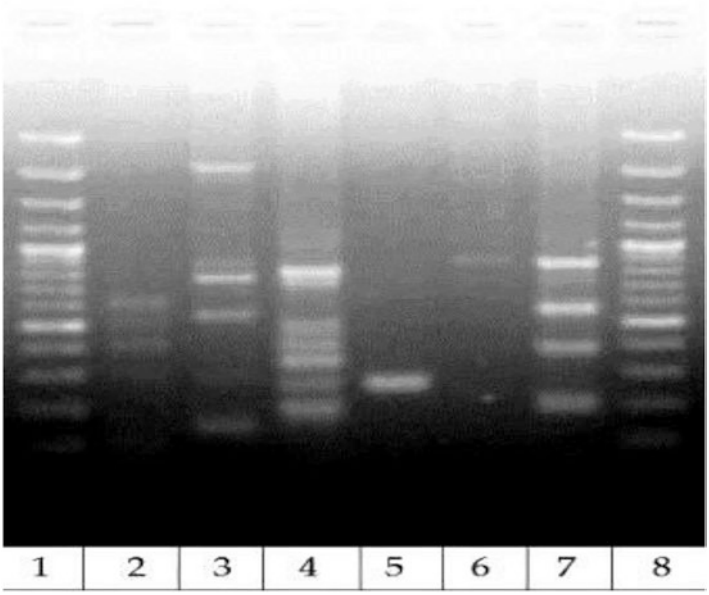



**Fig. 4** Amplification patterns obtained with single-family primer pairs in *C. annuum* variety 'Oranzheva Kapia'. Lanes 1 and 8: 100-bp DNA ladder (500 bp is the first intensive band). Lanes 2–7: SINE families SolS-Ia-F/R, SolS-Ib-F/R, SolS-II-F/R, SolS-IIIa-F/R, SolS-IIIb-F/R, and SolS-V-F/R

## ISAP Single-Family Reactions Performed with Primers Designed from the Same Sol-SINE Family

The next stage of the study involves single-family reactions conducted with two primers F and R from one SolS-SINE family.

We conducted ISAP analyses of multiple *Capsicum* species including *C. annuum, C. baccatum*, *C. fasciculatum*, *C. frutescens*, and *C. pubescens* in order to establish the informativeness of the single-family ISAP reactions (SolS-Ib-F/R, SolS-II-F/R, and SolS-V-F/R). The selected informative reactions were further performed with all samples. Single-family ISAP assays amplified different patterns. Primer pairs from SolS-SINE families IV, VI, and VII led to the amplification of high-molecular bands and were included in multiplex reactions.

The six single-family reactions (SolS-Ia-F/R, SolS-Ib-F/R, SolS-II-F/R, SolS-IIIa-F/R, SolS-IIIb-F/R, and SolS-V-F/R) generated 43 fragments in total at 150–3000-bp range of lengths (Fig. 4). The three reactions with SolS-Ia-F/R, SolS-IIIa-F/R, and SolS-IIIb-F/R generated few fragments with low intensity (Fig. 4, lanes 2, 5, and 6).

Amplification with single-family primers in several genotypes generated reproducible patterns but with few polymorphisms. However, the single-family reaction with SolS-Ib-F/R (Figs. 5 and 6) led to polymorphic patterns between the representatives of the *Capsicum* species, with the exception of *C. baccatum* (Fig. 5, lanes 5,

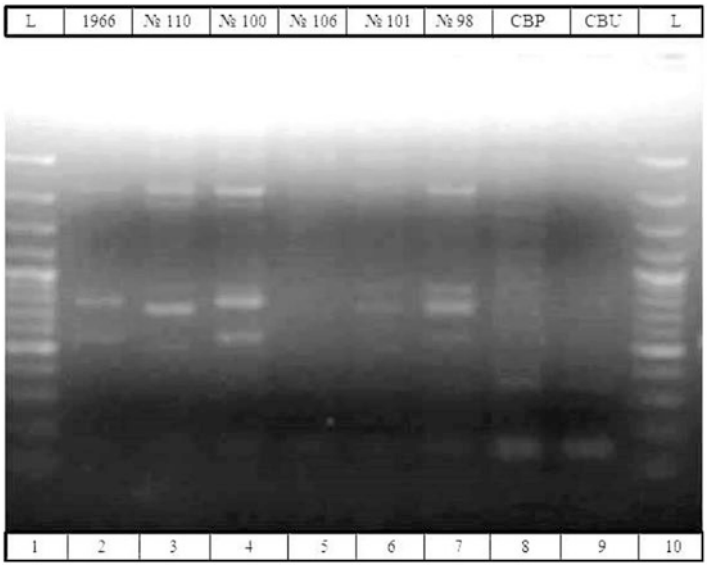



**Fig. 5** Amplification patterns obtained with primers SolS-Ib-SINE in *C. annuum (lane 2*, 'Pazardzhishka Kapia 794'), *C. frutescens* (lane 3, no 110, and lane 7, no 98), *C. fasciculatum* (lane 4, no 100), and *C. baccatum* (lane 5, no 106; lane 6, no 101; lane 8, CBP; and lane 9, CBU)

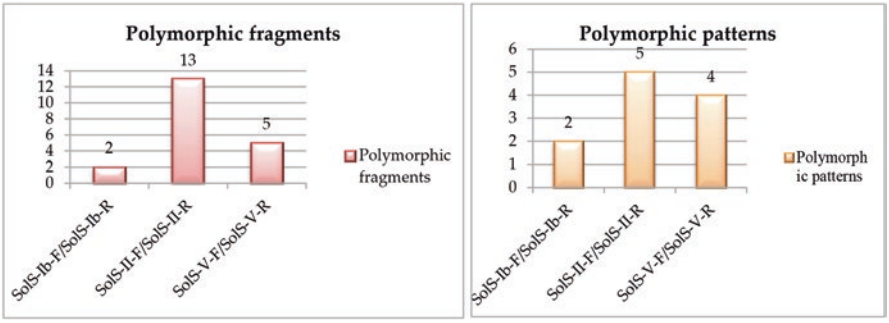



**Fig. 6** ISAP results, obtained with single-family primer pairs. (6a) (Left) Fragments differing by the length between accessions; (6b) (right) polymorphic patterns of pepper accessions

8, and 9), which showed no reliable amplification. The observed polymorphisms were reproducible.

The selected informative reactions (SolS-II-F/R and SolS-V-F/R) were performed with all the samples.

Generally, the application of single-family SINE primers demonstrates the applicability of potato-based ISAP to peppers. Single-family ISAP reactions with SolS-II and SolS-V primers generated the most polymorphic patterns and amplified the highest number of fragments (Fig. 7).

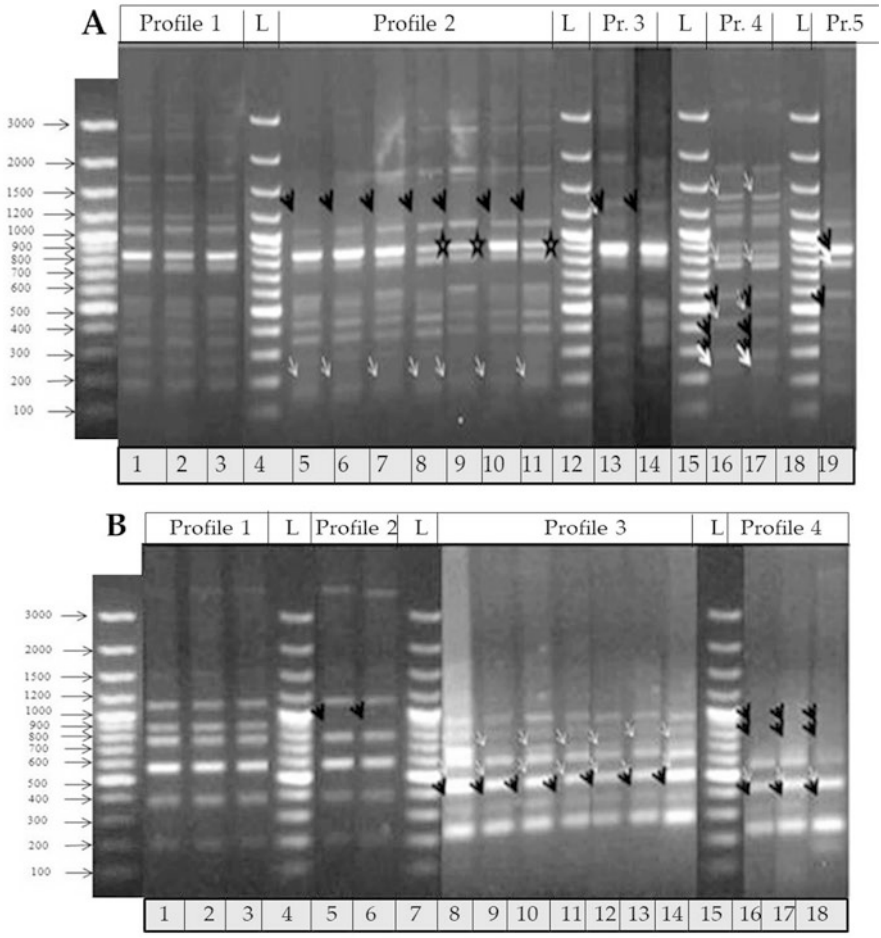



**Fig. 7** Amplification patterns with both primers of SolS-II SINE (panel A amplified with a primer pair SolS-II-F/R; lanes 1–3, *C. annuum*; lanes 5–11, *C. frutescens* from Peru; lanes 13 and 14, *C. annuum* 'Anglia 1'; lanes 16 and 17, *C. baccatum*; lane 19, *C. pubescens*) and of SolS-V SINE (panel B lanes 1–3, *C. annuum*; lane 5, 'Kurtovska Kapia 1619' and 6 'IZK Delicates'; lanes 8–14, *C. frutescens* from Peru; lanes 16–18, *C. baccatum* and *C. pubescens*) on different pepper genotypes. Polymorphic bands are indicated with arrows

Next, we analyzed different pepper genotypes with two ISAP primer combinations and observed recurrent profiles. Single-family reactions with primer pair SolS-II-F/R amplified 18 fragments; five were monomorphic and thirteen polymorphic, resulting in five profiles. The first profile was amplified on most specimens including *C. fasciculatum* and *C. annuum* (Fig. 7a, lanes 1, 2, and 3) with the exception of 'Anglia 1' (Fig. 7a, lanes 13 and 14). The second profile was amplified on specimens from Peru – 'Peru 1', 'Peru 2', 'Peru 4', 'Peru 5', 'Peru 7', 'Peru 8', and 'Peru 10' (Fig. 7a, lanes 5 to 11). The third profile was amplified on genotype 'Anglia 1'. The fourth profile was amplified on specimens from *C. baccatum* ('Peru 6', 'Peru

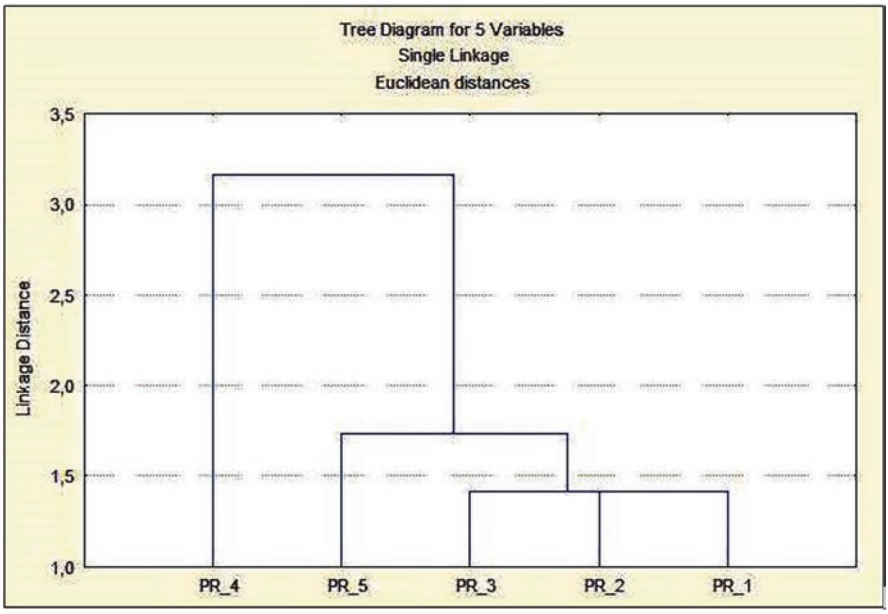



**Fig. 8** Dendrogram of polymorphic ISAP profiles amplified in pepper genotypes with primer pair SolS-II-F/R. PR: profiles

9', no. 106, CBP, and CBU) (Fig. 7a – lanes 16 and 17). The fifth profile was amplified on *C. pubescens* specimen (Fig. 7a – lane 19). Single-family reactions with primer pair SolS-V-F/R amplified seven fragments; two were monomorphic and five polymorphic, resulting in four profiles (Fig. 7b, Table 9). Profiles 1 (Fig. 7b, lanes 1–3) and 2 (variety 'Kurtovska Kapia 1619' and 'IZK Delicates'; Fig. 7b, lanes 5 and 6) were generated in *C. annuum* and *C. fasciculatum*. Profile 3 was amplified in some specimens from Peru ('Peru 1', 'Peru 2', 'Peru 4', 'Peru 5', 'Peru 7', 'Peru 8', 'Peru 10') (Fig. 7b, lanes 8–14). Profile 4 was amplified in *C. baccatum* specimens ('Peru 6', 'Peru 9', no. 106, no. 100, CBP, and CBU) and *C. pubescens* specimens (Fig. 7b, lanes 16–18).

According to cluster analysis, profiles 1, 2, and 3 include accessions of representatives of *C. annuum*, *C. frutescens* from Peru) amplified with primer pair SolS-II--F/R that were the most similar (Figs. 7a and 8). Profile 5 was generated on *C. pubescens* specimen (occupied an intermediate position) and the most different was profile 4 generated on *C. baccatum* specimens (Fig. 7a and 8).

Comparison of the patterns obtained by SolS-V family primers revealed that profiles 1 and 2 were the most similar. Profile 3 occupies an intermediate position, and the most distant is profile 4 (Figs. 7b and 9). According to amplified profiles, cluster analysis with primer pair SolS-V-F/R profiles 1 and 2 including accessions of *C. annuum* were the most similar. Profile 3 amplified in accessions from *C. frutescens* from Peru (occupied an intermediate position) and the most different was profile 4 amplified in *C. baccatum* and *C. pubescens* genotypes (Figs. 7b and 9).

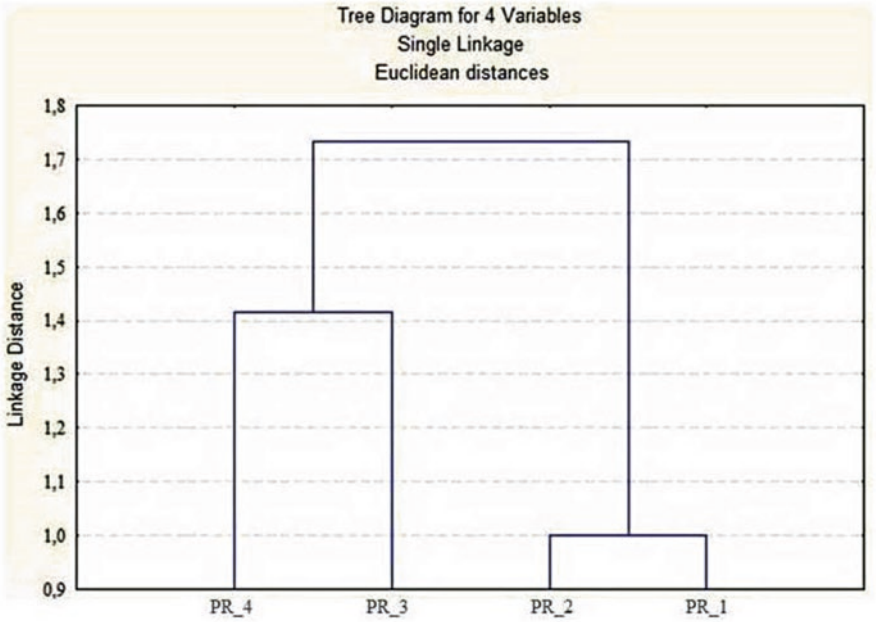



**Fig. 9** Dendrogram of polymorphic ISAP profiles amplified in pepper genotypes with primer pair SolS-V-F/R. PR: profiles

**Table 10** ISAP profiles, obtained with single-family primer pairs in the six groups of plant specimens

| Plant specimen group no. | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Primer pair | Polymorphic profiles amplified | | | | | |
| SolS-II-F/SolS-II-R | 1, 2, 4, 5 | 1 and 3 | 1 | 1 | 1 | 1 |
| SolS-V-F/SolS-V-R | 1, 3, 4 | 1 | 1 | 1 | 1 | 1 and 2 |

Reactions with primer pairs SolS-II-F/R and SolS-V-F/R generated distinctive profiles on *C. baccatum* specimens shown in Table 10.

Thus, the best results from pepper collection genotyping came from using primers from the families SolS-II and SolS-V. Our data suggest that SolS-IV, SolS-VI, and SolS-VII are not suitable for pepper genomic characterization by ISAP profiling since they amplify very long and irreproducible fragments.

Next, the applicability of four primers from different SolS families in different combinations in one PCR reaction was investigated (Fig. 10, Table 11).

Since single-family reactions with single-family primer pairs SolS-Ia, SolS-IIIa, and SolS-IIIb did not demonstrate potential for pepper genotyping, we performed multiplex (combinatorial between SINE families) reactions M1 (IaF + IbF), M3 (Ia + IIIa), M4 (Ia + IIIb), and M22 (IIIa+IIIb) on selected accessions from all six specimen groups (Fig. 10). These reactions produced polymorphic patterns mainly within different *Capsicum* species; however, they were weak and unclear, especially for M4 reaction (Fig. 10).

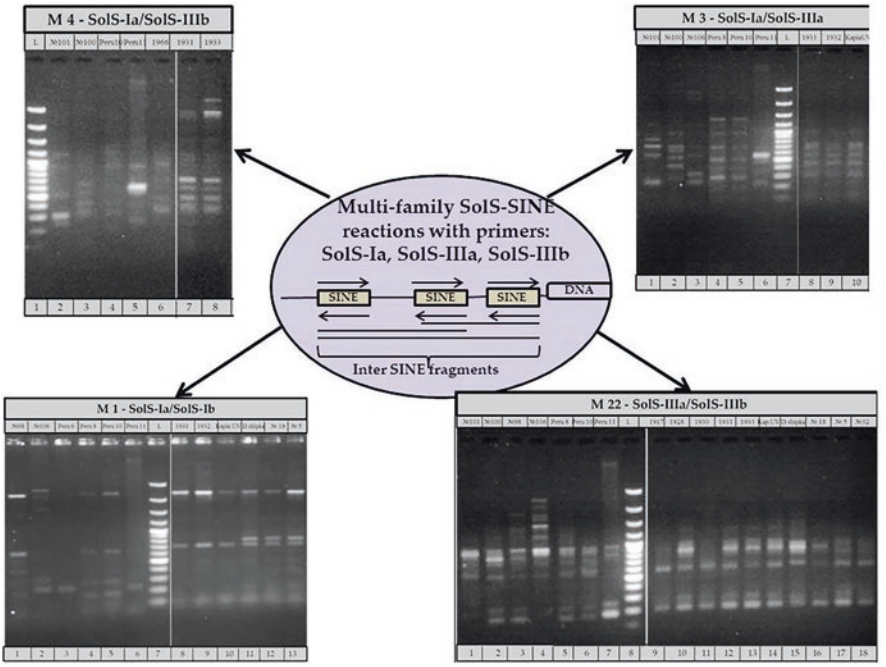



**Fig. 10** Amplification patterns of multifamily primer pairs SolS-Ia, SolS-IIIa, and SolS-IIIb with selected representatives of all six specimen groups. M1 (lanes 1, 4, and 5, *C. baccatum*; lane 2, *C. fasiculatum*; lanes 3, 6, and 7, *C. frutescens*; lane 8, *C. pubescens*; lanes 10–15, *C. annuum*), M3 (lanes 1, 4, and 5, *C. baccatum*; lane 2, *C. fasiculatum*; lanes 3, 6, and 7, *C. frutescens*; lane 8, *C. pubescens*; lanes 10, 11, and 12, *C. annuum*), M4 (lanes 2, 5, and 6, *C. baccatum*; lane 3, *C. fasiculatum*; lanes 4, 7, and 8, *C. frutescens*; lane 9, *C. pubescens*; and lanes 10, 11, and 12, *C. annuum*), M22 (lanes 1 and 4, *C. baccatum*; lane 2, *C. fasiculatum*; lanes 3, 5, and 6, *C. frutescens*; lanes 9–18, *C. annuum*)

For that reason, we performed 22 more combinatorial reactions (M5, M7, M8, M10–12, M14, M15, M18, M21, M23, M25–27, and M29–36) on two specimens – 'Pazardzhishka Kapia 794' and 'Oranzheva Kapia' varieties (Fig. 11). All those reactions produced monomorphic patterns in the two studied genotypes, and Fig. 11 shows only one of the two similar profiles. Performed multiplex combinatorial reactions amplified different numbers of fragments from 1 in reaction M32 and M33 to 18 fragments in reactions M2, M 9, M16, M17, M18, M20, and M21 (Table 11).

Combinatorial reactions that included one member of the primer pair SolS-II--R/F (M2, M9, M16–18, M20, and M21) generated patterns that are identical to those generated by single-family amplification with this pair. One exception is 'Anglia 1'. Patterns from reactions M2 and M20 lack 1100-bp and 1070-bp fragments (Fig. 7a, lane 14). The pattern generated by the M9 reaction is identical to profile 1 in *C. annuum* (Fig. 7a, lanes 1–3). M16 and M17 generated patterns identical to those in profile 2 from the single-family amplification using primer pair SolS-II-F/R (Fig. 7a, lane 13).

**Table 11** ISAP profiles of four multifamily SolS-SINE primers

| Primer set | Number of screened accessions | Number of amplified fragments |
|---|---|---|
| M1 – SolS-Ia/SolS-Ib | 26 | 5 |
| M2 – SolS-Ia/SolS-II | 73 | 18 |
| M3 – SolS-Ia/SolS-IIIa | 19 | 5 |
| M4 – SolS-Ia/SolS-IIIb | 14 | 8 |
| M5 – SolS-Ia/SolS-IV | 2 | 4 |
| M6 – SolS-Ia/SolS-V | 6 | 7 |
| M7 – SolS-Ia/SolS-VI | 2 | 4 |
| M8 – SolS-Ia/SolS-VII | 2 | 4 |
| M9 – SolS-Ib/SolS-II | 73 | 18 |
| M10 – SolS-Ib/SolS-IIIa | 2 | 6 |
| M11 – SolS-Ib/SolS-IIIb | 2 | 0 |
| M12 – SolS-Ib/SolS-IV | 2 | 4 |
| M13 – SolS-Ib/SolS-V | 6 | 7 |
| M14 – SolS-Ib/SolS-VI | 2 | 6 |
| M15 – SolS-Ib/SolS-VII | 2 | 4 |
| M16 – SolS-II/SolS-IIIa | 73 | 18 |
| M17 – SolS-II/SolS-IIIb | 73 | 18 |
| M18 – SolS-II/SolS-IV | 2 | 18 |
| M19 – SolS-II/SolS-V | 6 | 8 |
| M20 – SolS-II/SolS-VI | 73 | 18 |
| M21 – SolS-II/SolS-VII | 2 | 18 |
| M22 – SolS-IIIa/SolS-IIIb | 19 | 15 |
| M23 – SolS-IIIa/SolS-IV | 2 | 8 |
| M24 – SolS-IIIa/SolS-V | 73 | 7 |
| M25 – SolS-IIIa/SolS-VI | 2 | 3 |
| M26 – SolS-IIIa/SolS-VII | 2 | 0 |
| M27 – SolS-IIIb/SolS-IV | 2 | 8 |
| M28 – SolS-IIIb/SolS-V | 73 | 7 |
| M29 – SolS-IIIb/SolS-VI | 2 | 8 |
| M30 – SolS-IIIb/SolS-VII | 2 | 8 |
| M31 – SolS-IV/SolS-V | 2 | 7 |
| M32 – SolS-IV/SolS-VI | 2 | 1 |
| M33 – SolS-IV/SolS-VII | 2 | 1 |
| M34 – SolS-V/SolS-VI | 2 | 7 |
| M35 – SolS-V/SolS-VII | 2 | 7 |
| M36 – SolS-VI/SolS-VII | 2 | 7 |

Our data suggest that SolS-IV, SolS-VI, and SolS-VII were not suitable for pepper ISAP profiling since they amplified very long and irreproducible fragments. The same can be said for the SolS-IIIa reaction, which results in the amplification of a small number of fragments (three fragments) and is, therefore, inappropriate. Multiplex combinatorial reactions performed by participation of one member of the primer pair SolS-V-F/R (M6, M13, M31, and M34–36) also amplified patterns,

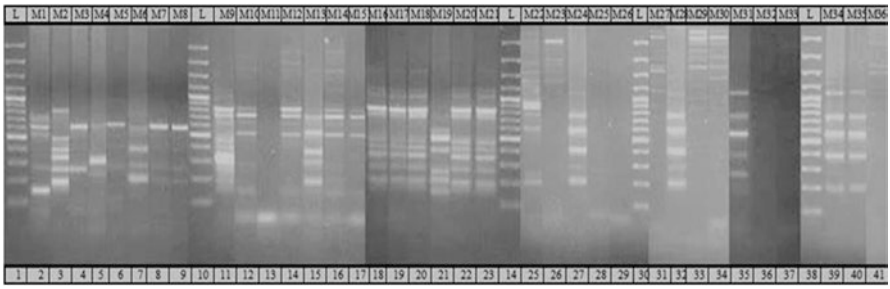


**Fig. 11** Amplification patterns obtained with multifamily primer pairs for the pepper variety 'Pazardzhishka Kapia 794' with primer sets listed in Table 11. Lanes 1, 10, 24, 30, and 38, DNA ladder; lanes 2–9, primer sets M1–8; lanes 11–23, primer sets M9–21; lanes 25–29, primer sets M22–26; lanes 31–37, primer sets M27–33; lanes 39–41, primer sets M34–36

which were identical to single-family amplifications by primers from this pair. In 'Kurtovska Kapia 1619', sets M28 (primer pair SolS-V/SolS-IIIb) and M24 (primer pair SolS-V/SolS-IIIa) generated a weak fragment with length of 900 bp and the patterns were identical to profile 1 of all Bulgarian *C. annuum* varieties. The best results were obtained with primer pairs SolS-II-F/R and SolS-V-F/R. SolS-II-F/R generated patterns that are clear, reproducible, and most polymorphic (Fig. 7). Combinatorial reactions with representative of these pairs generate similar patterns.

### 1.6.4 Discussion

Nowadays, efficient breeding programs have evolved into marker-assisted breeding programs. There are a number of requirement criteria for molecular marker methods: informativeness, reproducibility, and experimental requirements regarding time and equipment. One way to respond to breeders' needs is to adapt novel methods to a particular species. ISAP was developed for potatoes, but has potential for the related pepper as it is evident from the results obtained.

Markers, based on SINE or other mobile elements, are often designated as the "two-in-one" type and can be used not only for "simple" genotyping but also for more detailed research (Schmidt & Heslop-Harrison, 1998). When implemented in breeding programs, these markers make it possible to genetically characterize plants, regardless of environmental conditions and development stage (Ray, 2007). ISAP genotyping is based on the detection of SINE presence/absence at a particular locus (Tomlekova et al., 2017).

Annotation of 8.5 Gbp revealed 82,983 SINE copies related to all known Solanaceae SINE families. Thirty percent of SINEs is associated with genes (mostly in introns and non-translated regions). Some 10% of annotated genes contain at least one SINE insertion and one gene contained up to 16 SINE copies. In potato, SINEs contribute 0.32% to the genome, whereas in other species and varieties, SINEs account for approximately 0.15% (Seibt et al., 2016).

Seven SolS-SINE families and two subfamilies were identified in potato and nine SINE-specific primer pairs were designed (Seibt et al., 2016; Wenke et al., 2011). When applied on pepper, almost all SINE-based ISAP reactions with specific primers generated amplification products, but few pairs were generating clear polymorphic patterns. The results obtained, in the present study, demonstrate that three Sol-SINE families (SolS-IV, SolS-VI, and SolS-VII) are not useful in pepper because they amplify long unreproducible fragments. The single-family primer pairs with the most potential in pepper are SolS-V-F/SolS-V-R and SolS-II-F/SolS-II-R. This is different from potato, in which the most informative patterns are from SolS-IIIa-F/SolS-IIIa-R, SolS-IIIa-F/SolS-IV-F, and SolS-IIIa-F/SolS-IV-R (Seibt et al., 2012; Tomlekova et al., 2017b). The bioinformatic analysis of the genomes of four representatives of the Solanaceae family of potato (*S. tuberosum*), tomato (*S. lycopersicum*), wild tomato (*S. pennellii*), and pepper (*C. annuum* variety 'Zunla-1' and variety 'CM334') showed the presence of 82,983 SINE copies belonging to families SolS-I to SolS-VII and Au. The highest number of copies (21,398 SINEs) were found in *Capsicum* in variety 'CM334' and 26,204 in variety 'Zunla-1'. In all investigated Solanaceae species, the SolS-II family is represented with the highest copy number. Their number of copies varies from 2479 (tomatoes) to 7044 (for variety 'Zunla-1'). The maximum number of SINE elements averaged per 5 Mbp of the genome is about 180 in tomato, 270 in potato, and more than 340 in pepper. The highest number of SINEs associated with genes were found in pepper 'Zunla-1' – 15.0%. The highest number of SINEs (16 copies) accumulated in a single gene was observed in gene Capana12g002506. The two most abundant families are SolS-IIIa and SolS-IV in potato in comparison with their distribution in the wild potato species, tomato, pepper, and tobacco established by southern hybridization (Seibt et al., 2012; Wenke et al., 2011). In the cultivated potato, these two SINE families have had recent activity. ISAP-derived molecular markers are therefore highly polymorphic (Seibt et al., 2012). The obtained results showed that, in pepper, three of the SolS-SINE families – SolS-IV, SolS-VI, and SolS-VII – were not suitable for genotyping pepper because they lead to the amplification of excessively long fragments that are not reproducible. The SolS-IIIa reaction resulted in the amplification of a small number of fragments (three fragments). Families SolS-IIIa, SolS-IV, and SolS-VI burst in potato. Family SolS-V is promising and abundant in pepper from our results as well as in potato from the results obtained by Seibt et al. (2016).

Sol-SINE application on 17 pepper specimens that do not belong to *C. annuum* generated polymorphic patterns suitable for genotyping purposes. For *C. annuum* specimens, the primer pairs SolS-II-F/SolS-II-R and SolS-V-F/SolS-V-R appear to be the most appropriate for genetic characterization.

We believe that potato-derived SINE-based ISAP primers can be used to identify pepper species but not for intraspecies discrimination for identification. Primer pairs SolS-II-F/SolS-II-R and SolS-V-F/SolS-V-R generate distinctive patterns for *C. baccatum*. These primer pairs also generate the most informative patterns for *C. annuum*. In particular, primer pair SolS-II-F/SolS-II-R generates five distinctive amplification profiles and 13 polymorphic fragments in *Capsicum* spp. In

investigated Solanaceae species, the SolS-II family is represented with the highest copy number that ranges from 2479 (tomato) to 7044 in pepper (Wenke et al., 2011).

These results agree with bioinformatic data showing that the SolS-II family has the highest copy number in *Solanaceae*. Most likely, members of the SolS-II family were active during the genus' evolution and especially after species divergence. Furthermore, such activity appears to be linked to species-specific genome rearrangements (Seibt et al., 2016).

ISAP-based genotyping depends on the SINE presence at a particular locus. The biology of SINE, as well as other mobile elements, makes it possible to genotype and track evolutionary connections (Schmidt & Heslop-Harrison, 1998). In pepper, the adapted potato SINE-based ISAP reactions generate less polymorphism than in the source organism. This limits the method's applicability for a more detailed comparison between breeding lines. To overcome this limitation, pepper-specific ISAP primers need to be designed based on available pepper genome sequences (Kim et al., 2014; Tomlekova et al., 2016). Here, the SINE family Au might be included, as it is both highly abundant in the pepper genome and frequently associated with genes. Thus, pepper-specific ISAP could become an important tool in breeding programs in the future.

### 1.6.5 Conclusions

The potato-based ISAP method can be applied to pepper, but with limitations. The direct use of potato-specific primers generates polymorphism mainly at interspecies, but not at the intraspecies level. One could expect that development of primers based on pepper SINE elements might greatly improve the applicability of this robust method in pepper breeding programs.

**Acknowledgments** The authors acknowledge financial support from the International Atomic Energy Agency, grant numbers BUL/5/016 and RER/5/024. We are grateful for the contribution of Prof. Thomas Schmidt (Dresden University of Technology, Germany) in the development and introduction of the ISAP technique as a means of identifying and studying members of the family Solanaceae.

**Funding** This research was funded by International Atomic Energy Agency, Technical Cooperation projects BUL/5/016 and RER/5/024.

## References

Adetula, O. (2005). Genetic diversity of capsicum using random amplified polymorphic DNAs. *African Journal of Biotechnology, 5*, 120–122.

Ahn, Y., Tripathi, S., Kim, J., Cho, Y., Lee, H., Kim, D., Woo, J., & Yoon, M. (2014). Microsatellite marker information from high-throughput next-generation sequence data of *Capsicum annuum* varieties Mandarin and Blackcluster. *Scientia Horticulturae, 170*, 123–130.

Alzohairy, A. M., Gyulai, G., Mustafa, M., Edris, S., Sabir, J., Jansen, R. K., & Bahieldin, A. (2015). Retrotransposon-based plant DNA barcoding. In M. Ajmal Ali, G. Gyulai, & F. A. Hemaid (Eds.), *Plant DNA barcoding and Phylogenetics* (pp. 1–14). Lambert Academic Publishing.

Arnedo-Andrés, M., Gil-Ortega, R., Luis-Arteaga, M., & Hormaza, J. (2002). Development of RAPD and SCAR markers linked to the Pvr4 locus for resistance to PVY in pepper (*Capsicum annuum* L.). *Theoretical and Applied Genetics, 105*, 1067–1074.

Bahurupe, J. V., Sakhare, S. B., Kulwal, P. L., Akhare, A. A., & Pawar, B. D. (2013). Genetic diversity analysis in chilli (*Capsicum annuum* L.) using RAPD markers. *The Bioscan, An International Quarterly Journal of Life Sciences, 8*, 915–918.

Ballester, J., & Carmen de Vicente, M. (1998). Determination of $F_1$ hybrid seed purity in pepper using PCR-based markers. *Euphytica, 103*, 223–226.

Baoxi, Z., Sanwen, H., Guimei, Y., & Jiazhen, G. (2000). Two RAPD markers linked to a major fertility restorer gene in pepper. *Euphytica, 113*, 155–161.

Bello-Bello, J., Iglesias-Andreu, L., Avilés-Viñas, S., Gómez-Uc, E., Canto-Flick, A., & Santana-Buzzy, N. (2014). Somaclonal variation in habanero pepper (*Capsicum chinense* Jacq.) as assessed ISSR molecular markers. *Hort Science, 49*, 481–485.

Biedler, J., & Tu, Z. (2003). Non-LTR retrotransposons in the African malaria mosquito, Anopheles gambiae: Unprecedented diversity and evidence of recent activity. *Molecular Biology and Evolution, 20*, 1811–1825.

Cost, G. J., & Boeke, J. D. (1998). Targeting of human retrotransposon integration is directed by the specificity of the L1 endonuclease for regions of unusual DNA structure. *Biochemistry, 37*, 18081–18093.

Deragon, J., & Zhang, X. (2006). Short Interspersed Elements (SINEs) in plants: Origin, classification, and use as phylogenetic markers. *Systematic Biology, 55*, 949–956.

Eickbush, T. H., & Malik, H. S. (2002). In N. L. Craig, R. Craigie, M. Gellert, & A. M. Lambowitz (Eds.), *Mobile DNA II* (pp. 1111–1146). ASM, Herndon.

Ferguson, A., Zhao, D., & Jiang, N. (2013). Selective acquisition and retention of genomic sequences by Pack-Mutator-like elements based on guanine-cytosine content and the breadth of expression. *Plant Physiology, 163*, 1419–1432.

Feschotte, C., Jiang, N., & Wessler, S. (2002). Plant transposable elements: Where genetics meets genomics. *Macmillan Magazines, 3*, 329–341.

Geleta, L. F., Labuschagne, M. Y., & Viljoen, C. D. (2005). Genetic variability in pepper (*Capsicum annuum* L.) estimated by morphological data and amplified fragment length polymorphism markers. *Biodiversity and Conservation, 14*, 2361–2375.

Gozukirmizi, N., Yilmaz, S., Marakli, S., & Temel, A. (2015). *Retrotransposon-based molecular markers; tools for variation analysis in plants. Applications of molecular markers in plant genome analysis and breeding, Eds*. Ksenija Taški-Ajduković Research Signpost.

Hazarika, R., & Neog, B. (2014). Investigation of intraspecific diversity in *Capsicum chinense* using morphological and molecular markers. *Indian Journal of Genetics and Plant Breeding, 74*, 392–395.

Hedges, D. J., & Batzer, M. A. (2005). From the margins of the genome: Mobile elements shape primate evolution. *BioEssays, 27*, 785–794.

Ibarra-Torresa, P., Valadez-Moctezumab, E., Pérez-Grajalesb, M., Rodríguez-Camposc, J., & Jaramillo-Floresa, M. (2015). Inter- and intraspecific differentiation of *Capsicum annuum* and *Capsicum pubescens* using ISSR and SSR markers. *Scientia Horticulturae, 181*, 137–146.

Ilbi, H. (2003). RAPD markers assisted varietal identification and genetic purity test in pepper, *Capsicum annuum*. *Scientia Horticulturae, 97*, 211–218.

Jang, I., Moon, J. H., Yoon, J. H., Yang, T. J., Kim, Y. J., & Park, H. G. (2004). Application of RAPD and SCAR markers for purity testing of $F_1$ hybrid seed in chili pepper (*Capsicum annuum*). *Molecular Cell, 18*, 295–299.

Kajikawa, M., Ichiyanagi, K., Tanaka, N., & Okada, N. (2005). Isolation and characterization of active LINE and SINEs from the Eel. *Molecular Biology and Evolution, 22*, 673–682.

Kalendar, R., & Schulman, A. H. (2006). IRAP and REMAP for retrotransposon-based genotyping and fingerprinting. *Nature Protocols*, 12478–12484.

Kalendar, R., Vicient, C. M., Peleg, O., Anamthawat-Jonsson, K., Bolshoy, A., & Schulman, A. (2004). Large retrotransposon derivatives: Abundant, conserved but nonautonomous retroelements of barley and related genomes. *Genetics, 166*, 1437–1450.

Kalendar, R., Flavell, A. J., Ellis, T., Sjakste, T., Moisy, C., & Schulman, A. H. (2011). Analysis of plant diversity with retrotransposon-based molecular markers. *Heredity (Edinburgh), 106*, 520–530.

Kim, S., Park, M., Yeom, S., Kim, Y. M., Lee, J. M., Lee, A. H., Seo, E., Choi, J., Cheong, K., Kim, T. K., Lee, W. G., Oh, K. S., Bae, C., Kim, S. B., Lee, Y. H., Kim, S. Y., Kim, M. S., Kang, C. C., Jo, D. Y., Yang, H. B., Jeong, J. H., Kang, H. W., Kwon, J. K., Shin, C., Lim, Y. J., Park, H. J., Huh, H. J., Kim, S. J., Kim, D. B., Cohen, O., Paran, I., Suh, C. M., Lee, B. S., Kim, K. Y., Shin, Y., Noh, J. S., Park, J., Seo, S. Y., Kwon, Y. S., Kim, A. H., Park, M. J., Kim, J. H., Chio, S. H., Lee, S. M., Yu, Y., Choi, D. Y., Park, S. B., Deynze, A., Ashrafi, H., Hill, T., Kim, T. W., Pai, S. H., Ahn, K. H., Yeam, I., Giovannoni, J. J., Rose, K. J., Sorensen, T. W., Lee, J. S., Kim, W. R., Choi, Y. I., Choi, S. B., Lim, S. J., Lee, H. Y., & Choi, D. (2014). Genome sequence of the hot pepper provides insights into the evolution of pungency in capsicum species. *Nature Genetics, 46*, 270–278.

Kumar, A., Pearce, S., McLean, K., Harrison, G., Heslop-Harrison, J. S., Waugh, R., & Flavell, A. J. (1997). The Ty1-copia group of retrotransposons in plants: Genomic organisation, evolution, and use as molecular markers. *Genetica, 100*(1–3), 205–217. https://doi.org/10.1023/A:1018393931948

Kumar, S., Singh, V., Singh, M., Raia, S., Kumar, S., Rai, S., & Rai, M. (2007). Genetics and distribution of fertility restoration associated RAPD markers in inbreds of pepper (*Capsicum annuum* L.). *Scientia Horticulturae, 111*, 197–202.

Lee, H., Ayarpadikannan, S., & Kim, H. (2015). Role of transposable elements in genomic rearrangement, evolution, gene regulation and epigenetics in primates. *Genes and Genetic Systems, 90*, 245–257.

Li, G., & Quiros, C. F. (2001). Sequence-related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: Its application to mapping and gene tagging in Brassica. *Theoretical Applied Genetics, 103*, 455–461.

Lijun, O., & Xuexiao, Z. (2012). Inter simple sequence repeat analysis of genetic diversity of five cultivated pepper species. *African Journal of Biotechnology, 11*, 752–757.

Lippman, Z., Gendrel, A. V., Black, M., Vaughn, M. W., Dedhia, N., McCombie, W. R., Colot, V., & Martienssen, R. (2004). Role of transposable elements in heterochromatin and epigenetic control. *Nature, 430*, 471–476.

Livingstone, K. D., Lackney, K., Blauth, R. J., Van Wijk, R., & Jahn, M. K. (1999). Genome mapping in capsicum and evolution of genome structure in Solanaceae. *Genetics, 155*, 1183–1202.

Min, W. K., Han, J. H., Kang, W. H., Lee, H. R., & Kim, B. D. (2008). Reverse random amplified microsatellite polymorphism reveals enhanced polymorphisms in the 3' end of simple sequence repeats in the pepper genome. *Molecules and Cells, 26*, 250–257.

Minamiyama, Y., & Tsuro, M. H. (2006). An SSR-based linkage map of *Capsicum annuum*. *Molecular Breeding, 18*, 157–169.

Moodley, V., Ibaba, J., Naidoo, R., & Gubba, A. (2014). Full-genome analyses of a Potato Virus Y (PVY) isolate infecting pepper (*Capsicum annuum* L.) in the Republic of South Africa. *Virus Genes, 49*, 466–476.

Moore, J. K., & Haber, E. J. (1996). Capture of retrotransposon DNA at the sites of chromosomal double-strand breaks. *Nature, 383*, 644–646.

Munoz-Lopez, M., & Perez-Garacia, J. (2010). DNA transposons: Nature and applications in genomics. *Current Genomics, 11*, 115–128.

Murray, M. G., & Thompson, W. F. (1980). Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research, 8*, 4321–4325.

Ogundiwin, E. A., Berke, T. F., Massoudi, M., Black, L. L., Huestis, G., Choi, D., Lee, S., & Prince, J. P. (2005). Construction of 2 intraspecific linkage maps and identification of resistance QTLs for *Phytophthora capsici* root-rot and foliar-blight diseases of pepper (*Capsicum annuum* L.). *Genome, 48*, 698–711.

Paran, I. (2013). Molecular linkage maps of capsicum. In B. Kang & C. Kole (Eds.), *Genetics, genomics and breeding of peppers and eggplants* (pp. 40–55). Taylor & Francis Group, 161 p.

Park, M., Jo, S., Kwon, J.-K., & Choi, D. (2011). Comparative analysis of pepper and tomato reveals euchromatin expansion of pepper genome caused by differential accumulation of Ty3/Gypsy-like elements. *BMC Genomics, 12*, 85.

Poryazov, I., Petkova, V., & Tomlekova, N. (2013). *Vegetable production*. "DIMI 99" Ltd. 318 p. /Bulgarian/.

Pradeepkumar, T., Karihaloo, J. L., & Archak, S. (2001). Molecular characterization of *Piper nigrum* L. cultivars using RAPD markers. *Current Sciences, 81*, 245–248.

Qin, C., Yu, C., Shen, Y., Fang, X., Chen, L., Min, J., Cheng, J., Zhao, S., Xu, M., Luo, Y., Yang, Y., Wu, Z., Mao, L., Wu, H., Ling-Hu, C., Zhou, H., Lin, H., González-Morales, S., Trejo-Saavedra, D., Tian, H., Tang, X., Zhao, M., Huang, Z., Zhou, A., Yao, X., Cui, J., Li, W., Chen, Z., Feng, Y., Niu, Y., Bi, S., Yang, X., Li, W., Cai, H., Luo, X., Montes-Hernández, S., Leyva-González, M., Xiong, Z., He, X., Bai, L., Tan, S., Tang, X., Liu, D., Liu, J., Zhang, S., Chen, M., Zhang, L., Zhang, L., Zhang, Y., Liao, W., Zhang, Y., Wang, M., Lv, X., Wen, B., Liu, H., Luan, H., Zhang, Y., Yang, S., Wang, X., Xu, J., Li, X., Li, S., Wang, J., Palloix, A., Bosland, P., Li, Y., Krogh, A., Rivera-Bustamante, R., Herrera-Estrella, L., Yin, Y., Yu, J., Hu, K., & Zhang, Z. (2014). Whole-genome sequencing of cultivated and wild peppers provides insights into Capsicum domestication and specialization. *Proceeding of the National Academy of Sciences of the United States of American, 111*, 5135–5140.

Ravindran, S. (2012). Barbara McClintock and the discovery of jumping genes. *Proceeding of the National Academy of Sciences of the United States of America, 109*, 20198–20199.

Ray, D. A. (2007). SINEs of progress: Mobile element applications to molecular ecology. *Molecular Ecology, 16*, 19–33.

Rodriguez, J. M., Berke, T., Engle, L., & Nienhuis, J. (1999). Variation among and within Capsicum species revealed by RAPD markers. *Theoretical Applied Genetics, 99*, 147–156.

Saghai-Maroof, M. A., Soliman, K. M., Jorgensen, R. A., & Allard, R. W. (1984). Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. *Proceedings of the National Academy of Sciences of the United States of America, 81*, 8014–8018.

Schmidt, T. (1999). LINEs, SINEs and repetitive DNA: Non-LTR retrotransposons in plant genomes. *Plant Molecular Biology, 40*, 903–910.

Schmidt, T., & Heslop-Harrison, J. S. (1998). Genomes, genes and junk: The large-scale organization of plant chromosomes. *Trends in Plant Science, 3*, 195–199.

Schulman, A. (2007). A unified classification system for eukaryotic transposable elements. *Genetics, 8*, 973–982.

Seibt, K. M., Wenke, T., Wollrab, C., Junghans, H., Muders, K., Dehmer, K. J., Diekmann, K., & Schmidt, T. (2012). Development and application of SINE-based markers for genotyping of potato varieties. *Theoretical Applied Genetics, 125*, 185–196.

Seibt, K. M., Wenke, T., Muders, K., Trubergm, B., & Schmidt, T. (2016). Short interspersed nuclear elements (SINEs) are abundant in Solanaceae and have a family-specific impact on gene structure and genome organization. *The Plant Journal, 86*, 268–285.

Sundaram, V., Cheng, Y., Ma, Z., Li, D., Xing, X., Edge, P., Snyder, M., & Wang, T. (2014). Wide spread contribution of transposable elements to the innovation of gene regulatory networks. *Cold Spring Harbor Laboratory Press, 26*, 1–14.

Tam, S. M., Mhiri, C., Vogelaar, A., Kerkveld, M., Pearce, S. R., & Marie-Angèle, G. (2005). Comparative analyses of genetic diversities within tomato and pepper collections detected by retrotransposon-based SSAP, AFLP and SSR. *Theoretical and Applied Genetics, 110*, 819–831.

Tanksley, S. D., Bernatzky, R., Lapitan, N. L., & Prince, J. P. (1988). Conservation of gene repertoire but not gene order in pepper and tomato. *Proceedings of the National Academy of Sciences of the United States of America, 85*, 6419–6423.

Thul, S., Darokar, M., Shasany, A., & Khanuja, S. (2012). Molecular profiling for genetic variability in Capsicum species based on ISSR and RAPD markers. *Molecular Biotechnology, 51*, 137–147.

Todorova, V., Boteva, H., Masheva, S., Cholakov, T., Kostova, D., Yankova, V., & Dincheva, T. S. (2014). Technologies for field pepper production. In S. Masheva, M. Mihov, V. Todorova, E. Nacheva, V. Yankova, & H. Boteva (Eds.), *Technologies for production of vegetable crops and potatoes* (pp. 41–66). Bulgarian, first edition, circulation 300, Blakom printing house – Plovdiv. 245 p. /Bulgarian/.

Tomlekova, N., Spasova-Apostolova, V., & Panchev, I. (2016). RAPD analysis of Bulgarian pepper induced mutants. *Comptes rendus de l'Académie bulgare des sciences, 69*(6), 731–738.

Tomlekova, N., Spasova-Apostolova, V., Nacheva, E., Stoyanova, M., Teneva, A., Petrov, N., & Schmidt, T. (2017a). Genotyping of Bulgarian potato varieties by SINE–based ISAP markers. *Comptes rendus de l'Académie bulgare des Sciences, 70*, 61–70.

Tomlekova, N. B., White, P. J., Thompson, J. A., Penchev, E., & Nielen, S. (2017b). Mutation affecting fruit colour increases β-carotene concentration in sweet pepper. *PLoS One, 12*(2), e0172180. https://doi.org/10.1371/journal.pone.0172180

Tomlekova, N., Spasova-Apostolova, V., Pantchev, I., & Sarsu, F. (2021). Mutation associated with orange fruit colour increases concentrations of β-carotene in a sweet pepper variety (*Capsicum annuum* L.). *Food, 10*(6), 1225. https://doi.org/10.3390/foods10061225

Toquica, S., Rodríguez, F., Martínez, E., Duque, M., & Tohme, J. (2003). Molecular characterization by AFLPs of *capsicum* germplasm from the Amazon. *Genetic Resources and Crop Evolution, 50*, 639–647.

Van de Lagemaat, L. N., Gagnier, L., Medstrand, P., & Mager, D. L. (2005). Genomic deletions and precise removal of transposable elements mediated by short identical DNA segments in primates. *Genome Research, 15*, 1243–1249.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., & Zabeau, M. (1995). AFLP: A new technique for DNA fingerprinting. *Nucleic Acids Research, 23*, 4407–4414.

Votava, E., Nabhan, G., & Bosland, P. (2002). Genetic diversity and similarity revealed via molecular analysis among and within an *in-situ* population and *ex situ* accessions of chiltepín (*Capsicum annuum* var. *glabriusculum*). *Conservation Genetics, 3*, 123–129.

Wang, T., Zeng, J., Lowe, C. B., Sellers, R. G., Salama, S. R., Yang, M., Burgess, S. M., Brachmann, R. K., & Haussler, D. (2007). Species-specific endogenous retroviruses shape the transcriptional network of the human tumor suppressor protein p 53. *Proceedings of the National Academy of Sciences of the United States of America, 104*, 18613–18618.

Weiner, A. M., Deininger, P. L., & Efstratiadis, A. (1986). Nonviral retroposons: Genes, pseudogenes, and transposable elements generated by the reverse flow of genetic information. *Annual Review of Biochemistry, 55*, 631–661.

Welsh, J., & McClelland, M. (1990). Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research, 18*, 7213–7218.

Wenke, T., Döbel, T., Sörensen, T. R., Junghans, H., Weisshaar, B., & Schmidt, T. (2011). Targeted identification of short interspersed nuclear element families shows their widespread existence and extreme heterogeneity in plant genomes. *Plant Cell, 23*, 3117–3128.

Wessler, S. (1998). Transposable elements associated with normal plant genes. *Physiologia Plantarum, 103*, 581–586.

Wessler, S. (2006). Transposable elements and the evolution of eukaryotic genomes. *Proceedings of the National Academy of Sciences of the United States of America, 103*, 17600–17601.

Wicker, T., Sabot, F., Hua-Van, A., Bennetzen, J. L., Capy, P., Chalhoub, B., Flavel, A., Leroy, P., Morgante, M., Panaud, O., Paux, E., Miguel, S., & Schulman, A. H. (2007). A unified classification system for eukaryotic transposable elements. *Nature Reviews Genetics, 8*, 973–982.

Williams, J., Kubelik, A. R., Livak, K., Rafalski, J. A., & Tingey, S. V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research, 18*, 6531–6535.

Winkfein, R. J., Moir, R. D., Krawetz, S. A., Blanco, J., States, J. C., & Dixon, G. H. (1988). A new family of repetitive, retroposon-like sequences in the genome of the rainbow trout. *European Journal of Biochemistry, 176*, 255–264.

Wu, F., & Tanksley, S. D. (2010). Chromosomal evolution in the plant family Solanaceae. *BMC Genomics, 11*, 182.

Wu, F., Eannetta, N. T., Xy, Y., Durrett, R., Mazourek, M., Jahn, M. M., & Tanksley, S. D. (2009). A COSII genetic map of the pepper genome provides a detailed picture of synteny with tomato and new insights into recent chromosome evolution in the genus Capsicum. *Theoretical Applied Genetics, 118*, 1279–1293.

Xiong, Y., & Eickbush, T. H. (1990). Origin and evolution of retroelements based upon their reverse transcriptase sequences. *The EMBO Journal, 9*, 3353–3362.

Xu, X., Zeng, L., Li, Y., & Wang, H. (2014). Inheritance of resistance to *Phythophtora capsici* in *Capsicum annuum* and analysis of relative SRAP markers. *Journal of Chemical and Pharmaceutical Research, 6*, 1967–1972.

# Improved Breeding of High-Carotene Carrots Through Marker-Assisted Paternity Selection and Raman Spectroscopy

**Sabine K. Clausen, Steffen Dahlke, Bjarne Jørnsgård, and Søren K. Rasmussen**

**Abstract** Polycross is a functional and low-cost breeding method but the missing paternal pedigree data is a disadvantage for the use of polycross breeding in Apiaceae including carrot (*Daucus carota* L.). The present study describes a paternity test for carrot breeding using 14 previously described SSR markers. Phenotyping of harvested roots was done using a non-destructive and fast screening method to determine total carotenoid concentration by Raman spectroscopy. Genetic relationship between the parent cultivars was estimated using Nei's genetic distance and cluster analysis by POPGENE software. Cluster analysis divided the parent cultivars into two major groups according to geographic origin. The mean pairwise genetic distance between the cultivars was 0.096, an indication of very great genetic difference. The software program CERVUS was used for parentage analysis. A total of 82 progenies from a polycross of nine cultivars were genotyped with simple sequence repeat (SSR) markers and paternity was assigned successfully for 81.7% of the offspring at a 99% confidence level, with 58.2% being the result of self-fertilization. These results show that application of a marker-assisted paternity test in carrot polycross breeding allows the rapid assessment of genetic diversity and targeted selection of desired individuals for the next generation of breeding. This was shown by an increase in average carotenoid concentration of 200 ppm (range of 104–441 ppm) in the parent genotypes to an average of 245 ppm in the progeny ranging from 97 to as high as 553 ppm.

**Keywords** Carotenoids · *Daucus carota* L. · Polycross breeding · SSR markers · Raman spectroscopy · Food colourants

S. K. Clausen · S. Dahlke · S. K. Rasmussen (✉)
Molecular Plant Breeding Research Group, Department of Plant and Environmental Sciences, University of Copenhagen, Frederiksberg, Denmark
e-mail: skr@plen.ku.dk

B. Jørnsgård
Natural Colours, Chr. Hansen, Hørsholm, Denmark



A. Raina et al. (eds.), *Advanced Crop Improvement, Volume 2*,
https://doi.org/10.1007/978-3-031-26669-0_4

# 1 Introduction

Food colouring is widely used in order to influence the perceived flavour of food and make it more attractive to consumers. However, due to concerns about food safety, there is an increasing interest among both consumers and manufacturers in replacing synthetic dyes with natural colours (Coultate & Blackburn, 2018), and carotenoids are the most widely used natural pigments in industrial food production (Mortensen, 2006). The development of crop cultivars with a high concentration of pigments such as beta-carotene therefore has huge economic potential as raw material in the production of natural food colourants.

Carrot taproots are an attractive source of phytochemicals in general (Ahmad et al., 2019) and in particular considered for providing pro-vitamin A with the diets (Giuliano, 2017). Carrots belong to the Apiaceae botanical family, they are allogamous and biennial, requiring vernalization to induce flowering, and the edible taproot produced the first year can be white or coloured due to accumulation of either anthocyanins or beta-carotene. Carrots has been cultivated for centuries but the first carrot breeding efforts have been dated to France in the 1930s (Stein & Nothnagel, 1995). The outcrossing carrots are pollinated by wind or flies and synthetic lines can be produced by hand pollination. Modern varieties of carrots are in general hybrids involving several crosses (Stein & Nothnagel, 1995; Que et al., 2019) and self-pollination leads to inbreeding depression. Carrots can be vegetatively propagated from the green top. They may require adaptation to local agro-climatic conditions due to day length sensitivity, and chilling may initiate flowering at early sowing.

## *1.1 Breeding for Pigments in Carrots*

In order to develop new cultivars of carrot with a high concentration of pigments (beta-carotene and anthocyanin) in the taproots, a wide screen of carrot lines was initiated. Carrots were developed through selection between and within existing cultivars over several generations, as we initially observed a significant difference in carotenoid concentration within roots of the same cultivar. Carotenoids are fat soluble, which often makes determination of their concentration in plant tissue both difficult and time-consuming and involves the use of hazardous chemicals. Raman spectroscopy is an attractive method for the analysis of phytochemicals in many crops (de Oliveira et al., 2010; Baranska et al., 2013) and it has been widely used to determine carotenoids in carrots (Quilitzsch et al., 2005; Withnall et al., 2003; Schulz et al., 2005). Raman spectroscopy differs from traditional quantitation methods, such as HPLC and capillary electrophoresis, by being extremely fast, simple, and non-destructive. Following these studies, a new colour screening method has been developed for the rapid determination of carotenoid concentration in orange carrots (Lawaetz et al., 2016). This method allows the detection of single plants having a significantly increased content of dyes based on Raman spectroscopy (Lawaetz

et al., 2016). Furthermore, it is possible to regenerate the specific taproot by planting the green top, and therefore, this Raman screening method is considered to be non-destructive. Following flower initiation and hybridization, seeds can be produced for further selection as carrots are not vegetatively propagated.

## *1.2 Polycrossing*

The success of a breeding program is largely dependent on the extent of genetic diversity present in the population. Polycrossing is widely used in the breeding of outcrossing species such as forage crops, vegetables and trees (Varghese et al., 2015; Bohanec et al., 2020). In outcrossing species, pollination is done by insects or by wind and a recent example of polycross breeding is the spice caraway, which like carrots belongs to the Apiaceae plant family (von Maydell et al., 2021). However, one of the major limitations to this strategy is the lack of genetic control, with a complete loss of paternity information among the progeny. In this study, the polycross approach was used to maximize the number of hybrid combinations that can be represented within the progeny. Selected taproots with a high concentration of carotenoid were polycrossed by open pollination, and the progeny grown and compared in order to identify new cultivars with improved colour concentration.

Simple sequence repeat (SSR) marker-based paternity analysis is a cost-effective molecular tool for identifying paternity (Varshney et al., 2005). Previous studies have indicated that SSR markers are helpful in evaluating genetic diversity between cultivars and landraces in carrot and separating them into distinct groups (Clotault et al., 2010; Baranski et al., 2012). Since Niemann (2001) initiated the identification of SSR loci in carrot for linkage mapping, the availability of large amounts of SSRs in carrots has been reported. A set of 23 SSR markers has been used to study historical and contemporary gene dispersal in wild carrot populations (Rong et al., 2010; Cavagnaro et al., 2011), which in particular have contributed to the number of SSR markers that are publicly available by developing 300 SSR markers for the carrot genome.

The objective of this study was first to create a carrot breeding nursery for improving carotenoid concentration, by polycrossing a wide range of genebank accession, to create new variation. The second objective was to demonstrate that the genetic diversity and paternity of individual progeny of carrots from a polycross can be determined using polymorphic SSR markers. The ability to identify paternity information allows a rapid assessment of diversity at the genome level and a targeted selection of parental plants in carrot breeding programs. The third objective was to demonstrate that significant improvements in beta-carotene concentrations could be obtained by selection in a polycross population.

## 2 Materials and Methods

### 2.1 Plant Material

A carrot (*Daucus carota* L.) field trial with 132 different cultivars was carried out during 2014 on the Højbakkegård experimental farm of the University of Copenhagen in Taastrup (55.6717°N; 12.3003°E), Denmark. Carrot seed samples were obtained from the Nordic Genetic Resource Centre (NordGen) and small populations (OP) from the Daucus collection of the United States Department of Agriculture (USDA) (see Table 1b). The selected cultivars from NordGen originated from Denmark. Approximately, 100 seeds from each cultivar were sown in the field and five roots were harvested from each cultivar for further investigation. Harvested roots, parental and progeny, were measured using Raman spectroscopy (Lawaetz et al., 2016). Raman spectra were measured on carrot discs from a cross section of the taproot 5–10 cm from the top using a RamanRxn1 instrument (Kaiser Optical Systems Inc., MI, USA). The probe had a 3-mm spot size and Raman spectra were measured on three different spots in the secondary phloem of the disc. Reference values were obtained by UV–visible spectrophotometry from extracts of the same taproot (Lawaetz et al., 2016). Cultivars were only selected based on the amount of pigmentation at this stage of evaluation. Carrot cultivars with high amounts of pigments were selected for the establishment of a nine-parent polycross in March 2016 in a greenhouse at the University of Copenhagen in Frederiksberg, Denmark. All roots from the selected parent cultivars were harvested and stored for vernalization. Not all roots could be propagated after storage, hence the different amount of clones from the parent cultivars. A total of 96 parent plants from the nine cultivars were pollinated with bluebottle flies (Table 1a). Upon seed ripening, seeds from each

**Table 1a** Paternal material from *Daucus carota* L. used in polycross. Per cultivar: origin, DNA sampled maternal clones and progeny counts, paternity success rate (%), outcrossing and selfed progeny counts and self-fertilization rate (%)

| Cultivars (parents) | Origin country ISO 3166 | Maternal clone count | Progeny sampled count | Progeny paternity count | Paternity success rate (%) | Outcrossing progeny count | Selfed progeny count | Self-fertilization rate (%) |
|---|---|---|---|---|---|---|---|---|
| Cultivar 1 | DNK | 13 | 32 | 28 | 87.5 | 15 | 13 | 46.4 |
| Cultivar 2 | DNK | 26 | 20 | 16 | 80.0 | 6 | 10 | 62.5 |
| Cultivar 3 | NLD | 3 | 10 | 7 | 70.0 | 4 | 3 | 42.9 |
| Cultivar 4 | USA | 8 | 11 | 10 | 90.9 | 2 | 8 | 80.0 |
| Cultivar 5 | USA | 9 | 9 | 6 | 66.7 | 1 | 5 | 83.3 |
| Cultivar 6 | DNK | 6 | 0 | – | – | – | – | – |
| Cultivar 7 | DNK | 10 | 0 | – | – | – | – | – |
| Cultivar 8 | DNK | 11 | 0 | – | – | – | – | – |
| Cultivar 9 | DNK | 10 | 0 | – | – | – | – | – |
| Total | – | 96 | 82 | 67 | 81.7 | 28 | 39 | 58.2 |

See also Table 1b for cultivar details

**Table 1b** Paternal material from *Daucus carota* L. used in polycross

| Cultivars (parents) | Origin country ISO 3166 | Name | Accession no | Source |
|---|---|---|---|---|
| Cultivar 1 | DNK | Nina | NGB11856 | NordGen |
| Cultivar 2 | DNK | Flaron | NGB11867 | NordGen |
| Cultivar 3 | NLD | High Carotene Carrot | HRI:13:010316 | Warwick University, Genetic Resources Unit |
| Cultivar 4 | USA | USDA Ped, 2327B | | Phil Simon, University of Wisconsin |
| Cultivar 5 | USA | USDA Ped, 3363B | | Phil Simon, University of Wisconsin |
| Cultivar 6 | DNK | Nicco | NGB13527 | NordGen |
| Cultivar 7 | DNK | Ninet | NGB13534 | NordGen |
| Cultivar 8 | DNK | Nimbus | NGB15873 | NordGen |
| Cultivar 9 | DNK | Cortez | NGB18126 | NordGen |

individual parent plant were harvested in August 2016. An equal number of progeny seeds from the parent plants were then sown in the fields. A sample of the harvested roots was analysed by Raman spectroscopy, and progeny roots with the highest amounts of carotenoid were selected for further propagation in the greenhouse (a total of 82 progeny roots). Leaf tissue from parents and progeny plants in the greenhouse was collected for DNA analysis (a total of 178 plants).

## *2.2 DNA Extraction*

Total genomic DNA was isolated from 200 mg of freeze-dried leaf material using an SDS-based extraction protocol with minor modifications (Sreelakshmi et al., 2010). DNA concentration and purity (A260/A280 ratios) were determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific).

## *2.3 Microsatellite Assay*

Genotyping was performed with 14 previously described primer pairs (Table 2). Tails containing a portion of the plasmid M13 were added to forward primers (CACGACGTTGTAAAACGACC) for detection of amplicons by fluorescence with one of three dyes: FAM/NED/VIC (Oetting et al., 1995). PCR amplification was conducted in a final reaction volume of 10 µL containing 20 ng template DNA, 10× Key Buffer (VWR Chemicals), 25 mM MgCl2, 2 mM dNTPs, 10 µM each of forward and reverse primers, 10 µM M13 primer with label and 0.5 U *Taq* DNA polymerase (VWR Chemicals). PCR reactions were amplified with an Applied Biosystems 2720 Thermal Cycler with an initial denaturation step at 94 °C for

**Table 2** Simple sequence repeat (SSR) primer sequences for genotyping of carrot

| Locus | Forward primer sequence (5′ to 3′) | Reverse primer sequence (5′ to 3′) | References |
|---|---|---|---|
| DCM-2 | CGACGAATAAGATGCGAGAGA | CACTCTTGAGCCACCACCTATAC | Niemann (2001) |
| SSR-2-7A | AATCGAATTGTTTCTGTGAT | AAGACGATGTTGATGATAATAGT | Rong et al. (2010) |
| SSR-2-10H | GTCGTCTCGTCAACACTA | CGGAAGAGGAGCTGTAA | Rong et al. (2010) |
| GSSR-4 | CAATCTTGCCACTAAAAGAGCA | CAGATACAATAGACAGGAAACATCG | Cavagnaro et al. (2011) |
| GSSR-6 | TCTCCTCTTGATTCTTCTTCGC | CCAATAAGCGTAAGCGTTTCTC | Cavagnaro et al. (2011) |
| GSSR-14 | CCACCTTGGACAAAGCAAAC | GCCCAGTTCTTCTTAATTGCAG | Cavagnaro et al. (2011) |
| GSSR-16 | ATGCAAACGACAATATCCACAG | GCCCAGCCACTTCCTAGAT | Cavagnaro et al. (2011) |
| GSSR-107 | TTCTGGTCTTTTGACATGAAGG | CGGATTTGAGGTGAGTTGAATA | Cavagnaro et al. (2011) |
| GSSR-134 | CGGATTTGAGGTGAGTTGAATA | TTGGTCTTTTGACATGAAGGG | Cavagnaro et al. (2011) |
| GSSR-19 | CCGAGTTGGATTCGGAGAG | GTAAATTGAGGATTGCGAGTTG | Cavagnaro et al. (2011) |
| GSSR-35 | AATTCACAATCACCGACTCTCC | ACGTCAAAGCTCCTGTTCATTT | Cavagnaro et al. (2011) |
| GSSR-42 | CAGCACTACTCGAAGATTGGC | ACCTAGTTCTGTCCAAAGTGCG | Cavagnaro et al. (2011) |
| GSSR-85 | TGACTCGGTGGATGAATTAAGA | CACTGCTTTGCCATTGTTTT | Cavagnaro et al. (2011) |
| GSSR-91 | ATTCACCTTCAGTGCCTCCTAA | GAATTGTGTGTGGTGCCTTCTA | Cavagnaro et al. (2011) |

4 min, followed by 18 cycles of 94 °C for 1 min, 64 °C for 1 min (−0.5 °C per cycle), and 72 °C for 1 min, followed by 20 cycles of 94 °C for 1 min, 55 °C for 30 s, and 72 °C for 1 min, with a final extension of 10 min at 72 °C. PCR products labelled with one of each of the three dyes (FAM/NED/VIC) were pooled before the products were separated by capillary electrophoresis on an ABI PRISM® 3130xl Genetic Analyzer (Applied Biosystems, USA).

### *2.4 Data Treatment and Paternity Analysis*

Data were analysed using the software GeneMarker (SoftGenetics, State College, PA, USA). The SSR alleles were named in accordance with their specific size in base pairs for the paternity analysis. SSR fragments were also scored and entered into a binary data matrix, with '1' indicating the presence and '0' indicating the absence of peaks. The resulting presence/absence data matrix was analysed using POPGENE version 1.32 (Francis & Yang, 2019) to examine the genetic relationship between populations of parent cultivars, and a dendrogram was constructed from Nei's (1978) genetic distances.

The software program CERVUS 3.0.7 (Kalinowski et al., 2007) was used for parentage analysis. The allele frequency module of the program was used to calculate allele frequency, observed heterozygosity (HO), expected heterozygosity (HE) and polymorphic information content (PIC) for each locus. These data were then used to run the simulation module and estimate the threshold log-likelihood scores for paternity analysis. The simulation was conducted using the default genotype error rate of 1% and the default levels of confidence (strict confidence 95% and relaxed confidence 80%). The proportion of loci genotyped was derived from the output of allele frequency analysis. Finally, based on the genotype data of offspring and candidate parents, the parentage assignment module was used to run the paternity analysis using the generated allele frequency and simulation results.

## 3 Results

### *3.1 Single Sequence Repeat (SSR) Markers*

The 14 primers used for the analysis of 178 plants generated a total of 81 alleles, which ranged from 5 to 8 (Table 3). The PIC value reflecting the genetic diversity of the 14 microsatellite loci ranged from 0.49 to 0.73 with an average of 0.61, indicating that the least informative primer was SSR-2-7A and the most informative was DCM-2 (Table 3). Except for primer SSR-2-7A, all the primers were highly polymorphic, with PIC values greater than 0.50. The 178 genotypes of parent cultivars and their polycross offspring were clearly distinguished by the 14 SSR primers, confirming that the selected SSR markers have good discriminatory power for genotyping carrot germplasm in polycross populations.

**Table 3** Locus name, chromosome location (Cavagnaro et al., 2011), observed size range, number of alleles and polymorphism information content (PIC) of 14 SSR markers analysed in the polycross population with a total of 178 parent and progeny genotypes

| Locus | Linkage group | Fragment size range | Number of alleles | Individuals typed | PIC |
|---|---|---|---|---|---|
| DCM-2 | LG-9 | 181–212 | 6 | 178 | 0.73 |
| SSR-2-7A | – | 219–270 | 6 | 178 | 0.49 |
| SSR-2-10H | – | 202–214 | 5 | 176 | 0.64 |
| GSSR-4 | LG-6 | 254–287 | 5 | 175 | 0.65 |
| GSSR-6 | LG-4 | 282–310 | 5 | 172 | 0.59 |
| GSSR-14 | LG-1 | 211–232 | 5 | 176 | 0.68 |
| GSSR-16 | LG-9 | 229–253 | 5 | 177 | 0.56 |
| GSSR-107 | LG-8 | 272–322 | 5 | 177 | 0.57 |
| GSSR-134 | – | 274–326 | 5 | 175 | 0.58 |
| GSSR-19 | LG-5 | 221–332 | 8 | 166 | 0.61 |
| GSSR-35 | LG-8 | 183–210 | 7 | 175 | 0.66 |
| GSSR-42 | LG-2 | 303–331 | 8 | 164 | 0.54 |
| GSSR-85 | LG-7 | 216–261 | 5 | 177 | 0.67 |
| GSSR-91 | LG-3 | 258–344 | 6 | 171 | 0.60 |

## *3.2 Genetic Relationship Between Parent Cultivars*

The generated UPGMA dendrogram based on Nei's unbiased genetic distances grouped the populations of the parent cultivars into two major clusters (Fig. 1) according to geographic origin, with the first cluster consisting of cultivars 1, 2, 6, 7, 8 and 9, which all originated from Denmark, and cultivar 3 from the Netherlands. The second cluster contained cultivars 4 and 5, which were genotypes from the Daucus collection of the USDA.

Population pairwise FST values varied from 0.134 to 0.785, and Nei's genetic distance varied from 0.026 to 0.199 (Table 4). Most of the cultivars could be distinguished by a great or very great genetic difference (0.15–0.25 or >0.25, respectively), as classified by Hartl and Clark (1997). The FST values between cultivar 1 and cultivars 2, 7 and 9 were classified with little genetic difference (<0.05) and were therefore co-clustered in the dendrogram, also indicating that cultivar 1 was interbreeding freely with those four cultivars. The potential for overlap between these cultivars was highly possible because of their similar origins. The inclusion of more markers would probably resolve this problem.

## *3.3 Parent Assignment*

SSR markers were successfully scored on 96 maternal clones and 82 progenies from the polycross. Some of the cultivars (cultivars 6–9) did not give seeds after pollination, and hence, no DNA samples from these progenies could be obtained. The

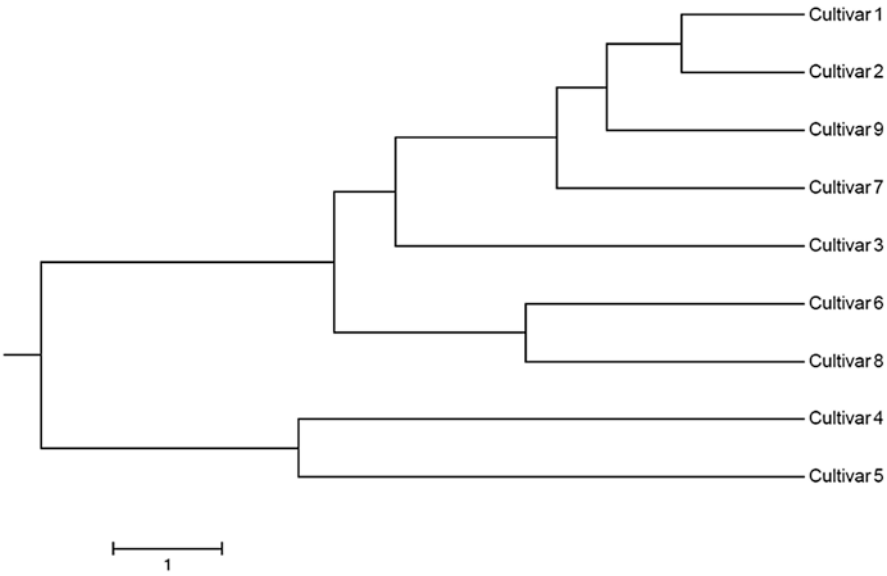



**Fig. 1** UPGMA dendrogram based on Nei's unbiased genetic distances between polycross breeding cultivars generated from SSR markers. Country of origin: cultivars 1, 2, 7, 8 and 9 DNK; cultivar 3, NLD; cultivar 4 and 5, USA. The numerical scale indicates genetic similarity

**Table 4** Unbiased measures of identity and genetic distance (Nei, 1978) among populations of carrot parent cultivars. Estimated FST is shown above the diagonal and Nei's genetic distance below the diagonal

| Cultivar | Origin country ISO 3166 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | DNK | – | 0.134 | 0.289 | 0.365 | 0.456 | 0.294 | 0.134 | 0.147 | 0.134 |
| 2 | DNK | 0.026 | – | 0.279 | 0.371 | 0.461 | 0.394 | 0.260 | 0.228 | 0.184 |
| 3 | NLD | 0.070 | 0.061 | – | 0.357 | 0.528 | 0.640 | 0.345 | 0.386 | 0.257 |
| 4 | USA | 0.099 | 0.104 | 0.073 | – | 0.452 | 0.674 | 0.420 | 0.483 | 0.355 |
| 5 | USA | 0.129 | 0.133 | 0.137 | 0.083 | – | 0.785 | 0.490 | 0.537 | 0.495 |
| 6 | DNK | 0.063 | 0.100 | 0.179 | 0.199 | 0.199 | – | 0.443 | 0.386 | 0.449 |
| 7 | DNK | 0.038 | 0.060 | 0.093 | 0.129 | 0.145 | 0.092 | – | 0.217 | 0.152 |
| 8 | DNK | 0.044 | 0.069 | 0.106 | 0.145 | 0.140 | 0.055 | 0.046 | – | 0.212 |
| 9 | DNK | 0.040 | 0.039 | 0.053 | 0.092 | 0.161 | 0.119 | 0.048 | 0.074 | – |

paternity success rate is the percentage of offspring, which were successfully assigned to a parent in the parentage analysis. Progeny where the parents were found to be the same as the progeny was counted as a selfed progeny and the self-fertilization rate was hence the percentage of progeny, which were the result of self-fertilization. Of the 82 progenies, a strict exclusion analysis classified 67 progenies (81.7%) with a paternal assignment, of which 39 progenies were classified as possible self-fertilizations (58.2%). The paternal assignment success rate varied from 66.7% to 90.9% among maternal clones (Table 1a).

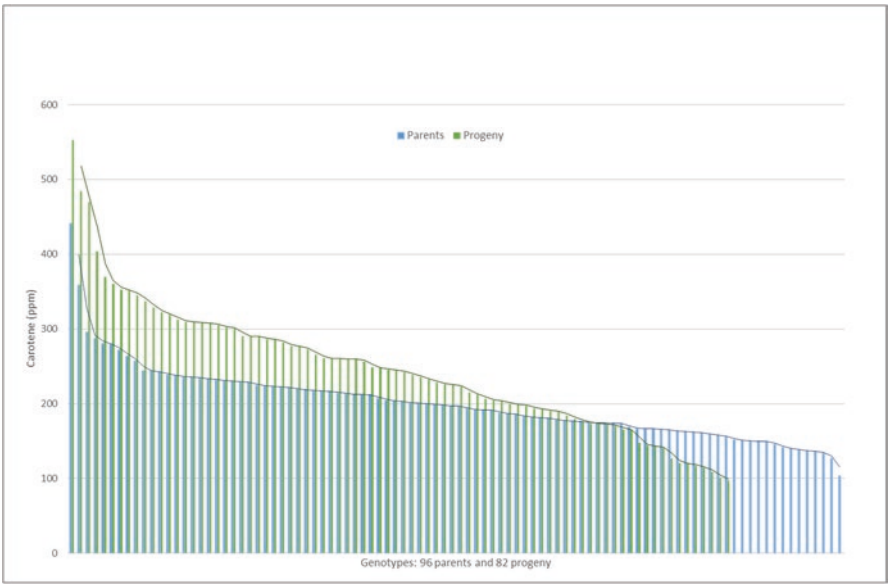



**Fig. 2** Total content of carotenoid (ppm) measured by Raman spectroscopy in a polycross population with a total of 96 parents and 82 progeny genotypes (sd not shown for clarity)

**Table 5** Average content of carotenoid (ppm) measured by Raman spectroscopy in the paternal material

| Cultivar | Total no. of roots | Avg. content of carotenoid (ppm) | SEM |
|---|---|---|---|
| Cultivar 1 | 13 | 173 | 31 |
| Cultivar 2 | 26 | 181 | 27 |
| Cultivar 3 | 3 | 341 | 86 |
| Cultivar 4 | 8 | 226 | 25 |
| Cultivar 5 | 9 | 201 | 45 |
| Cultivar 6 | 6 | 205 | 34 |
| Cultivar 7 | 10 | 184 | 71 |
| Cultivar 8 | 11 | 218 | 18 |
| Cultivar 9 | 10 | 215 | 41 |

Harvested roots were measured by fast screening of total carotenoids using Raman spectroscopy (Lawaetz et al., 2016). The measurements showed that the polycross experiment resulted in progenies with a generally higher content of carotenoid compared to the parents (Fig. 2). The nine parent genotypes had an average carotenoid content of 200 ppm (Table 5), ranging from 173 ppm (cultivar 1, 'Nina') to 341 (cultivar 3, 'High Carotene Carrot'), and the progeny had an average of 245 ppm, with a range of 97–553 ppm.

The origin of the progeny with the greatest increase in total carotenoid (above 300 ppm) was distributed between all of the parent genotypes in the polycross

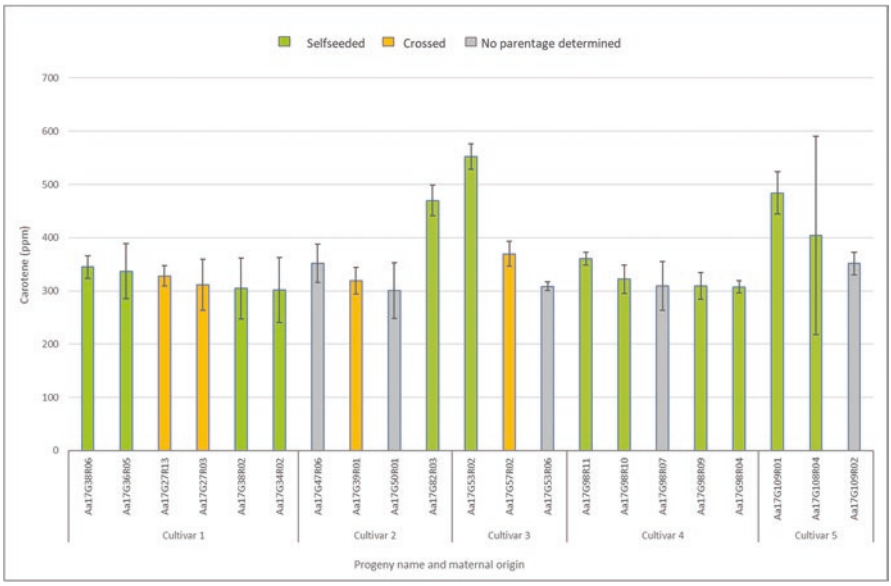



**Fig. 3** Parental information of selected progenies with a high content of carotenoid (above 300 ppm)

(Fig. 3). In general, some of the cultivars performed better than others with regards to pigment content. However, the carotenoid data suggest that it would be wise not to exclude entire cultivars based on general assumptions, as there would be a risk of valuable high-performing plant material being discarded.

Out of the 21 selected high-carotenoid progenies, the paternity analysis classified 16 progenies with a paternal assignment, of which 12 progenies were classified as possible self-fertilizations (57%). Paternity could not be determined in 24% of the progeny (Table 1a). These data indicate that there is a general tendency for self-fertilized plants to produce a higher carotenoid content in the progeny.

## 4 Discussion

This study investigated the utility of molecular markers to determine the genetic diversity and paternity of individual progeny of carrots from a polycross population. Fourteen previously described pairs of primers were used for the analysis (Niemann, 2001; Rong et al., 2010; Cavagnaro et al., 2011). All the SSR loci were polymorphic and there were no duplicates in the collection. The effective number of 81 alleles was identified with a mean of 5.8 per locus (Table 3), which is very similar to the data obtained for the carrot collection by Baranski et al. (2012). The marker developed by Niemann (2001) was the most discriminating, with the highest mean polymorphic information content (PIC).

Clustering accessions in a UPGMA dendrogram based on Nei's unbiased genetic distances were applied to investigate the genetic relationship in the parent material. The results revealed that the nine cultivars could be separated into two major clusters (Fig. 1). The first pool comprised the cultivars originating from Denmark, while the second cluster comprised one cultivar each from America and the Netherlands. This close genetic connection is not surprising as American carrots for human consumption originate from Europe.

A total of 82 progenies from the nine-cultivar polycross was genotyped with SSR markers and paternity was assigned successfully for 81.7% of the offspring at a 99% confidence level, while 58.2% were the result of self-fertilization. This study demonstrated that the 14 SSR markers were useful for identifying paternity among polycross progeny in carrots. However, when using a limited number of SSR loci, the choice of markers is very important and several factors should be considered to improve assignment accuracy such as marker diversity, population size, and gene frequency.

Selection of carrot phenotypes of interest has been going on for centuries and the first recording of breeding for beta-carotene content might be from Germany 1942 by Schuphan (1942). Since the 1980s, a large carrot breeding program was led by P. W. Simon, Wisconsin, to improve beta-carotene (Ellison et al., 2017) by using visual assessment of orange pigment in taproot for ranking offspring carrots. The overall target for our breeding efforts is to produce natural food colourants being anthocyanin (Meng et al., 2020) or beta-carotene (Lawaetz et al., 2016), which can be extracted from taproots. This exclude selection for sweetness and non-carbohydrate fibres such as lignin, which is important for consumption but requires adaptation to Danish agro-climatic conditions. The main goal of the present breeding investigation is to develop carrots with a high carotenoid content and to investigate the possibility of improving the breeding strategy by utilizing molecular markers. After only a single generation of breeding, it was possible to obtain an increase of carotenoids in the roots (Fig. 2), showing that colour content can be improved by using strain breeding in a polycross experiment. Comparing the paternity results and the Raman measurements from the roots with the highest carotenoid content (Fig. 3), most of the high-carotenoid roots were found to be the result of self-pollination. The offspring from the polycross with a carotenoid content above 400 ppm were all the result of selfing according to the SSR data, suggesting that further breeding with bulked selections of plants from the same cultivar would probably be the best way forward to further increase the colour content of carrot roots in the breeding program.

## 5 Conclusion

This study demonstrated that molecular markers are a cost-effective method for conducting paternity testing in carrots and a useful tool for selecting against self-pollination in breeding programs, while polycrossing increases the genetic

variation. The data reported here suggest that a relatively small number of carefully chosen markers may be adequate to meet most breeding needs for determining parents with high breeding value and candidate individuals for the next generation of breeding.

**Author Contributions** S.K.C, B.J and S.K.R; methodology, S.K.C; formal analysis, S.K.C and S.D.; investigation, S.K.C. and S.D.; data curation, S.K.C, S.D. and S.K.R.; writing–original draft preparation, S.K.C.; writing–review and editing, S.K.C, S.D., B.J. and S.K.R; project administration, S.K.C., B.J. and S.K.R.; funding acquisition, B.J. and S.K.R.

**Conflicts of Interest** The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

**Funding** This research was funded by the Danish Agricultural Agency, Green Development and Demonstration Programme, grant number 34009-13-0659.

## References

Ahmad, T., Cawood, M., Iqbal, Q., Ariño, A., Batool, A., Tariq, R. M. S., Azam, M., & Akhtar, S. (2019). Phytochemicals in *Daucus carota* and their health benefits. *Food, 8*, 424.

Baranska, M., Roman, M., Dobrowolski, J., Schulz, H., & Baranski, R. (2013). Recent advances in Raman analysis of plants: Alkaloids, carotenoids, and Polyacetylenes. *Current Analytical Chemistry, 9*, 108–127.

Baranski, R., Maksylewicz-Kaul, A., Nothnagel, T., Cavagnaro, P. F., Simon, P. W., & Grzebelus, D. (2012). Genetic diversity of carrot (*Daucus carota* L.) cultivars revealed by analysis of SSR loci. *Genetic Resource Crop Evolution, 59*, 163–170.

Bohanec, B., Jakše, J., Pilih, K. R., Štajner, N., & Murovec, J. (2020). Innovative F1 hybrid method enables testing of large number of line to line crossings for combining ability. *Acta Horticulture, 1282*, 179–186.

Cavagnaro, P. F., Chung, S. M., Manin, S., Yildiz, M., Ali, A., Alessandro, M. S., Iorizzo, M., Senalik, D. A., & Simon, P. W. (2011). Microsatellite isolation and marker development in carrot – Genomic distribution, linkage mapping, genetic diversity analysis and marker transferability across Apiaceae. *BMC Genomics, 12*, 386.

Clotault, J., Geoffriau, E., Linneton, E., Briard, M., & Peltier, D. (2010). Carotenoid biosynthesis genes provide evidence of geographical subdivision and extensive linkage disequilibrium in the carrot. *Theoretical Applied Genetics, 121*, 659–672.

Coultate, T., & Blackburn, R. S. (2018). Food colourants: Their past, present and future. *Colouration Technology, 134*, 165–186.

de Oliveira, V. E., Castro, H. V., Edwards, H. G. M., & de Oliveira, L. F. C. (2010). Carotenes and carotenoids in natural biological samples: A Raman spectroscopic analysis. *Journal of Raman Spectroscopy, 41*, 642–650.

Ellison, S., Iorizzo, M., Senalik, D., & Simon, P. W. (2017). The next generation of carotenoid studies in carrot (*Daucus carota* L.). Proceedings of the international symposium on carrot and other apiaceae, Ed.: M. Briard. *Acta Horticulture, 1153*, 93–99.

Francis, C. Y., & Yang, R. C. (2019). *Popgene version 1.32*. Available online: https://sites.ualberta.ca/~fyeh/popgene.html. Accessed on 26 June 2019.

Giuliano, G. (2017). Provitamin a biofortification of crop plants: A gold rush with many miners. *Current Opinion in Biotechnology, 44*, 169–180.

Hartl, D. L., & Clark, A. G. (1997). *Principles of population genetics*. Sinauer Associates. 519 pp.

Kalinowski, S. T., Taper, M. L., & Marshall, T. C. (2007). Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Molecular Ecology, 16*, 1099–1106.

Lawaetz, A. J., Christensen, S. M. U., Clausen, S. K., Jørnsgaard, B., Rasmussen, S. K., Andersen, S. B., & Rinnan, Å. (2016). Fast, cross cultivar determination of total carotenoids in intact carrot tissue by Raman spectroscopy and Partial Least Squares calibration. *Food Chemistry, 204*, 7–13.

Meng, D., Clausen, S. K., & Rasmussen, S. K. (2020). Transcriptome analysis reveals candidate genes related to anthocyanin biosynthesis in different carrot genotypes and tissues. *Plants, 9*, 344.

Mortensen, A. (2006). Carotenoids and other pigments as natural colourants. *Pure Applied Chemistry, 78*, 1477–1491.

Nei, M. (1978). Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics, 89*, 583–590.

Niemann, M. (2001). *Entwicklung von Mikrosatelliten Markern bei der Möhre (Daucus carota L.) und die Markierung eines Alternaria-Resistenzgens*. Dissertation, University Hannover, Shaker Verlag, Aachen. 116 pp.

Oetting, W. S., Lee, H. K., Flanders, D. J., Wiesner, G. L., Sellers, T. A., & King, R. A. (1995). Linkage analysis with multiplexed short tandem repeat polymorphisms using infrared fluorescence and M13 tailed primers. *Genomics, 30*, 450–458.

Que, F., Hou, X. L., Wang, G. L., Xu, Z. S., Tan, G. F., Li, T., Wang, Y. H., Khadr, A., & Xiong, A. S. (2019). Advances in research on the carrot, an important root vegetable in the *Apiaceae* family. *Horticulture Research, 6*, 69.

Quilitzsch, R., Baranska, M., Schulz, H., & Hoberg, E. (2005). Fast determination of carrot quality by spectroscopy methods in the UV-VIS, NIR and IR range. *Journal Applied Botany and Food Quality, 79*, 163.

Rong, J., Janson, S., Umehara, M., Ono, M., & Vrieling, K. (2010). Historical and contemporary gene dispersal in wild carrot (*Daucus carota* ssp. *Carota*) populations. *Annals Botany, 106*, 285–296.

Schulz, H., Baranska, M., & Baranski, R. (2005). Potential of NIR-FT-Raman spectroscopy in natural carotenoid analysis. *Biopolymers, 77*, 212–221.

Schuphan, W. (1942). Biochemische Sortenprüfung an Gartenmöhren als neuzeitliche Grundlage für planvolle Züchtungsarbeiten. *Züchter, 14*, 25–43.

Sreelakshmi, Y., Gupta, S., Bodanapu, R., Chauhan, V. S., Hanjabam, M., Thomas, S., Mohan, V., Sharma, S., Srinivasan, R., & Sharma, R. (2010). NEATTILL: A simplified procedure for nucleic acid extraction from arrayed tissue for TILLING and other high-throughput reverse genetic applications. *Plant Methods, 6*, 1–11.

Stein, M., & Nothnagel, T. (1995). Some remarks on carrot breeding (*Daucus carota sativus* Hoffm.). *Plant Breeding, 114*, 1–11.

Varghese, C., Varghese, E., Jaggi, S., & Bhowmik, A. (2015). Experimental designs for open pollination in polycross trials. *Journal of Applied Statistics, 42*, 2478–2484.

Varshney, R. K., Graner, A., & Sorrells, M. E. (2005). Genetic microsatellite markers: Features and applications. *Trends Biotechnology, 23*, 48–55.

von Maydell, D., Brandes, J., Lehnert, H., et al. (2021). Breeding synthetic varieties in annual caraway: Observations on the outcrossing rate in a polycross using a high-throughput genotyping system. *Euphytica, 217*, 1–15.

Withnall, R., Chowdhry, B. Z., Silver, J., Edwards, H. G. M., & de Oliveira, L. F. C. (2003). Raman spectra of carotenoids in natural products. *Spectrochimal Acta, Part A, 59*, 2207–2212.

# Traditional and Modern Molecular Cytogenetic Approaches to the Study of Mutagen-Induced DNA Damage: A Case of *Fagopyrum* Species

**J. Kwasniewska and A. Betekhtin**

**Abstract** This chapter summarises the optimisation experiments of the mutagenic treatment applied to two buckwheat species: *Fagopyrum esculentum* (common buckwheat) cv. Kora and Panda and *Fagopyrum tataricum* (Tartary buckwheat). Chemical mutagen-maleic acid hydrazide (MH) was used for mutagenic treatment. Based on the responses of buckwheat species to MH, the genome sensitivities were compared and discussed. Traditional and modern molecular cytogenetic approaches to study MH-induced damage on chromosome and DNA levels were applied.

**Keywords** Buckwheat · DNA damage · Maleic acid hydrazide · Micronuclei · TUNEL assay

## 1 Introduction

Buckwheat is a dicotyledonous grain crop plant of the family Polygonaceae. Most of the 26 buckwheat species are wild. Among them, *Fagopyrum esculentum* (common buckwheat) and *Fagopyrum tataricum* (L.) Gaertn. (Tartary buckwheat) are the most cultivated ones (Sytar et al., 2018). These species, categorised as pseudocereals, are essential for food production and are valuable for medicine purposes (Zhang et al., 2012; Kreft et al., 2020). Buckwheat seeds are also valuable because they do not contain gluten (Skerritt, 1986). The protein in buckwheat is of better quantity and quality than in wheat, rice and maize (Fabian & Ju, 2011; Khan & Shewry, 2009).

*F. esculentum* (2n = 16) is more widely distributed than Tartary buckwheat. It is cultivated in Asia and central and eastern Europe (Wijngaard & Arendt, 2006). Common buckwheat is the traditional crop with a short vegetation period of

J. Kwasniewska (✉) · A. Betekhtin
Plant Cytogenetics and Molecular Biology Group, Faculty of Natural Sciences, University of Silesia in Katowice, Katowice, Poland
e-mail: jolanta.kwasniewska@us.edu.pl



A. Raina et al. (eds.), *Advanced Crop Improvement, Volume 2*,
https://doi.org/10.1007/978-3-031-26669-0_5

90–100 days. This insecticidal plant, highly dependent on bees, is also a good honey plant. Its groats contain protein, saccharides, lipids, and vitamins and thus have health benefits. The preventive action of common buckwheat groats to different diseases is connected with the content of dietary fibre.

*F. tataricum* (2n = 16) is a wild buckwheat species, which is cultivated in East Asia for consumption purposes (Brian et al., 2004). It contains a large number of flavonoids, especially rutin, which has a known beneficial medicinal effect (Kreft, 2016). The rutin content in the groats of Tartary buckwheat is 100-fold higher than that in common buckwheat (Steadman et al., 2001). Interestingly, no rutin in cereals and pseudocereals except buckwheat was found (Hagels, 1999).

Because of the importance of buckwheat species, the improvement of some nutritional status and resistance features is desirable. Few examples of buckwheat mutagenesis are known (Tang et al., 2002). Recently, common buckwheat cultivars with high antioxidative activity using gamma ray irradiations were developed (Morishita et al., 2019). Also, the high rutin cultivars of common buckwheat using physical mutagen were previously demonstrated (Minami et al., 2001; Ito et al., 2005). A Tartary buckwheat dwarf mutant line (*ftdm*) by large-scale screening of an ethyl methanesulfonate (EMS)-mutagenised population was obtained recently (Sun et al., 2021).

One of the methods for genetic improvement is chemical mutagenesis. The characterisation of the cytogenetic effects of chemical treatment is the most important for optimising the appropriate mutagenic treatment conditions. Among the factors that determine the cytogenetic effects of mutagenic treatments are the conditions of mutagenic treatment: the concentration of the mutagen and treatment time. Also, the physiological stage of the plant material is crucial for the cytogenetic effect of mutagenic treatment. It would have to be especially emphasised that different plant species or even varieties are characterised by different sensitivities to various chemical and physical agents. The final cytogenetic effect seems to be related to genome size (Underbrink et al., 1968).

Maleic acid hydrazide is one of the most frequently used chemical agents in plant mutagenesis. This clastogenic agent efficiently induced DNA fragmentation, which leads to chromosome aberrations (CA), observed as changes in chromosome structure. They can be detected both in mitotic cells and in the interphase cells, as micronuclei formed from a whole chromosome or its fragment. MH is also described as a cytotoxic-type mutagen, which leads to disturbances of the cell cycle, such as reducing the mitotic activity of the cells and delaying the cell division (Marcano et al., 2004).

The analysis of the meristematic cells of M1 generation by using cytogenetic tests serves as a fast evaluation of genetic effect after mutagenic treatment. The non-dividing cells and mitotic ones are convenient for the analyses of the cytogenetic effects of mutagens (Kwasniewska, 2014). The damage can be analysed on both DNA and chromosome levels. It must be underlined that chromosome aberrations arise from DNA breakage, which are not repaired or repaired improperly. Recent advances in DNA damage analysis made it possible to assign the direct effect observed as DNA fragmentation in a single nucleus. One method to analyse DNA

breakage after mutagenic treatment is the TUNEL test (terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling). It quickly and precisely enables the detection of the single and double DNA breakage in the non-dividing cell. TUNEL test has been used to estimate DNA breakage in various species in response to mutagenic treatment; however, it still is not widely applied in plant mutagenesis. Identifying the DNA double-strand breaks (DSBs), which are key DNA damage that leads to chromosomal aberrations, enables TUNEL to serve as a predictive test for the formation of chromosome aberrations (Kwasniewska, 2014).

This chapter aims to optimise the procedure of mutagenic treatment with maleic acid hydrazide (MH) by using different experimental designs for buckwheat species: *F. esculentum,* cv. Kora and cv. Panda, and *F. tataricum.* The traditional cytogenetics for the analysis of the mitotic activity and chromosomal aberrations were accompanied by the modern molecular cytogenetic techniques – TUNEL test – to quantify the damage on the DNA level. The results of the cytogenetic characterisation of the effects of MH treatment will enable us to compare and discuss the sensitivities of buckwheat genomes to mutagenic treatment. The results will be helpful in future breeding programmes for *Fagopyrum* species.

## 2 Material and Methods

### *2.1 Material*

The seeds of the *Fagopyrum esculentum* cv. Kora and Panda were sourced from the Malopolska Plant Breeding Company, Cracow, Poland. The seeds of *Fagopyrum tataricum* (L.) Gaertn (Tartary buckwheat), sample k-17, were gained from the collection of the N.I. Vavilov Institute of Plant Genetic Resources, Saint Petersburg, Russia.

### *2.2 Mutagenic Treatment*

Before mutagenic treatment with MH (maleic acid hydrazide), the seeds were presoaked with distilled water for 8 h, 24 h or 48 h. Also, the *F. esculentum* embryos, isolated from seeds previously presoaked with distilled water for 24 h, were used for treatment with MH. This procedure was not performed for Tartary buckwheat due to the small size of the seeds. For mutagenic treatment, the following concentrations of MH (Sigma, CAS 123-3301) were used: 1, 2, 3 and 4 mM. For the control, the water was used for the incubation. The seeds and isolated embryos were treated with MH for 2 h. The mutagenic treatment procedure was repeated twice. The plants for cytogenetic analyses were grown in the dark at 21 ± 1 °C in Petri dishes and collected after 72 h, in both control and following MH treatment. *Fagopyrum*

*esculentum* cv. Kora and Panda and *Fagopyrum tataricum* seeds, control and treated with MH, were also sown in the soil for the analysis of plant growth.

## 2.3 Analyses of the Mitotic Index and the Micronuclei

Roots of M1 buckwheat seedlings, not treated and treated with MH, were fixed for cytogenetic analyses. The mitotic indexes and the frequency of nuclei with micronuclei in the meristematic root cells of the *Fagopyrum esculentum* cv. Kora and cv. Panda seeds and *Fagopyrum tataricum* were analysed. Roots were fixed in AA – methanol: acetic acid (3:1 v/v) – for 4 h at room temperature (RT). Two biological repetitions were performed, with ten plants per each one. One meristem was used to make one preparation. Cytogenetic preparations were made using the enzymatic technique. The preparations were stained with DAPI. The mitotic indexes and the frequencies of nuclei with micronuclei were estimated for 1000 meristematic root cells on each slide. ANOVA ($p < 0.05$) and Tukey's test (Tukey HSD test, $p < 0.05$) were used for statistical analyses.

## 2.4 TUNEL Test

TUNEL test was carried for *Fagopyrum esculentum* cv. Kora and cv. Panda and *Fagopyrum tataricum* (L.) Gaertn. The root tips, after the root cap removal, were used in the TUNEL reaction. The samples were fixed with 4% paraformaldehyde (Fluka) in PBS for 1 h at RT. The material was washed three times for 5 min in PBS. The enzymatic digestion was not applied to make nuclei preparations. The preparations were made by squashing the root meristems in PBS. After freezing at −70 °C, preparations were stored at 4 °C. Cell permeabilisation was done by incubating the preparations in 0.1% Triton X-100 (Sigma) in 0.1% sodium citrate for 2 min at 4 °C. Then slides were washed with PBS. The nuclei with DNA breaks were detected with the TUNEL reaction mixture (in situ Cell Death Detection Kit, Fluorescein, Roche). A 50 µL TUNEL reaction mixture (enzyme solution/label solution, 1:9 v/v) was used for each slide and incubated for 1 h at 37 °C in a humid chamber in the dark. The positive control was a preparation of nuclei treated with DNase (1 U) for 30 min at 37 °C. Then the TUNEL reaction mixture was applied. For the negative control, a TUNEL mixture without enzyme was applied. Slides were washed three times with PBS and stained with DAPI (2 µg/mL), air-dried and then mounted in Vectashield.

We used a Zeiss AxioImager.Z.2 wide-field fluorescence microscope, equipped with filters for FITC and DAPI for the examination of preparations. One thousand

cells on three slides for each treatment group and species/cultivar were analysed for the estimation of the frequencies of TUNEL-positive nuclei. Student's t-test, with $p < 0.05$ indicating significance, was used for the assessment of the significant differences between experimental groups.

## 3 Results

### *3.1 Plant Growth and Morphology*

The analyses of the germination capacity and the root length of seedlings of *Fagopyrum esculentum* cv. Panda and cv. Kora and *F. tataricum* that were grown in Petri dishes were made. No reduction in the germination capacity was observed following the mutagenic treatment with 1, 2, 3 or 4 mM MH for all buckwheat species and cultivars (data not presented on the figures). However, all applied concentrations of MH caused a substantial, MH-dose dependent reduction of the root growth (Figs. 1, 2, and 3).

However, all concentrations of MH have reduced the *Fagopyrum* plant growth (Figs. 4, 5, and 6).

This reduction was dependent on the dose of MH used in the experiments. The growth reduction was observed on 10th day and 20th day after treatment.

The weakest growth reduction as a result of MH treatment was observed for *F. tataricum* plants. Only slight differences in plant height were observed after MH treatment for *F. esculentum* cultivars Panda and Kora.

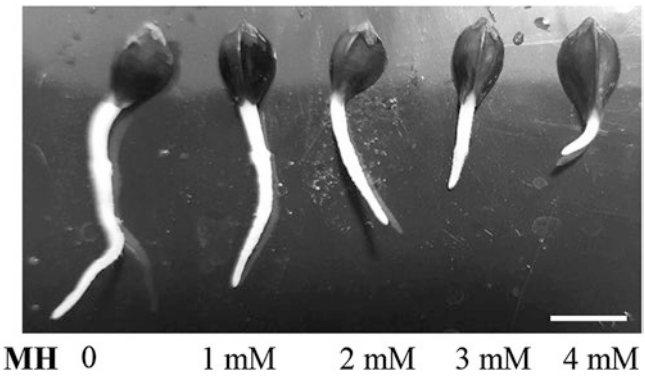



**Fig. 1** *Fagopyrum esculentum* cv. Kora seedlings: control and treated with maleic acid hydrazide (MH). The bar represents 1 cm

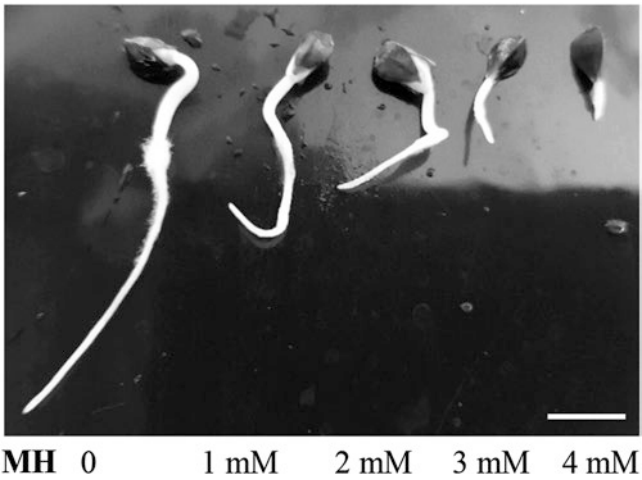



**Fig. 2** *Fagopyrum esculentum* cv. Panda seedlings: control and treated with maleic acid hydrazide (MH). The bar represents 1 cm

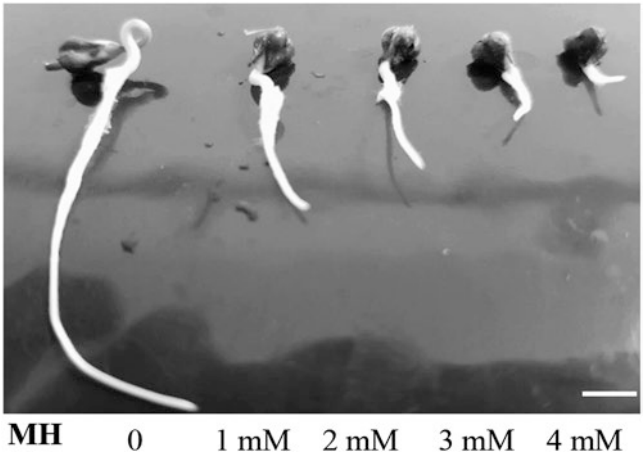



**Fig. 3** *Fagopyrum tataricum* seedlings: control and treated with maleic acid hydrazide (MH). The bar represents 1 cm

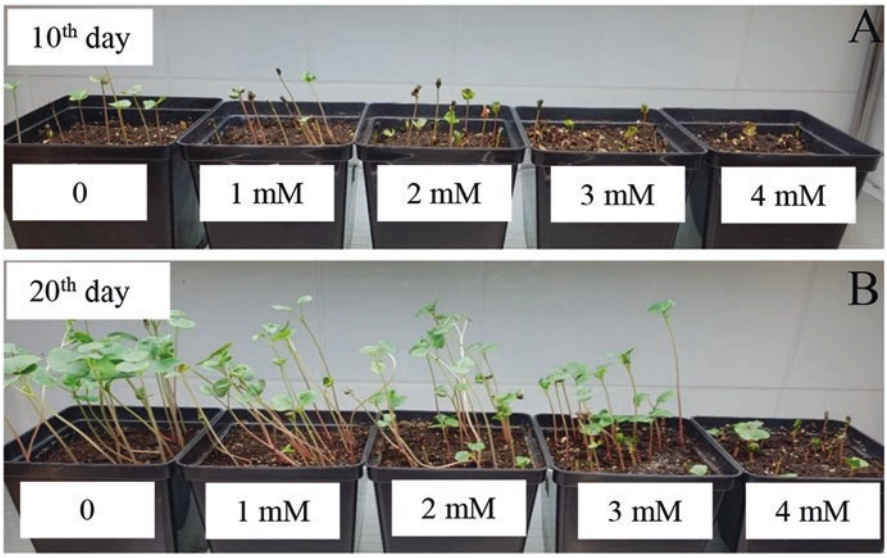



**Fig. 4** The comparison of the growth of *Fagopyrum esculentum* cv. Kora seedlings: 10 days (**a**) and 20 days (**b**) after the treatment with maleic acid hydrazide (MH)

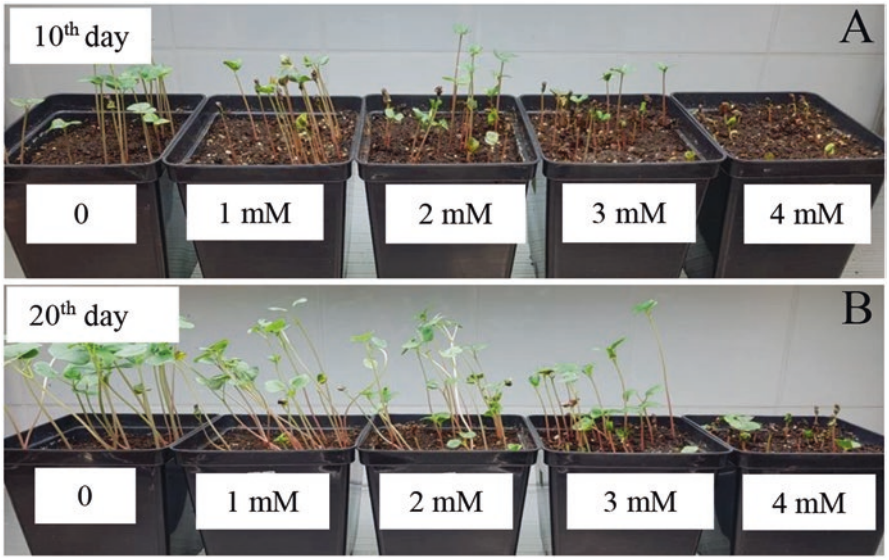



**Fig. 5** The comparison of the growth of *Fagopyrum esculentum* cv. Panda seedlings 10 days (**a**) and 20 days (**b**) after the treatment with maleic acid hydrazide (MH)

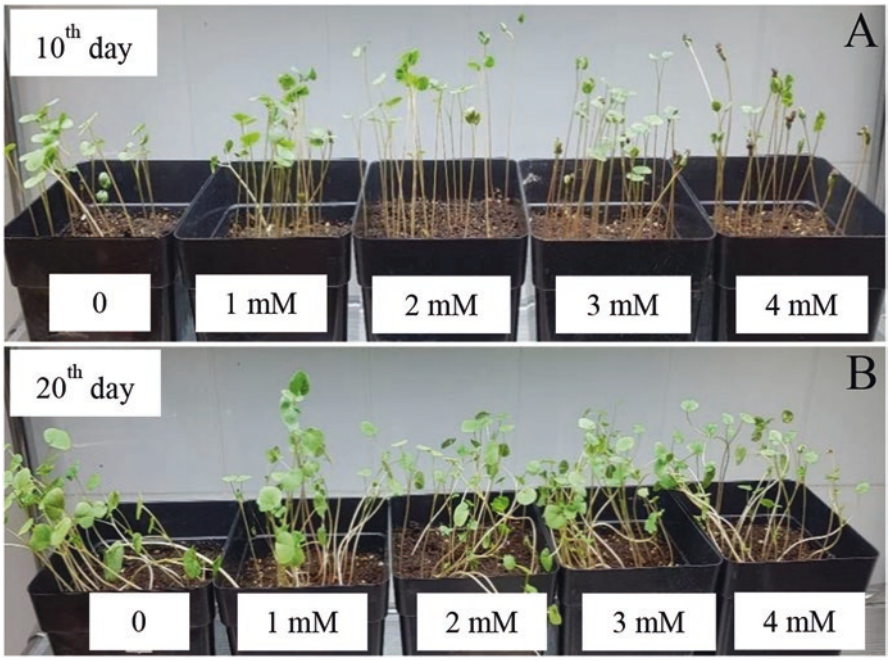



**Fig. 6** The comparison of the growth of *Fagopyrum tataricum* seedlings 10 days (**a**) and 20 days (**b**) after the treatment with maleic acid hydrazide (MH)

## 4 Cytological Analyses

### *4.1 Mitotic Activity*

The differences in the responses of *F. esculentum* and *F. tataricum* as a reduction of MI were observed. The mitotic activity in control, non-treated meristematic root cells was 8.98–10.1% for *F. esculentum*, depending on the time and the method of soaking the seeds prior to the mutagenic treatment. The mitotic index value after MH treatment was also dependent on these conditions. A significant dependence of the mitotic index on the concentration of MH was observed. No differences were observed in the mitotic index for the Panda and Kora cultivars tested, in both control and MH-treated ones; therefore, the results were polled (Fig. 7a). MH caused a significant reduction of even the total inhibition of the mitotic index. The complete blockage of the mitotic activity of the root cells was observed after application of 4 mM MH, after soaking the seeds for 48 h and 24 h followed by the isolation of embryos.

The mitotic activity for the control *F. tataricum* root meristematic cells was 9.9–10.53%. The reduction of MI after 1, 2 and 3 mM MH was not as statistically significant as in *F. esculentum.* Similarly, 4 mM MH had the strongest effect on the reduction of MI; however, it did not stop it completely (Fig. 7b).

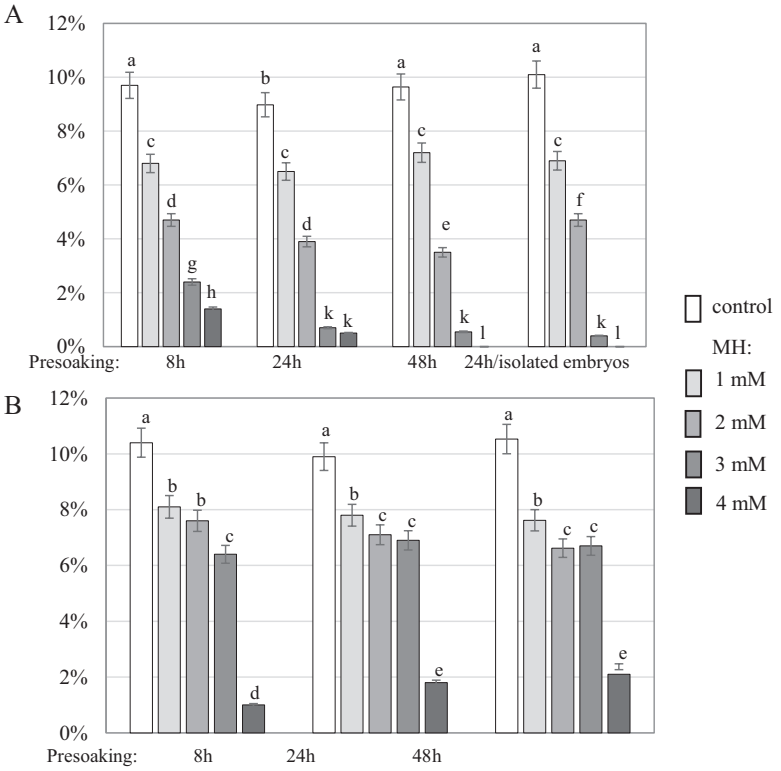



**Fig. 7** Mitotic index in *F. esculentum* cv. Panda and Kora (**a**) and *F. tataricum* (**b**) root meristematic cells: control and MH treated. In different experimental conditions: seeds were presoaked for 8 h, or 24 h, or 48 h, or presoaked for 24 h, and isolated embryos were used for treatment. Statistically significant differences are indicated by different letters (ANOVA followed by the Tukey HSD test, $p < 0.05$; mean ± SD)

## *4.2 Micronuclei*

Treatment with MH led to the formation of micronuclei (MN) in *Fagopyrum esculentum* (Fig. 8a) and *F. tataricum* (Fig. 8b). The micronuclei in buckwheat cells are extremely small. The number of micronuclei in one cell ranged from 0 to 2.

The frequencies of root meristematic cells with micronuclei, both in control and after MH treatment, were analysed (Fig. 9). No micronuclei were observed in control cells. No differences were observed in the frequency of micronuclei for the *F. esculentum* cultivars used, both control and MH-treated ones; therefore, the results were polled (Fig. 9a).

The response to MH was significantly dependent on the dose of MH. Interestingly, only 2 and 3 mM MH induced formation of MN in *F. esculentum*, while for *F. tataricum* only 3 and 4 mM MH were effective in MN induction. The highest frequency of *F. esculentum* cells with MN was 2.9% followed by the treatment of isolated

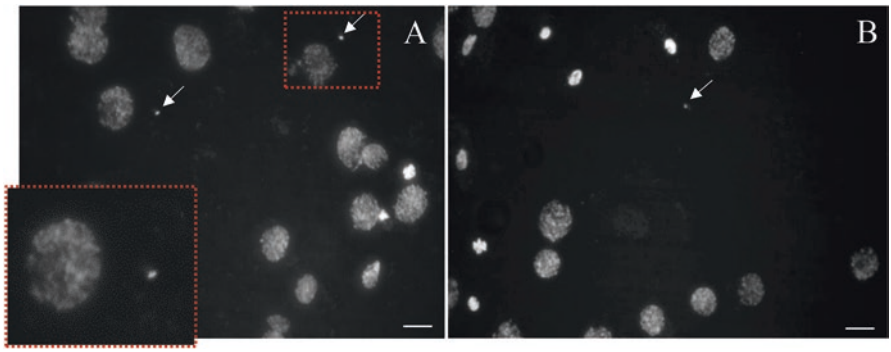



**Fig. 8** The nuclei of *Fagopyrum esculentum* cv. Kora (**a**) and *Fagopyrum tataricum* (**b**) after treatment with MH; micronuclei are indicated by arrows. Enlarged nucleus with micronuclei is shown on the left below. DAPI staining. Bars represent 10 µm

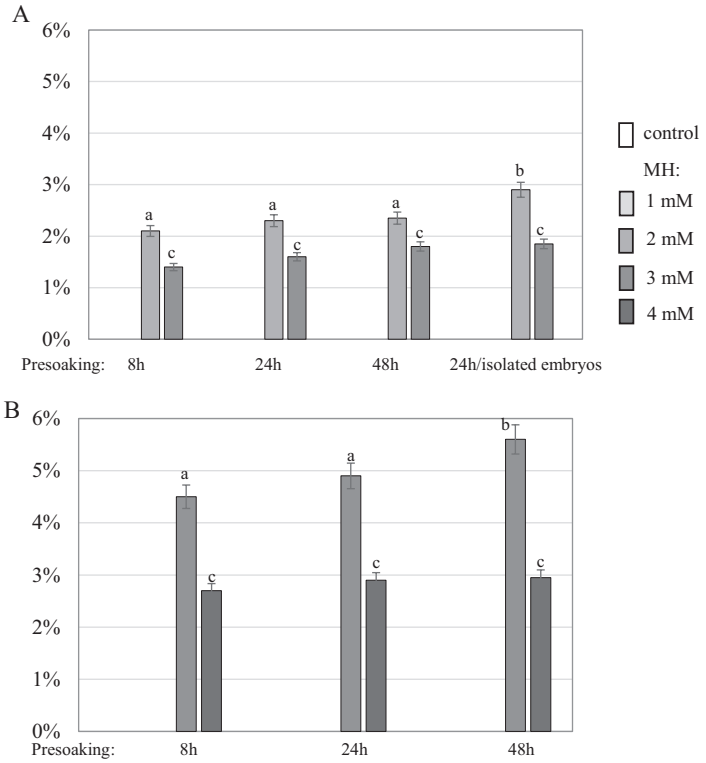



**Fig. 9** The frequency of root meristematic cells with micronuclei in *F. esculentum* cv. Panda and Kora (**a**) and *F. tataricum* (**b**) root meristematic cells: control and MH treated. In different experimental conditions: seeds were presoaked for 8 h, or 24 h, or 48 h, or presoaked for 24 h and isolated embryos were used for treatment. Statistically significant differences are indicated by different letters (ANOVA followed by the Tukey HSD test, $p < 0.05$; mean ± SD)

embryos with 3 mM MH, whereas it was 5.6% in *F. tataricum* after 48 h of soaking. For both buckwheat species, the time of soaking the seeds did not affect the frequency of cells with micronuclei.

### *4.3 DNA Damage*

To detect the nuclei with DNA damage in roots, control and following MH treatment, the TUNEL test was used. To analyse the frequency of TUNEL-positive nuclei, DAPI stainings were used (Fig. 10A–D). The same nuclei, showing green fluorescence, were observed in the FITC channel that are characterised by DNA breaks (Fig. 10A′–D′).

In control *F. esculentum* and *F. tataricum* cells, TUNEL-specific nuclei with relatively weak fluorescence (Fig. 10A, A′, C, C′), were observed with the very low frequency of maximum 1% (Fig. 11). The material that had been treated with DNase showed TUNEL-positive signals in 92% of the nuclei for the positive control. No FITC-labelled nuclei were observed in the negative control. The conditions of *F. esculentum* seeds soaked prior to treatment with MH, except the 2 mM dose, did not affect the frequency of cells with DNA damage. Only statistically significant differences were found for 2 mM MH. In *F. esculentum* TUNEL-positive nuclei were observed with the highest frequency after treatment with 2 mM MH, from 24.3% to 32.4%, depending on the presoaking conditions. The use of lower doses of MH, 3 and 4 mM, resulted in the lower frequency of TUNEL-positive nuclei. In *F. tataricum* the similar frequency of TUNEL-positive nuclei of about 30% was induced by 3 mM MH. In case of *F. tataricum* damaged nuclei were induced with the highest frequency by 3 mM MH, and the use of a higher dose, 4 mM, even tends to lower this frequency. No dependence of the TUNEL-positive frequency on the seeds' soaking conditions was found. Only a significant increase in the frequency of nuclei detected by the TUNEL test was demonstrated for 3 mM MH when 48 h of soaking was applied.

## 5 Discussion

Currently, there is an increased focus on pseudocereals, including buckwheats, to improve their diversity in response to climate changes (Zhang et al., 2017). It has to be underlined that knowledge in the field of the buckwheat mutagenesis is not wide at present. Here, we report on the characterisation of the cytogenetic responses of *Fagopyrum esculentum* and *F. tataricum* to maleic acid hydrazide (MH). For the common buckwheat we compare the effect of treatment with MH in two cultivars of *F. esculentum* – Kora and Panda. MH is widely applied in plant mutagenesis; hence, we used it as a model mutagen for comparative studies of different buckwheat species and cultivars (Swietlinska & Zuk, 1978).

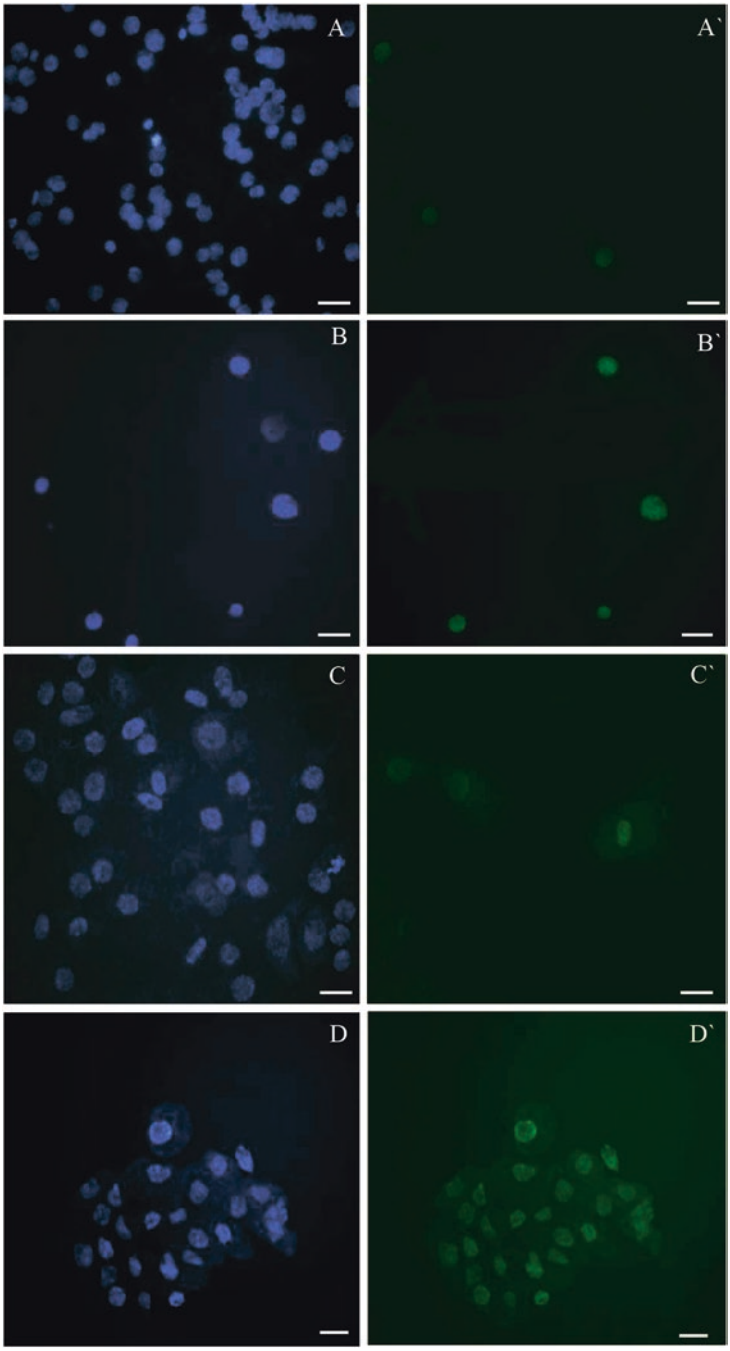



**Fig. 10** The results of TUNEL assay – in situ detection of DNA fragmentation in the *F. esculentum* cv. Panda (**A**, **A′**–**B**, **B′**) and *F. tataricum* (**C**, **C′**–**D**, **D′**) root tips: control and treated with 3 mM MH. Blue fluorescence, DAPI staining (**A**–**D**); green fluorescence, FITC showing the TUNEL-positive nuclei (**A′**–**D′**), control (**A**, **A′**, **C**, **C′**), 3 mM MH (**B**, **B′**, **D**, **D′**). Scale bars: 20 μm

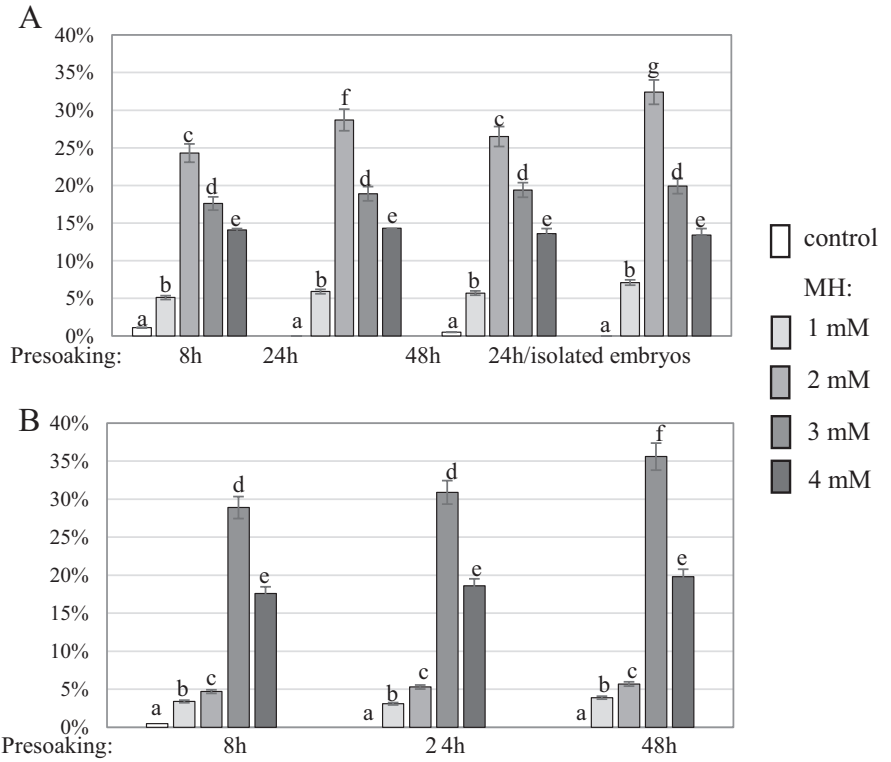



**Fig. 11** The frequency of TUNEL-positive *F. esculentum* cv. Panda and Kora (**a**) *and F. tataricum* (**b**) root meristematic cells: control and MH treated. In different experimental conditions: seeds were presoaked for 8 h, or 24 h, or 48 h, or presoaked for 24 h and isolated embryos were used for treatment. Statistically significant differences are indicated by different letters (ANOVA followed by the Tukey HSD test, $p < 0.05$; mean ± SD)

For the first time, the significant differences in the response of genomes of *F. esculentum* and *F. tataricum* to mutagenic treatment were demonstrated. All cytogenetic parameters used for describing the genotoxic effect of MH showed that *F. tataricum* genome is less sensitive to MH than *F. esculentum*. Interestingly, we did not notice the difference on the sensitivities of *F. esculentum* cv. Panda and Kora to mutagenic treatment with MH. A number of factors influence the cytogenetic effect of mutagens. It is well known that the somatic and genetic effects of mutagens are not the same in different species and their varieties. The reasons for these differences may be nuclear volume and DNA content (Underbrink et al., 1968). However, data on the genome size of buckwheat species indicates that the genome of *F. esculentum* is 2.5 times larger than that of *F. tataricum* (1.2 Gb vs. 489.3 Mb) (Yasui et al., 2016; Zhang et al., 2017). Available data on the identification of various genes potentially involved in the biosynthesis of rutin, resistance to aluminium and stresses associated with drought and cold indicate that *F. tataricum* tolerates high levels of abiotic stresses (Zhang et al., 2017).

It is possible that the harsh conditions in which the Tartary buckwheat grows have an impact on its sensitivity to mutagens. Dose-dependent responses to MH were observed on both the common buckwheat and Tartary buckwheat; however, interestingly, the reduction of growth was not correlated to the dose of MH for Tartary buckwheat.

Among the many factors important for the final mutagenic effect, the ability of mutagens to penetrate plant tissue and nucleus is important. The concentration of the mutagen and the treatment time determine the mutagenic effect – a stronger genetic and somatic effect is observed after the use of higher doses of the mutagen (Shu, 2009). The final mutagenic effect is also determined by the physiological state of the plants and the conditions of mutagenic treatment. Moreover, the efficiency of DNA repair processes influences the final response to the mutagen (Maluszynski et al., 2003).

Nuclear breaks are the crucial DNA damage that is induced by numerous mutagens (Kumari et al., 2008; Juchimiuk-Kwasniewska et al., 2011). DNA double-strand breaks (DSBs) are required lesions for the production of chromosome rearrangements (Pfeiffer et al., 1996; Schubert et al., 2004). We evaluated DNA damage in a buckwheat nucleus using the TUNEL test. The advantage of the TUNEL test is its possibility to detect DNA breaks in non-dividing cells. Dose dependencies and presoaking condition dependencies were observed for the frequencies of micronuclei and DNA-damaged nuclei, detected by the TUNEL test. Thus, we postulate that in case of *Fagopyrum* species, using the TUNEL test for the estimation of cytogenetic effect is faster and easier than time-consuming analysis of the dot-like micronuclei.

The ability of mutagens to penetrate plant tissue, cells and nucleus is crucial for the final mutagenic effect. The time of seed presoaking has no significant influence on the genotoxic effects of *F. esculentum* and *F. tataricum.* However, the application of MH to isolated embryos increased the cytogenetic effect. The most effective mutagenic treatment in common buckwheat was observed if the MH has been applied to isolated embryos, isolated from previously presoaked seeds for 24 h.

## 6 Conclusion and Future Perspective

Our study demonstrated the differences in the cytogenetic effects caused by MH for *F. esculentum* and *F. tataricum*. No differences regarding the sensitivities of analysed cultivars to MH – *F. esculentum* Kora and Panda – were demonstrated.

Future studies, using other chemical and physical mutagens, will be applied to determine the sensitivity of the genome of buckwheat species. Looking to the future, this knowledge will greatly contribute to the applied improvement of *Fagopyr*um breeding programmes as efficient biological tools for food production.

Our data will provide valuable resources that can have an impact on successfully modulating yield productivity and yield stability for future buckwheat breeding programmes.

**Acknowledgements** The authors wish to acknowledge the support of the International Atomic Energy Agency under the framework of the RER5024 project.

## References

Brian, J., Shan, F., Tian, Z., Xu, G., Lin, R., Chunsheng, X., et al. (Eds.). (2004). Study on new health foods of Tartary buckwheat. In *Advances in buckwheat research proceed of the 9th international symposium on buckwheat, Prague, Czech Republic*, p. 23.

Fabian, C., & Ju, Y. H. (2011). A review on rice bran protein: Its properties and extraction methods. *Critical Reviews in Food Science and Nutrition, 51*(9), 816–827.

Hagels, H. (1999). *Fagopyrum esculentum* Moench. Chemical review. *Zbornik BFUL, 73*, 29–38.

Ito, S., Ohsawa, R., Tsutsumi, T., Baba, T., Arakawa, A., Hayashi, K., & Nakamura, E. (2005). A new common buckwheat variety "Toyomusume" with high yield and higher rutin content. *Bulletin of the National Agricultural Research Center, 6*, 1–13.

Juchimiuk-Kwasniewska, J., Brodziak, L., & Maluszynska, J. (2011). FISH in analysis of gamma ray-induced micronuclei formation in barley. *Journal of Applied Genetics, 51*(1), 23–29.

Khan, K., & Shewry, P. R. (2009). *Wheat: Chemistry and technology* (4th ed.). AACC International.

Kreft, M. (2016). Buckwheat phenolic metabolites in health and disease. *Nutrition Research Reviews, 29*(1), 30–39.

Kreft, I., Zhou, M., Golob, A., Likar, M., Luthar, Z., Germ, M., & Dziedzic, K. (2020). Breeding buckwheat for nutritional quality. *Breeding Science, 70*, 67–73.

Kumari, S., Rastogi, R. P., Singh, K. L., Singh, S. P., & Sinha, R. P. (2008). DNA damage: Detection strategies. *EXCLI Journal, 7*, 44–62.

Kwasniewska, J. (2014). Mutagenic effects at DNA and chromosome level. In N. Tomlekova, I. Kozgar, & R. Wani (Eds.), *Mutagenesis: Exploring novel genes and pathways* (Vol. 17, pp. 333–354). Wageningen Academic Publishers/Enfield Publishing & Distribution Co.

Maluszynski, M., Szarejko, I., Maluszynska, J. (2003). *Mutation techniques. In: Pond, S., Cameron, S. (eds) Genetics of crop improvement. Elsevier*, pp. 1–16.

Marcano, L., Carruyo, I., Del Campo, A., & Montiel, X. (2004). Cytotoxicity and mode of action of maleic hydrazide in root tips of *Allium cepa* L. *Environmental Research, 94*, 221–226.

Minami, M., Kitabayashi, H., Nemoto, K., & Ujihara, A. (2001). Breeding of high rutin content common buckwheat, "SunRutin". In *Advances in buckwheat research, proceeding of the 8th international symposium buckwheat at Chunchon*, pp. 367–370.

Pfeiffer, P., Gottlich, B., Reichenberger, S., Feldmann, E., Daza, P., Ward, J. F., Milligan, J. R., Mullendres, L. H. F., & Natarajan, A. T. (1996). DNA lesions and repair. *Mutation Research, 366*, 69–80.

Schubert, I., Pecinka, A., Meister, V., Klatte, M., & Jovtchev, G. (2004). DNA damage processing and aberration formation in plants. *Cytogenetic and Genome Research, 104*, 104–108.

Shu, Q. Y. (2009). Induced mutations in the genomics era. Joint FAO/ IAEA Programme, Nuclear Techniques in Food and Agriculture, Vienna Austria, pp. 458.

Skerritt, J. H. (1986). Molecular comparison of alcohol-soluble wheat and buckwheat proteins. *Cereal Chemistry, 63*, 365–369.

Steadman, K. J., Burgoon, M. S., Lewis, B. A., Edwardson, S. E., & Obendorf, R. L. (2001). Minerals, phytic acid, tannin and rutin in buckwheat seed milling fractions. *Journal of the Science of Food and Agriculture, 81*, 1094–1100.

Sun, Z., Wang, X., Liu, R., Du, W., Ma, M., Han, Y., Li, H., Liu, L., & Hou, S. (2021). Comparative transcriptomic analysis reveals the regulatory mechanism of the gibberellic acid pathway of Tartary buckwheat (Fagopyrum tataricum (L.) Gaertn.) dwarf mutants. *BMC Plant Biology, 21*, 206.

Swietlinska, Z., & Zuk, J. (1978). Cytotoxic effects of maleic hydrazide. *Mutation Research, 55*(1), 15–30.

Sytar, O., Biel, W., Smetanska, I., & Brestic, M. (2018). Bioactive compounds and their biofunctional properties of different buckwheat germplasms for food processing. In M.-L. Zhou, I. Kreft, G. Suvorova, Y. Tang, & S. H. Woo (Eds.), *Buckwheat germplasm in the world* (pp. 191–204). Academic.

Tang, Y., Bin, Z. X., & Gang, Z. (2002). Improvement of tartary buckwheat by induced mutations with $^{60}$Co gamma rays. In M. Maluszynski & K. J. Kasha (Eds.), *Mutations, in vitro and molecular techniques for environmentally sustainable crop improvement*. Springer.

Morishita, T., Shimizu, A., Yamaguchi, H., & Degi, K. (2019). Development of common buckwheat cultivars with high antioxidative activity- 'Gamma no irodori', 'Cobalt no cikara', and Ruchiking. *Breeding Science, 69*, 514–520.

Underbrink, A. G., Sparrow, R. C., & Pond, D. V. (1968). Chromosome and cellular radiosensitivity II. Use of interrelationships among chromosome volume, nucleotide content and Do of 120 diverse organisms in predicting radiosensitivity. *Radiation Botany, 8*, 205–237.

Wijngaard, H. H., & Arendt, E. K. (2006). Buckwheat. *Cereal Chemistry, 83*(4), 391–401.

Yasui, Y., Hirakawa, H., Ueno, M., Matsui, K., Katsube-Tanaka, T., Yang, S. J., Aii, J., Sato, S., & Mori, M. (2016). Assembly of the draft genome of buckwheat and its applications in identifying agronomically useful genes. *DNA Research, 23*(3), 215–224.

Zhang, Z.-L., Zhou, M.-L., Tang, Y., Li, F.-L., Tang, Y.-X., shao, J.-R., Xue, W.-T., & Wu, Y.-M. (2012). Bioactive compounds in functional buckwheat food. *Food Research International, 49*, 389–395.

Zhang, L., Li, X., Ma, B., Gao, Q., Du, H., Han, Y., Li, Y., Cao, Y., Qi, M., Zhu, Y., Lu, H., Ma, M., Liu, L., Zhou, J., Nan, C., Qin, Y., Wang, J., Cui, L., Liu, H., Liang, C., & Qiao, Z. (2017). The Tartary buckwheat genome provides insights into rutin biosynthesis and abiotic stress tolerance. *Molecular Plant, 10*, 1224–1237.

# Improvement of Yield in Cowpea Varieties Using Different Breeding Approaches


**Aamir Raina, Rafiul Amin Laskar, Mohammad Rafiq Wani, and Samiullah Khan**

**Abstract** Cowpea is an important warm-season legume growing in arid and semi-arid regions. The cowpea productivity is low compared to other legumes such as chickpea, lentil, faba bean and mung bean. The low productivity is attributed to different abiotic and biotic stresses, therefore, different breeding strategies have been introduced and implemented to alleviate the negative impact of environmental stresses. In this chapter, we reviewed the contributions of conventional approaches and modern breeding strategies and their role in the improvement of cowpea genotypes. This chapter discusses in detail challenges and landmark achievements of hybridization, tissue culture, mutation breeding and molecular breeding and its role in mitigating the abiotic and biotic stresses, improving yield and nutritional traits, mining of QTLs associated with economically important traits, development and fine tuning of genetic maps in cowpea.

**Keywords** Cowpea · Yield improvement · Hybridization · Mutation breeding · Tissue culture · Molecular breeding

A. Raina (✉)
Mutation Breeding Laboratory, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

Botany Section, Women's College, Aligarh Muslim University, Aligarh, Uttar Pradesh, India
e-mail: araina@myamu.ac.in

R. A. Laskar
Department of Botany, Pandit Deendayal Upadhyaya Adarsha Mahavidyalaya (PDUAM), Eraligool, Karimganj, Assam, India

M. R. Wani
Department of Botany, Abdul Ahad Azad Memorial Degree College Bemina, Cluster University, Srinagar, Jammu and Kashmir, India

S. Khan
Mutation Breeding Laboratory, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India



A. Raina et al. (eds.), *Advanced Crop Improvement, Volume 2*,
https://doi.org/10.1007/978-3-031-26669-0_6

## 1 Introduction

Cowpea (*Vigna unguiculata* (L) Walp.) is a diploid warm season legume with 22 chromosomes (2n = 2x = 22), grown in the semi-arid tropics, belonging to family Fabaceae, subfamily Faboideae (syn. Papillionoideae), tribe Phaseoleae, subtribe Phaseolinae, genus *Vigna*, and section Catiang (Maréchal et al., 1978; Verdcourt, 1970). In Asia, Africa, Southern Europe, the Southern United States and Central and South America, cowpea is a major food and forage legume (Singh, 2005; Timko et al., 2007a). It is a multipurpose crop used as human food and cattle feed and is a source of revenue for millions of resource-poor farmers (Langyintuo et al., 2003; Singh, 2002). The origin of cowpea is debatable and opinions vary in terms of its African and Asian origin. Padulosi and Ng (1997) proposed that cowpea originated in southern Africa and Baudoin and Maréchal (1985) considered east and southern Africa as the major region of diversity, with west and central Africa as the secondary centre of diversity. Cowpea seeds are rich in dietary proteins (23% to 32%; high in lysine and tryptophan), minerals, folic acid and vitamins B, consumed in a variety of forms such as cooked dishes or flour products (Nielsen et al., 1997; Ahenkora et al., 1998; Hall et al., 2003a, b). In addition to seeds and fresh green leaves, unripe pods are eaten as a dish in many regions of Africa and Asia (Tarawali et al., 1997, 2002). In many regions of West Africa, cowpea is also used as a source of protein-rich fodder for cattle (Singh & Tarawali, 1997; Tarawali et al., 1997, 2002). According to the FAO, 89,03,329 tonnes of dry cowpea grain is produced yearly on about 14,447,336 hectares of land across the world (Table 1).

Among the regions Africa produces more than 90% of total cowpea production (Fig. 1). However, the cowpea production is greater than FAO estimates as the FAO does not include the production statistics in Brazil, India and other countries (Singh et al., 2002). Nigeria is the world's leading producer of cowpea, with an annual production of 27,31,344.31 tonnes on 4,303,005 hectares of land (Table 2).

In Asia, Myanmar recorded 108,021 tonnes of cowpea in 2019 (Table 3). The production of cowpea is restricted by a wide range of biotic and abiotic factors (Singh, 2005; Timko et al., 2007a, b). Among the abiotic factors, water availability is one of the most critical abiotic limitations to development and output, even though cowpea is intrinsically more drought-tolerant than other crops. Among biotic factors, cowpea is vulnerable to a number of bacterial, fungal, and viral diseases, as well as a wide range of insect pests (Singh, 2005; Timko et al., 2007a, b). There exists a broad scope for cowpea breeders to generate cultivars with agronomic

**Table 1** Cowpea (dry) area harvested (ha), yield (hg/ha) and production (tonnes) by regions (2019)

| Region | Area harvested | Yield | Production |
|---|---|---|---|
| Africa | 14,205,204 | 6066 | 8,616,443 |
| Americas | 63,699 | 10,210 | 65,039 |
| Asia | 170,755 | 11,594 | 197,970 |
| Europe | 7678 | 31,098 | 23,877 |
| World | 14,447,336 | 6163 | 8,903,329 |

Source: FAOSTAT (2021)

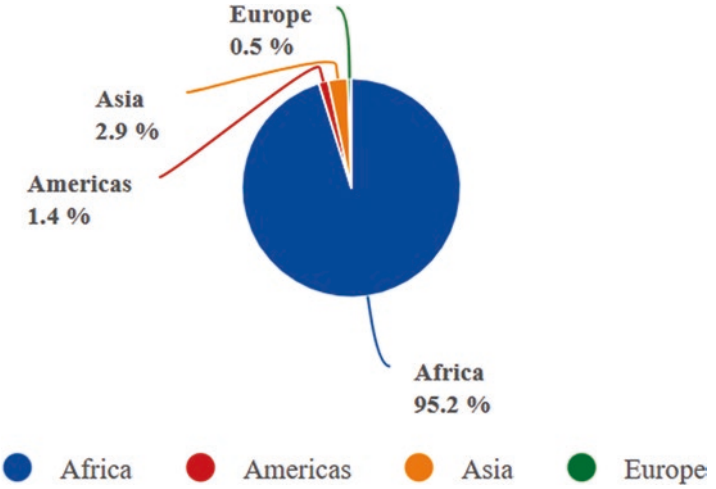



**Fig. 1** Production share of cowpeas (dry) by region (average 1994–2019). (Source: FAOSTAT, 2021)

**Table 2** Top ten producers of cowpeas (dry) (average 1994–2019)

| Country | Production (tonnes) |
|---|---|
| Nigeria | 27,31,344.31 |
| Niger | 10,60,251.77 |
| Burkina Faso | 4,23,806.5 |
| Ethiopia | 3,24,046.77 |
| Ghana | 2,11,205.33 |
| United Republic of Tanzania | 1,37,684.35 |
| Myanmar | 1,28,907.42 |
| Mali | 1,21,820.65 |
| Cameroon | 1,16,515.54 |
| Sudan | 1,13,862.5 |

Source: FAOSTAT (2021)

**Table 3** Major cowpea (dry) producers of Asia

| Country | Area harvested (ha) | Yield (hg/ha) | Production (tonnes) |
|---|---|---|---|
| China, mainland | 14,503 | 10,133 | 14,696 |
| Iraq | 64 | 53,594 | 343 |
| Myanmar | 122,637 | 8808 | 108,021 |
| Palestine | 46 | 37,826 | 174 |
| Philippines | 248 | 19,315 | 479 |
| Sri Lanka | 7195 | 11,212 | 8067 |
| Yemen | 26,062 | 25,397 | 66,190 |

Source: FAOSTAT (2021)

characteristics such as high yielding potential and resistance to abiotic and biotic stresses. Different breeding strategies have been employed to improve the agronomy of cowpea. In this chapter, we reviewed the conventional and modern breeding approaches used for improving the cowpea cultivars.

## 2 Hybridization

Hybridization is a process in which desirable traits such as high yield, better nutrient quality and tolerance to biotic and abiotic stresses are transferred from one or multiple parent crops into a single hybrid cultivar. In the process of hybridization, selection of parents plays a critical role in determining the success or failure of achieving the desired goals. For instance, parents that are tolerant to a particular stress are employed to transfer the tolerance traits into a susceptible variety with good yielding potential. In contrast, parents adapted to diverse environmental conditions are employed to develop hybrid varieties with enhanced genetic variability. Therefore, it is important to get the preliminary data on agronomically important traits of parents for a crossing programme. The source of desirable traits is important to screen and usually wild relatives of any crop are tolerant to diverse environmental stresses (Stalker, 1980). Therefore, wild relatives of cowpea that independently evolved within specific environments are valuable genetic resources and source of important traits that could be used for producing of climate-smart cowpea varieties in the context of climate change (Chheda & Fatokun, 1982). The production of existing cowpea varieties is severely hampered by some insect pests, particularly the pod borer (*Maruca vitrata),* and pod-sucking bugs (*Clavigralla tomentoscollis* Germ, and *C. shadabi*). However, the tolerance in these cultivated cowpea varieties could be improved by using wild relatives as parents in crossbreeding programmes. For instance, wild *Vigna* species, such as *V. oblongifolia* and *V. vexillata,* are tolerant to the legume pod borer and pod-sucking bugs and efforts are put together to transfer the stress tolerance traits from these species to cultivated cowpea (IITA, 1972; Singh et al., 1990). Several crosses were carried out between *Vigna unguiculata* and *V. vexillata* with the objective of transferring the desirable genes for resistance to insect pests from the wild to the cultivated species. Such wild relatives act as an important source of environmental stress tolerance and have contributed immensely in developing elite crop varieties. Till now cowpea has been successfully crossed only to genotypes belonging in section *Catiang.* Fatokun and Singh (1987) were successful in developing partially sterile but vigorously growing F1 hybrids by crossing *Vigna unguiculata* with *Vigna pubescence* (a hairy wild relative). Even though *V. vexillata* from the Plectrotropis section is the most phylogenetically close to cowpea yet failed to produce any interspecific hybrid (Barone & Ng, 1990; Fatokun, 2002), the failure had been attributed to a lack of fertilization and degeneration of 7-day-old fertilized ovules.

## *2.1 Interspecific Hybridization in the Subgenus Vigna*

Interspecific hybridization involving non-cultivated but insect-resistant *Vigna* species (*Vigna oblongifolia* x *V. luteola*) resulted into a partially fertile F1 interspecific hybrid. The F hybrids were advanced to F2 plants which were crossed with cultivated cowpea. However, no hybrids were produced and interspecific hybridization attempts were not successful in transferring insect resistance genes from any of *Vigna* species to cultivated cowpea. The successful crossing of *V. luteola* and *V. oblongifolia* produced hybrid plants, which can be employed as bridges for crosses to cowpea (Schnapp et al., 1990). Several workers have made effort to overcome the cross-incompatibility between *Vigna unguiculata* and *V. vexillata* (Fatokun, 2002) These efforts include the following: (a) cross-breeding among accessions of both species and none of crosses succeeded to produce interspecific hybrids. (b) Spraying of growth hormones such as 2,4-D and NAA on the flowers of *V. vexillata* before and after pollination; however, pod retention was improved but no hybrid seeds were produced (Fatokun, 2002). (c) Embryos extracted from ovules and placed in the MS culture media revealed no development after the globular stage (Fatokun, 1991; Fatokun, 2002). (d) Polyploidization of both species was carried out in both the species. However, polyploid lines were produced only in the accessions of cultivated cowpea and even these lines failed to produce any hybrid when crossed with *V. vexillata* in both directions (Fatokun, 2002). The author also crossed parthenocarpic cowpea line (R1 36) with accessions of *V. vexillata* with no success in the hybrid production. Based on these attempts, it can be concluded that cultivated cowpea are incompatible with its wild relatives outside the section Catiang that prevented the transfer of useful genes in *V. vexillata* to cultivated cowpea. Moreover, crosses between *V. vexillata* with various cultivated and non-cultivated cowpeas were not feasible (Fatokun, 1991). A cross between members of section *Catiang*, viz., cowpea variety (IT84S-2246-4) and a genotype of *V. unguiculata* ssp. *dekindtiana* var. *pubescens* (TVNu IlO-3A), resulted into partially fertile F1 hybrids. This was another attempt of transferring insect resistance or hairiness from var. *pubescens* to cowpea. Another cross was made between *V. unguiculata* and *V. unguiculata* ssp. *Rhomboidea* that resulted into partially F1 fertile plants. The cytological analysis of pollen mother cells of F1 plants revealed an unequal distribution of chromosomes to the microspores at late telophase II and this may be attributed to the increasing sterility of pollens. Another cross between yard-long bean (*V. unguiculata* ssp. *sesquipedalis*) and *V. unguiculata* ssp. *tenuis* resulted in F1 plants that were robust in growth. However, pollen fertility was low and that has been attributed to a lack of complete homology between chromosomes. Considering the higher rate of failure of interspecific or even intraspecific hybridization in cowpea, it is important to research the environmental factors that could influence the success of hybridization. Among the environmental factors, temperature and humidity are important that affect flower initiation, pollen fertility and pod set in the hybrids. Amusa et al. (2022) reported that a moderate temperature and high humidity are appropriate to achieve the success of hybridization in cowpea. The low temperature and high humidity improve

the stigma reception and activity of the pollen down to the receptive style, thus producing viable hybrids from the cross. Hybridization has been employed to develop F1 hybrids with improved drought tolerance (Sherif et al., 1991). Previous workers have reported that cowpea hybrids exhibit significant heterosis for a number of pods per plant, seeds per pod seed yield per plant (Adu-Dapaah et al., 1988; Patil & Shete, 1987; Teofilo et al., 1984), pod length, number of clusters per plant, seed length and seed weight (Patil & Shete, 1987), green fodder (Lodhi et al., 1990) and yield protein content of seed (Emebiri, 1991).

## 3 Tissue Culture

The development of varieties using traditional breeding approaches are tedious, laborious and expensive. This necessitates the implementation of new modern breeding tools such as plant tissue culture, as most pulse crops are self-pollinated and possess a narrow genetic base that further hinders the variety development (Raina et al., 2016). Genetic variability is one of the main prerequisite in any crop improvement programme. Existing cowpea cultivars have been reported to possess a narrow genetic base due to monoculture farming by farmers and cross-incompatibility between wild *Vigna* species and cultivated cowpea. This has greatly hindered the transfer of desirable traits from wild cowpea to cultivated cowpea (Abdu Sani et al., 2015; Fang et al., 2007; Gomathinayagam et al., 1998; Latunde-Dada, 1990; Wamalwa et al., 2016). Therefore, it is very important to introduce new breeding strategies for widening the genetic base by the introgression of useful genes from CWR into cultivated species. Among the new breeding tools, plant tissue culture is a promising technique to overcome those constraints and play a central role in improving agronomic traits and developing varieties with higher yield and stress tolerance (Zaidi et al., 2005). The recalcitrant nature of cowpea necessitates the creation of reproducible tissue culture protocols to increase genetic variability and selection of improved plant varieties (Ochatt et al., 2010). Using plant tissue culture techniques, several attempts have been made to develop whole cowpea plants from various genotypes (Brar et al., 1999). Like other legumes, very little progress has been achieved in cowpea improvement using tissue culture techniques. Several workers had used different explants to regenerate whole cowpea plants, for instance, primary leaves (Muthukumar et al., 1995; Prem Anand et al., 2000; Ramakrishna et al., 2005), mature cotyledon (Brar et al., 1999; Muthukumar et al., 1995), cotyledonary node (Van Le et al., 2002; Chaudhury et al., 2007), embryonic axis (Popelka et al., 2006), epicotyl (Pellegrineschi, 1997) and mature embryo (Odutayo et al., 2005; Popelka et al., 2006). Aasim et al. (2008) employed shoot meristem excised from 3–4-day-old in vitro-grown seedlings on MS medium and succeeded in developing a reliable micropropagation system for Turkish cowpea cv. Akkiz. In another study, Aasim et al. (2010) employed preconditioned embryonic axes of the Turkish cowpea cultivar Akkiz and succeeded in developing a viable and reproducible approach for shoot regeneration in the cowpea in vitro multiplication

and shoot regeneration protocols. The same research group also developed a reliable, efficient and reproducible micropropagation system for multiplication of in vitro genetically transformed cowpea. Monti et al. (1997) reported 33% of explants (primary leaves and hypocotyl) developed some shoots cultured on a modified B5 medium containing fresh coconut water and high cytokinin concentration. The authors also subjected explants to histology analysis that depicted a strong cellular proliferation on the epidermis explant, where callus was formed. Other experiments involving in vitro culture of Italian local cowpea cultivar "Cornetto" in a medium containing natural Nigerian coconut water, commercial coconut water (Sigma C5915, deproteinized) and local coconut water revealed that Nigerian coconut water induced maximum healthy shoot production. In addition, it was concluded that only the basal part of immature leaflets produced shoots. Ganapathi and Anand (1998) employed cowpea seedling leaf explants and were successful in developing somatic embryos. After successful shoot differentiation from explants, scientists at Purdue University attempted to produce multiple bud proliferation from highly morphogenic cowpea tissues. They studied the effect of a low concentration of auxin and high concentration of benzyl amino purine (3–6 mg/L) on the regeneration capacity of cotyledon segments and embryonic axes from embryos of different stages of several cowpea species. The explants grown in a medium containing a low concentration of cytokinin under light conditions produced shoots from regenerated buds. After 21 days of in vitro culture, shoots developed at 50% frequency from the cotyledon explants (Monti et al., 1997). Scientists at the University of Naples studied the effect of different concentrations of thidiazuron, viz., 5 mM, 10 mM and 20 mM, on apical and lateral bud proliferation of cowpea cv Cornetto and other three lines, viz., TVu 9062, VITA3 and VITA4. The results revealed that the maximum average frequency of multiple bud proliferation was observed in Cornetto (87%) and the line TVu 9062 (85%). However, rooting was observed on transferring these buds into a basal medium lacking thidiazuron (Malik & Saxena, 1992). Therefore, it was concluded that thidiazuron is a growth regulator for induction of multiple bud proliferation from cotyledonary and apex nodes. Machuka et al. (2000) also attempted the induction of multiple shoot formation using different explants such as roots, leaves and stem apices. They were successful in inducing organogenesis of different cowpea genotypes. Popelka et al. (2006) developed the most effective genetic transformation of cowpea using regeneration by organogenesis of several explants grown on culture media containing moderate levels of cytokinin. The authors concluded that the best explants for multiple shoot formation are longitudinally bisecting seeds through their embryonic axes with removed shoot and root apices. By virtue of its recalcitrant nature, several protocols have been put forward for in vitro regeneration in cowpea using different explants (Kartha et al., 1981; Nagl et al., 1997; Le et al., 2002). Muthukumar et al. (1995) were able to induce somatic shoots from leaflet callus and Ramakrishnan et al. (2005) recommended somatic embryos for plantlet regeneration. However, little progress has been made in reporting a reproducible protocol for in vitro regeneration in cowpea (Prem Anand et al., 2000).

## 4 Mutation Breeding

Hugo de Vries (1901) first introduced the concept of using induced mutations for creating novel varieties and the first experimental proof that X-rays are mutagenic in *Drosophila* and maize was reported by Muller (1927) and Stadler (1928), respectively. Then several workers experimented and concluded that rays are mutagenic, Ganger and Blakeslee (1927) in *Datura stramonium* and Goodspeed (1929) in *Nicotiana.* The first mutant was developed in *Nicotiana tobacum* called "Chlorina" by treating flower buds with X-rays in the 1930s (Tollenaar, 1934; Konzak, 1957). Gustafsson (1947) is the pioneer to advocate the applicability of mutations in crop improvement programmes. This encouraged other workers to experiment with different mutagens and led to a broader understanding of induced mutations for the genetic improvement of crops (Brock, 1965; Gaul, 1965; Ilbas et al., 2005; Kharkwal, 1996; Nakagawa et al., 2011; Raina et al., 2017). Unlike other crop improvement means, mutation breeding offers a possibility of enhancing one or two traits without altering the genetic setup (Khan et al., 2009; Shu et al., 2012). Thousands of economically important varieties have been developed using mutation breeding techniques (Ahloowalia et al., 2004; Khan et al., 2009; Raina et al., 2016). Induced mutagenesis is the most efficient technique to greatly increase genetic variation in a short period of time and has been employed in various crops such as cowpea (Raina et al., 2020a; Rasik et al., 2022), lentil (Laskar et al., 2018a, b; Wani et al., 2021), faba bean (Khursheed et al., 2015), fenugreek (Hasan et al., 2018), mung bean (Wani et al., 2017), urdbean (Goyal et al., 2019a, b), chickpea (Laskar et al., 2015; Raina et al., 2019), black cumin (Tantray et al., 2017; Amin et al., 2019) and finger millet (Sellapillaibanumathi et al., 2022). Because natural mutations occur sporadically, artificial mutations are generated, and genetic gain is best achieved by using mutagens (Raina & Khan, 2020; Raina et al., 2018a, 2022a, b, c, d; Rasik et al., 2022; Sellapillai et al., 2022).

Among the physical mutagens, gamma rays are the most favoured in the crop improvement programmes (Celik & Atak, 2017; Khursheed et al., 2019) due to its superb penetration power that enables it to create ionization of atoms and excitation of electrons that react and influence the DNA structure and properties. This often leads to rupture of chemical bonds of the bases and the backbone of DNA molecules. Gamma rays are known to cause hydrolysis of water molecules in a process called radiolysis that leads to generation of free radicals, viz., H and OH. These free radicals are highly reactive and attack the DNA constituents, more vigorously in the presence of oxygen and induce alterations in structural and functional properties of DNA molecules. In addition, chemical mutagens are more advantageous than physical mutagens (Auerbach, 1965; Goyal et al., 2021a, b; Khursheed et al., 2016; Wani et al., 2014a). Among chemical mutagens, alkylating agents such as ethyl methanesulphonate, methyl methanesulphonate and sodium azide are most potent. By virtue of their properties, chemical mutagens have emerged as most preferable method of mutagenesis (Jain, 2002; Greene et al., 2003; Perry et al., 2003; Goyal et al., 2020a, b). Several workers also reported that chemical mutagenesis was more effective and efficient than physical mutagenesis in cowpea and other legumes (Ajayi et al., 2017;

Dhanavel et al., 2008; Gnanamurthy & Dhanavel, 2014; Khursheed et al., 2017; Ojomo & Cheda, 1975; Raina et al., 2022a, b). There are various reports available that support the effectiveness of combined mutagens over individual mutagens for the improvement of cowpea (Bind et al., 2016; Deepalakshmi & Anandakumar, 2003; Girija & Dhanavel, 2009; Singh et al., 2013). In mutagenesis experiments, it is important to evaluate the mutagenic potency; chlorophyll mutation is one of the reliable index to make this assessment. Several workers have reported chlorophyll mutations in cowpea (Girija & Dhanavel, 2009; John, 1999; Mohanasundaram et al., 2001). Further, mutagenic effectiveness and efficiency play a critical role in the success of crop improvement programmes and it is very imperative to assess this property of mutagens prior to experimentation. Mutagenic effectiveness and efficiency are two unique properties that determine the success of any mutagen. The effectiveness and efficiency of various mutagens were reported to vary to a greater extent in pulses such as cowpea (Dhanavel et al., 2008). Girija and Dhanavel (2009) treated seeds of *Vigna unguiculata* with different doses of gamma rays such as 15, 20, 25, 30 and 35 kR; EMS such as 5, 10, 15, 20 and 25 mM; and combined treatments of gamma rays and EMS such as 15 kR + 15 mM, 20 kR + 15 mM, 25 kR + 15 mM, 30 kR + 15 mM and 35 kR + 15 mM. Mutagenic effectiveness and efficiency were estimated on the basis of seedling injury, seed lethality in $M_1$ and frequency of chlorophyll and viable mutations in $M_2$ generation. They reported that mutagenic effectiveness and efficiency were higher at lower doses of both single and combination treatments of mutagens. EMS was found to be more effective and efficient compared to gamma rays and combined treatments. Nair et al. (2014) treated Pusa Komal and Arka Garima varieties of *Vigna unguiculata* with different doses of gamma rays such as 100, 200, 300, 400 and 500 Gy and EMS at 0.25%, 0.30%, 0.35%, 0.40% and 0.45%. Variety Arka Garima showed less sensitivity than Pusa Komal. With the increasing doses of mutagens, mutagenic effectiveness and efficiency decreased in the two varieties.

Plant breeders are usually interested in agronomic traits that happen to quantitative in nature controlled by multiple genes (Kalapchieva & Tomlekova, 2016; Laskar et al., 2015; Raina et al., 2021; Wani et al., 2014b). Gaul (1965) emphasized the worth of micromutations in plant breeding by reporting that micro mutations occur at higher frequency and affect morpho-physiological traits. Since mutagen-induced variability for quantitative traits in plants is heritable and the response of the selection seems fine, many workers such as Khan and Siddiqui (1992), Wani et al. (2011, 2014b), Raina et al. (2016), Javed et al. (2016) and Laskar and Khan (2017) have advocated that in quantitatively inherited traits, induced mutations can be exploited to create useful variations, where appropriate selection could be employed for further improvement. Homozygosity is a prerequisite for the expression of a mutated gene because induced mutations occur randomly in the genome and inheritance is almost always recessive (Micke, 1999).

The practical value of induced mutagenesis, in creating successful genetic variability for several desired traits in plant improvement programmes, has been well established and has been demonstrated by many workers in various pulse crops (Raina et al., 2021). In mutation breeding research programmes,

quantitative traits are considered as the most important attributes. These traits are governed by several genes with additive effects. After the studies of Brock (1965, 1967), it became a common practice to exert selection in M2 generation onwards and advance only healthy plants from $M_2$ to $M_3$ generation. Selection for quantitative traits, is effective in early generation as the combinations of favourable alleles may be lost in subsequent generations due to rigorous or even no selection for other traits (Raina et al., 2020b). Many researchers have recommended early generation selection for quantitative traits and some of the noteworthy are Sneep (1977), Micke (1999), and Clement et al. (2015). Kharkwal (1983) also hold the same opinion that the efficacy of early generation selection for isolating elite for polygenetically inherited traits. The efficiency of early generation selection in mutation breeding experiments has been reported in many crop species such as cowpea (Bhadru & Navale, 2012; Padi & Ehlers, 2008; Raina et al., 2022c).

Food insecurity and malnutrition are the most striking global threat, particularly in developing countries including India, where the population grows at an alarming rate. Thus, plant breeders are aiming to develop high-yielding crop varieties with improved mineral density. Since the beginning of agriculture, legumes have been part of the human diet. Many grain legume crops are still an irreplaceable source of dietary proteins, fibres, vitamins and minerals (Mitchell et al., 2009; Wang et al., 2003a) mostly in the vegetarian diets of Indian people. Cowpeas are the highest source of proteins, very palatable, very nutritious and comparatively free from metabolites or other toxins (Quass, 1995). Several environmental factors such as rainfall, light intensity, length of the day, length of growing season and temperature affect the protein content of the cowpea (Oluwatosin, 1997). Cowpeas contain a considerable amount of protein and mineral density. Chemical and nutritional compositions of cowpea, as well as its cooking properties, vary significantly according to environmental and genetic factors (Giami, 2005). Adekola and Oluleye (2007) observed that gamma ray-induced cowpea mutants possess a higher protein content in comparison to the control. The amino acid composition of cowpea protein revealed that similar to most legume proteins, cowpea protein is rich in arginine, leucine and lysine but poor in sulphur amino acids methionine and cysteine (Bressani, 1985; Farinu & Ingrao, 1991). The relatively high lysine content makes cowpea an excellent improver of the protein quality of cereal (Bressani, 1985). Wang et al. (2003b) suggested that the induced mutagenesis can be a potent methodology for a balanced increase in mineral elements in addition to yield and its attributing characters. The impact of mutagens on the genetics of trace element availability in the cell has not been extensively studied; thus, work on mineral elements like iron (Fe), zinc (Zn) and copper (Cu) for their enhancement in pulses needs to be undertaken. The Consultative Group on International Agriculture Research Micronutrient Project (CGIARMP) reported that combining the high micronutrient trait such as Fe, Zn and Cu content, with a high yield is possible, unlike the protein content and yield that are mostly negatively correlated, through breeding strategies (Gregoria, 2002). It is an established fact that the availability of adequate nutrition is essential for the

optimum quantitative and qualitative growth of crop and cowpea is not an exception to that. Mutants with altered seed mineral profiles have been identified in pea (Wang et al., 2003b), chickpea (Raina et al., 2017), faba bean (Khursheed et al., 2018a, b, c) and lentil (Laskar et al., 2019). Even though mutation breeding has contributed immensely in the development of thousands of elite mutant varieties. However, this breeding strategy has major drawbacks such as it is an extremely time-consuming process. This led scientists to adopt a much faster and more effective breeding strategy, that is molecular breeding.

## 5 Molecular Breeding

Plant breeders are interested in pyramiding desirable agronomic traits such as maturity time, photoperiod sensitivity, seed quality, insect pests and disease resistance into a single cultivar (Timko et al., 2007a, b; Timko & Singh, 2008). Conventional breeding has contributed a lot in improving the agronomy of cowpea. Several national and international research programmes implemented conventional breeding approaches to develop crops with high yielding potentials and enhanced tolerance to biotic and abiotic stresses. Nevertheless, such a process is cumbersome, time-consuming and expensive. The limitations of conventional breeding approaches necessitated the introduction and implementation of new breeding technologies such as DNA marker-based molecular plant breeding (Moose & Mumm, 2008; Xu & Crouch, 2008). DNA markers play a pivotal role in the screening of elite genotypes in less time and a cost-effective way and could accelerate the crop development process (Foolad, 2007). In cowpea development of genomic resources began almost one decade earlier compared to other crops. Earlier workers emphasized on molecular diversity and genetic linkage mapping using different markers such as Panella and Gepts (1992), Pasquet (1999, 2000) (allozymes), Fotso et al. (1994) (seed storage proteins), Vaillancourt and Weeden (1992) (chloroplast DNA polymorphism), Fatokun et al. (1993) (RFLP), Fatokun et al. (1997), Fang et al. (2007) (AFLP), Spencer et al. (2000), Simon et al. (2007) (DNA amplification fingerprinting), Mignouna et al. (1998), Fall et al. (2003), Nkongolo (2003), Ba et al. (2004), Diouf and Hilu (2005), Xavier et al. (2005), Zannou et al. (2008) (RAPD), Ogunkanmi et al. (2008), Uma et al. (2009), Xu et al. (2010) (SSR), Sawadogo et al. (2010) (cross-species SSRs from Medicago), Ghalmi et al. (2010) (ISSR), Li et al. (2001), He et al. (2003) (STMS) and Huynh et al. (2013) (SNP). Among the DNA markers, SNPs have gained popularity due to their abundance in the genomes and their amenability for high-throughput detection formats and platforms (Mammadov et al., 2012). SNP markers play a vital role in studies of genetic diversities in crops such as cowpea (Huynh et al., 2013; Egbadzor et al., 2014a, b). Teyiou et al. (2018) used 181 SNP markers for diversity analysis of 50 cowpea lines (collected from the University of California Riverside and IITA/Nigeria, Burkina Faso) and succeeded in the separation of the core collection of 20 elite lines that could be used in future breeding programmes. They also reported a panel of 20 genotypes that showed maximum variability of the germplasm.

### 5.1 Biotic Stress Resistance

Moreover, SNPs were also employed to screen disease-resistant cowpea genotypes. For instance, Muchero et al. (2011) identified strong sources of host resistance to *Macrophomina phaseolina*, a devastating fungal pathogen that causes substantial losses in crop productivity. They also mapped putative resistance loci on a cowpea genetic map comprising of SNPs and amplified fragment length polymorphisms (AFLPs). The cowpea mosaic virus (CPMV) causes a severe production loss in many cowpea-producing areas. Bhattarai et al. (2017) conducted GWAS on 333 cowpea germplasm accessions, collected from 39 different countries, and identified SNPs associated with CPMV resistance. Six SNP markers were found linked with CPMV resistance, viz., C35069548_1883, scaffold65342_6794, scaffold66293_6549, scaffold95805_2175, C350 81948_540 and scaffold17319_4417. These SNP markers would play a pivotal role in developing CPMV-resistant cultivars through MAS. Shi et al. (2016) used 1031 SNP markers on a panel of 400 cowpea accessions and reported SNP markers, viz., C35046071_ 1260, scaffold96328_ 3387, C35084634_455 and scaffold96765_4430, were found linked with bacterial blight resistance in cowpea. Cowpea aphid incurs a substantial loss in cowpea productivity (Nair et al., 2003). Proper strategies are required to mitigate the production loss and the best way to do this is the development of pest-resistant cultivars. Qin et al. (2017) conducted a genetic diversity analysis for aphid-resistant resources on 338 cowpea accessions collected from 40 countries using 1047 SNP markers. GWAS revealed SNP markers, viz., Scaffold30061_3363 and C35011941_894, were found linked with aphid resistance. In another study, CPA-resistant cowpea genotypes were identified in Africa (Hall et al., 2003a, b; Pathak, 1988) and India (Chari et al., 1976). Ombakho et al. (1987) reported two major genes, viz., Ac1 and Ac2 for controlling aphid resistance.

### 5.2 Abiotic Stress Resistance

Increasing salinity causes a substantial loss in cowpea productivity and alleviation of negative impacts of salinity stress requires a broad understanding of genetics of salt tolerance and susceptibility. Ravelombola et al. (2018) conducted association mapping for salt tolerance at germination and seedling stages to identify SNP markers linked with salt tolerance in cowpea. Ravelombola et al. (2018) analysed the salt tolerance index of 271 cowpea genotypes using 1049 SNPs. The study revealed significant variation in the salt tolerance index for the germination rate, and SNP markers, viz., Scaffold87490_622, Scaffold87490_630 and C35017374_128, were found to be linked with salt tolerance at the germination stage and seven SNPs, viz., Scaffold93827_270, Scaffold87490_ 633, Scaffold68489_600, Scaffold87490_640, Scaffold82042_3387, Scaffold93942_1089 and C35069468_1916, were highly linked with salt tolerance at the seedling stage. These SNP markers could be used in

future breeding programmes aimed at developing salt-tolerant cowpea cultivars. Ashebir et al. (2013) reported genetic variations in 19 cowpea genotypes evaluated for salt tolerance at the germination stage. Similarly, Win and Oo (2015) also reported genetic variations while assessing 21 cowpea genotypes for salt tolerance at the seedling stage.

Another factor that impacts the market value of cowpea is the high-temperature-induced browning (Hbs) of seed coats. The brown discoloration reduces the consumer acceptance and affects its commercial value. Pottorff et al. (2014a, b) identified three QTL linked with seed browning, viz., Hbs-1, Hbs-2 and Hbs-3. These QTLs could facilitate the development of cultivars resistant to heat-induced seed browning. In addition larger seed size is also crucial trait for yield with a high commercial value in Africa and other cowpea-producing regions (Mishili et al., 2009). A substantial studies are required to map the QTLs associated with the larger seed size in cowpea.

## *5.3 Cowpea Genetic Maps and Trait-Linked Markers*

Fatokun et al. (1993) developed the first cowpea linkage map using a mapping population of 58 F2 plants derived from a cross between TVNu 1963 and IT84S-2246-4. The map revealed 89 loci comprising 79 RFLP, 5 RAPD and 4 cDNA markers and 1 simply inherited morphological trait was assigned to 10 linkage groups spanning 680 cM of the cowpea genome. The second linkage map was developed by Menendez et al. (1997) using 94 F8 RILs derived from a cross between IT84S-2049 and 524B. This map revealed 181 loci, comprising 133 RAPDs, 19 RFLPs and 25 AFLPs. A third genetic map was created using 94 F8 RILs derived from the cross between IT84S-2246-4 and TVNu 110-3A (Ubi et al., 2000). This map revealed 80 loci comprising of 77 RAPD and three morphological loci were assigned to 12 LGs spanning 669.8 cM of the genome making an average distance of 9.9 cM between marker loci. In 2009, the first SNP consensus map was developed based on the genotyping of 741 members of six bi-parental RIL populations that revealed 928 SNP markers distributed over 11 LGs, covering a total genetic distance of 680 cM (Muchero et al., 2009a). Updated versions of cowpea consensus maps are accessible via HarvEST:Cowpea (http://harvest.ucr.edu/). These maps have been useful to screen QTLs for desirable traits in cowpea such as tolerance to flower bud thrips (Omo-Ikerodah et al., 2008), yellow mosaic virus (Gioi et al., 2012), race-specific *Striga gesnerioides* (Ouédraogo et al., 2001, 2002b; Boukar et al., 2004), root-knot nematode (Ouédraogo et al., 2002a; Huynh et al., 2016), bacterial blight (Agbicodo et al., 2010) and *Macrophomina* (Muchero et al., 2010). In addition, QTLs were also identified for agronomic traits such as days to flower and maturity; pod length; seeds per pod; leaf length and width and leaf area (Ubi et al., 2000); leaf shape (Pottorff et al., 2012a); seed weight (Fatokun et al., 1992); tolerance to seedling-stage drought (Muchero et al., 2009b); delayed senescence, biomass and grain yield (Muchero et al., 2013); heat-induced browning

of seed coats (Pottorff et al., 2014a, b); aphid resistance (Huynh et al., 2015); and *Fusarium oxysporum* f. sp. tracheiphilum (Pottorff et al., 2012b).

The combined efforts of all working for the Tropical Legumes I project, Generation Challenge Program team and LGC Genomics resulted in the conversion of SNP assays to the KASP system while working on the marker-assisted recurrent selection (MARS) and marker-assisted backcrossing (MABC) populations. The team mapped 1022 SNPs. With the advancement in molecular marker techniques and softwares such as "SNP Selector", "KBioConverter" and "Backcross Selector" (http://breedit.org/), and several improved cowpea consensus genetic maps have been developed such as version 4 (Lucas et al., 2012) and version 6 in HarvEST:Cowpea at http://harvest.ucr.edu/. The Breeding Management System (BMS) of the Integrated Breeding Platform (IBP) (https://www.integratedbreeding.net/) working with MARS lines is aiming at developing cowpea lines carrying all the desired traits such as grain yield, drought tolerance and *Striga* and *Macrophomina* resistance. At present, efforts are streamlined to improve the effectiveness of the genotyping and the applicability of the genetic map through a "Feed the Future" project entitled, "Innovation Lab for Climate Resilient Cowpea". The project is aimed at fingerprinting diverse cowpea accessions using 50,000 SNPs to facilitate mapping of trait-linked markers.

## *5.4 Quality and Nutritional Traits*

The genetic upgradation of existing cultivars is important for improving grain quantity and quality and tolerance to abiotic and biotic stresses. A minicore (the "UCR Minicore") composed of 368 domesticated cowpeas selected from a larger set of ~5000 accessions comprising the UC Riverside collection has been assembled (Muñoz-Amatriaín et al., 2021). High-density genotyping of minicore using 51,128 SNPs led to the identification of six subpopulations, mainly differentiated by cultivar group and geographic origin. This study also reported the identification of SNPs associated with important agronomic traits including flowering time. A cluster of four genes (*Vigun05g004000*, *Vigun05g004100*, *Vigun05g004200* and *Vigun05g004300*) annotated as *FLOWERING LOCUS T* (*FT*) are in flowering time regions termed as Vu05. Moreover, authors also reported one major QTL associated with pod load score, dry pod weight and dry fodder weight. High pod load reflects a high number of pods per plant, which is an indication of a low rate of flower abortion. The QTL comprised of genes such as *Vigun04g039300*, *Vigun04g039400*, *Vigun04g039800, Vigun04g039900 Vigun04g039500*, *Vigun04g039600* and *Vigun04g039700.*

A broad understanding of the genetic basis of cowpea seed size could equip breeders in developing varieties with larger seeds. To better understand the underlying genetic factors of seed size, Lo et al. (2019) conducted a genome-wide

association study (GWAS) and meta-analysis on a panel of 368 cowpea diverse accessions from 51 countries and identified 17 loci linked with seed weight, length, width and density using 51,128 SNPs. By integrating synteny-based analysis with common bean, six candidate genes were associated with seed weight, viz., *Vigun05g036000*, *Vigun05g039600*, *Vigun05g204200*, *Vigun08g217000*, *Vigun11g187000* and *Vigun11g191300*. *Vigun05g036000* encodes seed size (Cheng et al., 1996; Jin et al., 2009; Weber et al., 1996), while *Vigun05g039600* encodes a phosphate transporter PHO1 (a positive regulator of seed development) (Zhou et al., 2009). *Vigun05g204200* encodes the polycomb group protein FERTILIZATION-INDEPENDENT ENDOSPERM (FIE) that is associated with endosperm development and regulates seed size (Folsom et al., 2014; Ohad et al., 1996). *Vigun08g217000* encodes a histidine kinase 2 associated with increased organ size (Lonardi et al., 2019). Using GWAS, QTLs associated with root architecture (Burridge et al., 2017), pod length (Xu et al., 2017) and black seed coat colour (Herniter et al., 2018) have also been reported in cowpea. Egbadzor et al. (2013) evaluated 78 cowpea accessions and reported 18 SNP makers linked with seed size in cowpea.

In the improvement programmes, cowpea growth habit is an important trait with a wide variation such as erect, semi-prostrate and prostrate types. Therefore, understanding the genetics of plant growth habit in cowpea is gaining importance and helping breeders in developing suitable cowpea cultivars with desirable growth habits. Ravelombola et al. (2017) used 1031 SNPs for performing an association mapping study for plant habit in 487 cowpea genotypes. The study revealed ten SNP markers associated with growth habit, viz., C35060651_729, C35061339_799, C35062457_1855, C35072764_1384, C35080248_2355, Scaffold2771_4351, Scaffold29522_3213, Scaffold35913_2678, Scaffold53560_188 and Scaffold58098_4297. These makers could be employed for increasing marker-assisted selection (MAS) aimed at developing cowpea cultivars with a desired growth habit.

## 6 Conclusion

Different conventional and modern breeding approaches have contributed to the development of desired cowpea genotypes. However, conventional breeding approaches are laborious and cost-ineffective and could not meet the needs of the present era. On the other hand, modern breeding approaches are quick and cost-effective and are preferred in developing varieties that harbour a desired trait. However, further improvement in modern breeding approaches is required to overcome the drawbacks.

## References


Aasim, M., Khawar, K. M., & Özcan, S. (2008). In vitro micropropagation from shoot meristems of Turkish cowpea (*Vigna unguiculata* L.) cv. Akkiz. *Bangladesh Journal of Botany, 37*(2), 149–154.

Aasim, M., Khawar, M. K., & Özcan, S. (2010). Efficient in vitro propagation from preconditioned embryonic axes of Turkish cowpea (*Vigna unguiculata* L.) cultivar Akkiz. *Archives of Biological Sciences, 62*(4), 1047–1052.

Abdu Sani, L., Usman, I. S., Ishiaku Faguji, M., & Muhammad Bugaje, S. (2015). Towards efficient in vitro regeneration of cowpea (*Vigna unguiculata* L. Walp): A review. *British Biotechnology Journal, 7*, 174–182.

Adekola, O. F., & Oluleye, F. (2007). Influence of mutation induction on the chemical composition of cowpea *Vigna unguiculata* (L.) Walp. *African Journal of Biotechnology, 6*(18).

Adu-Dapaah, H., Singh, B. B., Chheda, H. R., & Fatokun, C. A. (1988). Heterosis and inbreeding depression in cowpea. *Tropical Grain Legume Bulletin, 35*, 23–27.

Agbicodo, E. M., Fatokun, C. A., Bandyopadhyay, R., Wydra, K., Diop, N. N., Muchero, W., et al. (2010). Identification of markers associated with bacterial blight resistance loci in cowpea [*Vigna unguiculata* (L.) Walp.]. *Euphytica, 175*, 215–226. https://doi.org/10.1007/s10681-010-0164-5

Ahenkora, K., Adu-Dapaah, H. K., & Agyemang, A. (1998). Selected nutritional components and sensory attributes of cowpea (*Vigna unguiculata* [L.]Walp.) leaves. *Plant Foods for Human Nutrition, 52*, 221–229.

Ahloowalia, B. S., Maluszynski, M., & Nichterlein, K. (2004). Global impact of mutation derived varieties. *Euphytica, 135*(2), 187–204.

Ajayi, A., Ologundudu, A., Azuh, V., Daramola, O., & Ar, K. (2017). Colchicine–induced genetic variations in M2 and M3 generations of cowpea (*Vigna unguiculata* L. Walp).

Amin, R., Wani, M. R., Raina, A., Khursheed, S., & Khan, S. (2019). Induced morphological and chromosomal diversity in the mutagenized population of black cumin (Nigella sativa L.) using single and combination treatments of gamma rays and ethyl methane sulfonate. *Jordan Journal of Biological Sciences, 12*(1), 23–30.

Amusa, O. D., Ogunkanmi, L. A., Adetumbi, J. A., Akinyosoye, S. T., Bolarinwa, K. A., & Ogundipe, O. T. (2022). Intraspecific-cross compatibility in cowpea (*Vigna unguiculata* (L.) Walp.). *Journal of Crop Improvement, 36*(2), 207–221.

Ashebir, G., Mebeasilassie, A., & Manikanidan, M. (2013). The response of some cowpea (*Vigna unguiculata* (L.) Walp.) genotypes for salt stress during germination and seedling stage. *Journal of Stress Physiology & Biochemistry, 9*, 73–84.

Auerbach, C. (1965). Past achievements and future task of research in chemical mutagenesis. *Proceedings of the 11th International Conference Genetics, 2*, 275–284.

Ba, F. S., Pasquet, R. S., & Gepts, P. (2004). Genetic diversity in cowpea [*Vigna unguiculata* (L.) Walp.] as revealed by RAPD markers. *Genetic Resources and Crop Evolution, 51*, 539–550. https://doi.org/10.1023/B:GRES.0000024158.83190.4e

Barone, A., & Ng, Q. (1990). Embryological study of crosses between *Vigna unguiculata* and *V. vexillata*. In N. Q. Ng & L. M. Monti (Eds.), *Cowpea genetic resources* (pp. 151–160). IITA.

Baudoin, J. P., & Maréchal, R. (1985). Genetic diversity in *Vigna*. In S. R. Singh & K. O. Rachie (Eds.), *Cowpea research, production and utilization* (pp. 3–9). Wiley.

Bhadru, D., & Navale, P. A. (2012). Early generation selection criteria for high yielding genotypes in two populations of cowpea. *Indian Journal of Agricultural Research, 46*(1), 65–69.

Bhattarai, G., Shi, A., Qin, J., Weng, Y., Bradley Morris, J., Pinnow, D. L., Buckley, B., Ravelombola, W., Yang, W., & Dong, L. (2017). Association analysis of cowpea mosaic virus (CPMV) resistance in the USDA cowpea germplasm collection. *Euphytica, 213*(10), 1–12.

Bind, D., Dwivedi, V. K., & Singh, S. K. (2016). Induction of chlorophyll mutations through physical and chemical mutagenesis in cowpea [*Vigna unguiculata* (L.) Walp.]. *International Journal of Advanced Research, 4*(2), 49–53.

Boukar, O., Kong, L., Singh, B. B., Murdock, L., & Ohm, H. W. (2004). AFLP and AFLP-derived SCAR markers associated with Striga gesnerioides resistance in cowpea. *Crop Science, 44*, 1259–1264. https://doi.org/10.2135/cropsci2004.1259

Brar, M. S., Al-Khayri, J. M., Morelock, T. E., & Anderson, E. J. (1999). Genotypic response of cowpea *Vigna unguiculata* (L.) to in vitro regeneration from cotyledon explants. *In Vitro Cellular & Developmental Biology, 35*, 8–12.

Bressani, R. (1985). Nutritive value of cowpea. In S. R. Singh & K. O. Rachie (Eds.), *Cowpea research production and utilisation* (pp. 353–359). Wiley.

Brock, R. D. (1965). Induced mutations affecting quantitative characters. In: The use of induced mutations in plant breeding. *Radiation Botany (Suppl.), 5*, 451–464.

Brock, R. D. (1967). Quantitative variation in *Arabidopsis thaliana* induced by ionizing radiations. *Radiation Botany, 7*, 193–203.

Burridge, J. D., Schneider, H. M., Huynh, B.-L., Roberts, P. A., Bucksch, A., & Lynch, J. P. (2017). Genome-wide association mapping and agronomic impact of cowpea root architecture. *Theoretical and Applied Genetics, 130*, 419–431.

Celik, O., & Atak, C. (2017). Applications of ionizing radiation in mutation breeding. In *New insights on gamma rays*. InTech.

Chari, M., Patel, G., Patel, P., & Raj, S. (1976). Evaluation of cowpea lines for resistance to aphid Aphis craccivora Koch. *Gujarat Agricultural University Research, 1*, 130–132.

Chaudhury, D., Madanpotra, S., Jaiwal, R., Saini, R., Ananda Kumar, P., & Jaiwal, P. K. (2007). Agrobacterium tumefaciens-mediated high frequency genetic transformation of an Indian cowpea (*Vigna unguiculata L. Walp.) cultivar and transmission of transgenes into progeny. Plant Sci. 172, 692–700.*

Cheng, W.-H., Taliercio, E. W., & Chourey, P. S. (1996). The miniature1 seed locus of maize encodes a cell wall invertase required for normal development of endosperm and maternal cells in the pedicel. *Plant Cell, 8*, 971–983.

Chheda, H. R., & Fatokun, C. A. (1982). Numerical analysis of variation patterns in okra (Abelmoschus esculentus [L.] Moench). *Botanical Gazette, 143*, 253–261. https://doi.org/10.1086/337298

Clement, J. D., Constable, G. A., Stiller, W. N., & Liu, S. M. (2015). Early generation selection strategies for breeding better combinations of cotton yield and fibre quality. *Field Crops Research, 172*, 145–152.

Deepalakshmi, A. J., & Anandakumar, C. R. (2003). Efficiency and effectiveness of physical and chemical mutagens in urdbean (*Vigna mungo* (L.) Hepper). *Madras Agricultural Journal, 90*(7–9), 485–489.

Dhanavel, D., Pavadai, P., Mullainathan, L., Mohana, D., Raju, G., Girija, M., & Thilagavathi, C. (2008). Effectiveness and efficiency of chemical mutagens in cowpea (*Vigna unguiculata* (L.) Walp). *African Journal of Biotechnology, 7*(22).

Diouf, D., & Hilu, K. W. (2005). Microsatellites and RAPD markers to study genetic relationship among cowpea breeding lines and local varieties in Senegal. *Genetic Resources and Crop Evolution, 52*, 1057–1067. https://doi.org/10.1007/s10722-004-6107-z

Egbadzor, K. F., Dadoza, M., Danquah, E. Y., Yeboah, M., Offei, S. K. & Ofori, K. (2013). Genetic control of seed size in cowpea (*Vigna unguiculata (L.) Walp). International Journal of Agricultural Science, 5(2), 367–371.*

Egbadzor, K. F., Ofori, K., Yeboah, M., Aboagye, L. M., & Opoku-Agyeman, M. O. (2014a). *Diversity in 113 cowpea [Vigna unguiculata* (L.) Walp] *accessions assessed with 458 SNP markers (p. 3). Springer.*

Egbadzor, K. F., Danquah, E. Y., Ofori, K. et al. (2014b). Diversity in 118 cowpea [*Vigna unguiculata (L.) Walp] accessions assessed with 16 morphological traits. International Journal of Plant Breeding and Genetics, 8, 13–24.* https://doi.org/10.1186/2193-1801-3-541.

Emebiri, L. E. (1991). Inheritance of protein content in seeds of selected crosses of cowpea *(Vigna unguiculata). Journal of the Science of Food and Agriculture, 54*, 1–7.

Fall, L., Diouf, D., Fall-Ndiaye, M. A., Badiane, F. A., & Gueye, M. (2003). Genetic diversity in cowpea [*Vigna unguiculata* (L.) Walp.] varieties determined by ARA and RAPD techniques. *African Journal of Biotechnology, 2*, 48–50. https://doi.org/10.5897/AJB2003.000-1009

Fang, J., Chao, C.-C. T., Roberts, P. A., & Ehlers, J. D. (2007). Genetic diversity of cowpea [*Vigna unguiculata* (L.) Walp.] in four West African and USA breeding programs as determined by AFLP analysis. *Genetic Resources and Crop Evolution, 54*, 1197–1209. https://doi.org/10.1007/s10722-006-9101-9

Farinu, G. O., & Ingrao, G. (1991). Gross composition, amino acid, phytic acid and trace element content of thirteen cowpea cultivars and their nutritional significance. *Journal of the Science of Food and Agriculture, 55*, 401–410.

Fatokun, C. A. (1991). Wide hybridization in cowpea: Problems and prospects. *Euphytica, 54*, 137–140. https://doi.org/10.1007/BF00039599

Fatokun, C. A. (2002). Breeding cowpea for resistance to insect pests: attempted crosses between cowpea and Vigna vexillata. In C. A. Fatokun, S. A. Tarawali, B. B. Singh, et al. (Eds), *Challenges and opportunities for enhancing sustainable cowpea production. Proceedings of the world cowpea conference III held* at IITA, Ibadan, Nigeria, 4–8 September 2000, pp. 52–61.

Fatokun, C. A., & Singh, B. B. (1987). Interspecific hybridization between *Vigna pubescens* and *V. unguiculata* through embryo rescue. *Plant Cell, Tissue and Organ Culture, 9*, 229–233.

Fatokun, C. A., Menancio-Hautea, D. I., Danesh, D., & Young, N. D. (1992). Evidence for orthologous seed weight genes in cowpea and mung bean based on RFLP mapping. *Genetics, 132*, 841–846.

Fatokun, C. A., Dariush, D., Menancio-Hautea, D. I., & Young, N. D. (1993). A linkage map for cowpea [*Vigna unguiculata* (L.) Walp.] based on DNA markers. In S. J. O'Brien (Ed.), *Genetic maps. Locus maps of complex genomes* (6th ed., pp. 256–258). Cold Spring Harbour Laboratory Press.

Fatokun, C. A., Young, N. D., and Myers, G. O. (1997). Molecular markers and genome mapping in cowpea in Advances in cowpea research, eds B. B. Singh, D. R. Mohan Raj, K. E. Dashiell, and L. E. N. Jackai (: Co-publication of International Institute of Tropical Agriculture (IITA); Japan International Research Center for Agricultural Sciences (JIRCAS)), 352–360.

Folsom, J. J., Begcy, K., Hao, X., Wang, D., & Walia, H. (2014). Rice fertilization- Independent Endosperm1 regulates seed size under heat stress by controlling early endosperm development. *Plant Physiology, 165*, 238–248.

Foolad, M. R. (2007). Genome mapping and molecular breeding of tomato. *International Journal of Plant Genomics, 2007*.

Fotso, M., Azanza, J. L., Pasquet, R. S., & Raymond, J. (1994). Molecular heterogeneity of cowpea (*Vigna unguiculata*, Fabaceae) seed storage proteins. *Plant Systematics and Evolution, 191*, 39–56. https://doi.org/10.1007/BF00985341

Ganapathi, A., & Anand, P. (1998). Somatic embryogenesis from young leaves of cowpea (*Vigna unguiculata* (L.) Walp. (Abstract) in Plant biotechnology and in vitro biology for the 21st century. IX International Congress on Plant Tissue and Cell Culture, 14–19 June 1998, Jerusalem, Israel.

Ganger, C. S., & Blakeslee, A. E. (1927). Chromosome and gene mutations in Datura following exposure to radium rays. *Proceedings of the National Academy of Sciences of the USA, 10*, 75–70.

Gaul, H. (1965). The concept of macro and micromutations in barley. *Radiation Biology (Suppl.), 5*, 407–428.

Ghalmi, N., Malice, M., Jacquemin, J. M., Ounane, S. M., Mekliche, L., & Baudoin, J. P. (2010). Morphological and molecular diversity within Algerian cowpea [*Vigna unguiculata* (L.) Walp.] landraces. *Genetic Resources and Crop Evolution, 57*, 371–386. https://doi.org/10.1007/s10722-009-9476-5

Giami, S. Y. (2005). Compositional and nutritional properties of selected newly developed lines of cowpea (*Vigna unguiculata* L. Walp). *Journal of Food Composition and Analysis, 18*(7), 665–673.

Gioi, T. D., Boora, K. S., & Chaudhary, K. (2012). Identification and characterization of SSR markers linked to yellow mosaic virus resistance genes in cowpea (*Vigna unguiculata*). *International Journal of Plant Research, 2*, 1–8. https://doi.org/10.5923/j.plant.20120201.01

Girija, M., & Dhanavel, D. (2009). Mutagenic effectiveness and efficiency of gamma rays, ethyl methane sulphonate and their combination treatment in Cowpea (*Vigna unguiculata* L. Walp). *Global Journal of Molecular Sciences, 4*(2), 68–75.

Gnanamurthy, S., & Dhanavel, D. (2014). Effect of EMS on induced morphological mutants and chromosomal variation in Cowpea (*Vigna unguiculata* (L.) Walp). *International Letters of Natural Sciences, 17*.

Gomathinayagam, P., Rathnaswamy, R., & Ramaswamy, N. M. (1998). Interspecific hybridization between *Vigna unguiculata* (L.) Walp. and *V. vexillata* (L.) A. Rich. through in vitro embryo culture. *Euphytica, 102*, 203–209.

Goodspeed, T. B. (1929). The effects of X-rays and radium on species of genus Nicotiana. *The Journal of Heredity, 20*, 243–259.

Goyal, S., Wani, M. R., Laskar, R. A., Raina, A., Amin, R., & Khan, S. (2019a). Induction of Morphological Mutations and Mutant Phenotyping in Black gram [*Vigna mungo* (L.) Hepper] using Gamma Rays and EMS. *Vegetos, 32*(4), 464–472.

Goyal, S., Wani, M. R., Laskar, R. A., Aamir, R., & Samiullah, K. (2019b). Assessment on cytotoxic and mutagenic potency of gamma rays and EMS in *Vigna mungo* L. Hepper. *Biotecnología Vegetal, 19*(3), 193–204.

Goyal, S., Wani, M. R., Laskar, R. A., Raina, A., & Khan, S. (2020a). Mutagenic effectiveness and efficiency of individual and combination treatments of gamma rays and ethyl methanesulfonate in black gram [*Vigna mungo* (L.) Hepper]. *Advances in Zoology and Botany, 8*(3), 163–168.

Goyal, S., Wani, M. R., Laskar, R. A., Raina, A., & Khan, S. (2020b). Performance evaluation of induced mutant lines of black gram (*Vigna mungo* (L.) Hepper). *Acta Fytotechn Zootechn, 23*(2), 70–77.

Goyal, S., Wani, M. R., Laskar, R. A., Raina, A., Amin, R., & Khan, S. (2021a). Quantitative assessments on induced high yielding mutant lines in urdbean [*Vigna mungo* (L.) hepper]. *Legume Science*. https://doi.org/10.1002/leg3.125

Goyal, S., Wani, M. R., Raina, A., Laskar, R. A., & Khan, S. (2021b). Phenotypic diversity in mutagenized population of urdbean (*Vigna mungo* (L.) Hepper). *Heliyon, 7*(5), e06356.

Greene, E. A., Codomo, C. A., Taylor, N. E., Henikoff, J. G., Till, B. J., Reynolds, S. H., Enns, L. C., Burtner, C., Johnson, J. E., Odden, A. R., Comai, L., & Henikoff, S. (2003). Spectrum of chemically induced mutations from a large scale reverse – Genetic screen in Arabidopsis. *Genetics, 164*, 731–740.

Gregoria, G. B. (2002). Progress in breeding for trace minerals in staple crops. *The Journal of Nutrition, 132*, 500S–502S.

Gustafsson, A. (1947). Mutations in agricultural plants. *Hereditas, 33*, 1–99.

Hall, A. E., Cisse, N., Thiaw, S., Elawad, H. O. A., Ehlers, J. D., et al. (2003a). Development of cowpea cultivars and germplasm by the Bean/Cowpea CRSP. *Field Crops Research, 82*, 103–134.

Hall, A. E., Cisse, N., Thiaw, S., Elawad, H. O. A., Ehlers, J. D., Ismail, A. M., Fery, R. L., Roberts, P. A., Kitch, L. W., Murdock, L. L., Boukar, O., Phillips, R. D., & McWatters, K. H. (2003b). Development of cowpea cultivars and germplasm by the bean/cowpea. *CRSP Field Crops Research, 82*, 103–134.

Hasan, N., Laskar, R. A., Raina, A., & Khan, S. (2018). Maleic hydrazide induced variability in fenugreek (Trigonella foenum-graecum L.) cultivars CO1 and Rmt-1. *Journal of Botanical Sciences, 7*(1), 19–28.

He, C., Poysa, V., & Yu, K. (2003). Development and characterization of simple sequence repeat (SSR) markers and their use in determining relationships among Lycopersicon esculentum cultivars. *Theoretical and Applied Genetics, 106*, 363–373. https://doi.org/10.1007/s00122-002-1076-0

Herniter, I. A., Muñoz-Amatriaín, M., Lo, S., Guo, Y.-N., & Close, T. J. (2018). Identification of candidate genes controlling black seed coat and pod tip color in Cowpea (*Vigna unguiculata* [L.] Walp). *Genes Genomes Genetics, 8*, 3347–3355.

Hugo de Vries. (1901). *Die mutation theorie* (1 Von, pp. 1–648). Viet and Co.

Huynh, B.-L., Close, T. J., Roberts, P. A., Hu, Z., Wanamaker, S., Lucas, M. R., et al. (2013). Gene pools and the genetic architecture of domesticated cowpea. *Plant Genome, 6*, 1–8. https://doi.org/10.3835/plantgenome2013.03.0005

Huynh, B.-L., Ehlers, J. D., Ndeye, N. N., Wanamaker, S., Lucas, M. R., Close, T. J., et al. (2015). Genetic mapping and legume synteny of aphid resistance in African cowpea (*Vigna unguiculata* L. Walp.) grown in California. *Molecular Breeding, 35*, 36. https://doi.org/10.1007/s11032-015-0254-0

Huynh, B.-L., Matthews, W. C., Ehlers, J. D., Lucas, M. R., Santos J. R. P., et al. (2016). A major QTL corresponding to the Rk locus for resistance to root-knot nematodes in cowpea (*Vigna unguiculata L. Walp.). Theoretical and Applied Genetics 129, 87–95.*

IITA. International Institute of Tropical Agriculture. (1972). *Grain legume program* (IITA Annual Report) (pp. 13–19). IITA.

Ilbas, A. I., Eroglu, Y., & Eroglu, H. E. (2005). Effect of the application of different concentrations of SA for different times on the morphological and cytogenetic characteristics of Barley (*Hordeum vulgare* L.) seedling. *Acta Botanica Sinica, 47*, 1101–1106.

Jain, S. M. (2002). *Feeding the world with induced mutations and biotechnology*. Proceedings of the International Nuclear Conference on Global Trends and Perspectives. Seminar 1: Agriculture and Bioscience, MINT, Bangi, Malaysia, pp. 1–14.

Javed, I., Ahsan, M., Ahmad, H. M., & Ali, Q. (2016). Role of mutation breeding to improve Mungbean (*Vigna radiata* L. Wilczek) yield: An overview. Nature. *Science, 14*(1), 63–77.

Jin, Y., Ni, D.-A., & Ruan, Y.-L. (2009). Posttranslational elevation of cell wall invertase activity by silencing its inhibitor in tomato delays leaf senescence and increases seed weight and fruit hexose level. *Plant Cell, 21*, 2072–2089.

John, S. A. (1999). Mutation frequency and chlorophyll mutations in parents and hybrids of cowpea following gamma irradiation. *Indian Journal of Genetics, 59*(3), 357–361.

Kalapchieva, S., & Tomlekova, N. B. (2016). Sensitivity of two garden pea genotypes to physical and chemical mutagens. *Journal of BioScience and Biotechnology, 5*(2), 167–171.

Kartha, K. K., Pahl, K., Leung, N. L., & Mroginski, L. A. (1981). Plant regeneration from meristems of grain legumes: soybean, cowpea, peanut, chickpea, and bean. *Canadian Journal of Botany, 59*, 1671–1679.

Khan, S., & Siddiqui, B. A. (1992). Effect of selection for improvement of quantitative characters in mutated population of mungbean (*Vigna radiata* (L.) Wilczek). *Journal of Indian Botanical Society, 71*, 69–71.

Khan, M. H., & Tyagi, S. D. (2009). Studied on induction of chlorophyll mutations in soybean (*Glycine max* (L.) Merrill). *Frontiers of Agriculture in China, 3*(3), 253–258.

Kharkwal, M. C. (1983). New selection technique for micro mutations. Abstract XV international congress of genetics. New Delhi, India, p 298.

Kharkwal, M. C. (1996). Accomplishments of mutation breeding in crop improvement in India. In M. S. Sachdev, P. Sachdev, & D. L. Deb (Eds.), *Isotopes and radiations in agriculture and environment research* (pp. 196–218). Indian Society for Nuclear Techniques in Agriculture and Biology, Nuclear Research Laboratory, I.A.R.I..

Khursheed, S., Laskar, R. A., Raina, A., Amin, R., & Khan, S. (2015). Comparative analysis of cytological abnormalities induced in *Vicia faba* L. genotypes using physical and chemical mutagenesis. Chromosome. *Science, 18*(3–4), 47–51.

Khursheed, S., Raina, A., & Khan, S. (2016). Improvement of yield and mineral content in two cultivars of *Vicia faba* L. through physical and chemical mutagenesis and their character association analysis. *Archives of Current Research International, 4*(1), 1–7.

Khursheed, S., Raina, A., Parveen, K., & Khan, S. (2017). Induced phenotypic diversity in the mutagenized populations of faba bean using physical and chemical mutagenesis. *Journal*

*of the Saudi Society of Agricultural Sciences, 18*, 113–119. https://doi.org/10.1016/j.jssas.2017.03.001

Khursheed, S., Raina, A., Laskar, R. A., & Khan, S. (2018a). Effect of gamma radiation and EMS on mutation rate: their effectiveness and efficiency in faba bean (*Vicia faba* L.). *Caryologia: International Journal of Cytology, Cytosystematics and Cytogenetics, 71*(4), 397–404. https://doi.org/10.1080/00087114.2018.1485430

Khursheed, S., Raina, A., & Khan, S. (2018b). Physiological response of two cultivars of faba bean using physical and chemical mutagenesis. *International Journal of Advance Research in Science and Engineering, 7*(4), 897–905.

Khursheed, S., Raina, A., Amin, R., Wani, M. R., & Khan, S. (2018c). Quantitative analysis of genetic parameters in the mutagenized population of faba bean (*Vicia faba* L.). *Research on Crops, 19*(2), 276–284.

Khursheed, S., Raina, A., Parveen, K., & Khan, S. (2019). Induced phenotypic diversity in the mutagenized populations of faba bean using physical and chemical mutagenesis. *Journal of the Saudi Society of Agricultural Sciences, 18(2), 113–119.*

Konzak, C. F. (1957). III. Genetic effects of radiation on higher plants. *The Quarterly Review of Biology, 32(1), 27–45.*

Langyintuo, A. S., Lowenberg-DeBoer, J., Faye, M., Lamber, D., Ibro, G., et al. (2003). Cowpea supply and demand in West Africa. *Field Crops Research, 82*, 215–231.

Laskar, R. A., & Khan, S. (2017). Assessment on induced genetic variability and divergence in the mutagenized lentil populations of microsperma and macrosperma cultivars developed using physical and chemical mutagenesis. *PLoS One, 12*(9), e0184598. https://doi.org/10.1371/journal.pone.0184598

Laskar, R. A., Khan, S., Khursheed, S., Raina, A., & Amin, R. (2015). Quantitative analysis of induced phenotypic diversity in chickpea using physical and chemical mutagenesis. *Journal of Agronomy, 14*(3), 102–111.

Laskar, R. A., Laskar, A. A., Raina, A., Khan, S., & Younus, H. (2018a). Induced mutation analysis with biochemical and molecular characterization of high yielding lentil mutant lines. *International Journal of Biological Macromolecules, 109*, 167–179.

Laskar, R. A., Wani, M. R., Raina, A., Amin, R., & Khan, S. (2018b). Morphological characterization of gamma rays induced multipodding mutant (mp) in lentil cultivar Pant L 406. *International Journal of Radiation Biology, 94*(11), 1049–1053.

Laskar, R. A., Khan, S., Deb, C. R., Tomlekova, N., Wani, M. R., Raina, A., & Amin, R. (2019). Lentil (Lens culinaris Medik.) diversity, cytogenetics and breeding. In J. M. Al-Khayri et al. (Eds.), *Advances in plant breeding: Legumes* (pp. 319–369). Springer. https://doi.org/10.1007/978-3-030-23400-3_9

Latunde-Dada, A. O. (1990). Genetic Manipulation of the Cowpea (*Vigna unguiculata [L.] Walp.) for Enhanced Resistance to Fungal Pathogens and Insect Pests. In N. C. Brady (Ed), Advances in Agronomy (pp. 133–154). Academic Press.*

Le, B. V., Cruz de Carvalho, M. H., Zuily-Fodil, Y., Thi, A. T. P., Van, K. T. T. (2002). Direct whole plant regeneration of cowpea (*Vigna unguiculata (L.) Walp) from cotyledonary node thin cell layer explants. Journal of Plant Journal of Plant Physiology, 159, 1255–1258.*

Li, C. D., Fatokun, C. A., Ubi, B., Singh, B. B., & Scoles, G. J. (2001). Determining genetic similarities and relationships among cowpea breeding lines and cultivars by microsatellite markers. *Crop Science, 41, 189–197.*

Lo, S., Muñoz-Amatriaín, M., Hokin, S. A., Cisse, N., Roberts, P. A., Farmer, A. D., Xu, S., & Close, T. J. (2019). A genome-wide association and meta-analysis reveal regions associated with seed size in cowpea [*Vigna unguiculata* (L.) Walp]. *Theoretical and Applied Genetics, 132*(11), 3079–3087.

Lodhi, G. P., Boora, K. S., Jain, S., & Balchand. (1990). Heterosis for fodder yield and quality characters in cowpea *(Vigna unguiculata* [L.] Walp.). *Crop Research, 3*, 66–73.

Lonardi, S., Muñoz-Amatriaín, M., Liang, Q., Shu, S., Wanamaker, S. I., Lo, S., Tanskanen, J., Schulman, A. H., Zhu, T., Luo, M. C., & Alhakami, H. (2019). The genome of cowpea (*Vigna unguiculata [L.] Walp.). The Plant Journal, 98(5), 767–782.*

Lucas, M. R., Ehlers, J. D., Roberts, P. A., & Close, T. J. (2012). Markers for quantitative resistance to foliar thrips in cowpea. *Crop Science, 52*, 2075–2081. https://doi.org/10.2135/cropsci2011.12.0684

Machuka, J., Adesoye, A., & Obembe, O. O. (2000). *Regeneration and genetic transformation in cowpea. Proceedings of World Cowpea Conference III.* (pp. 185–196). IITA.

Malik, K. A., & Saxena, P. K. (1992). Thidiazuron induces high frequency of shoot regeneration in intact seedlings of pea (Pisum sativum) chickpea (Cicer arietinum) and lentil (Lens culinaris Medik). *Australian Journal of Plant Physiology, 19*, 731–740.

Mammadov, J., Aggarwal, R., Buyyarapu, R., & Kumpatla, S. (2012). SNP markers and their impact on plant breeding. *International Journal of Plant Genomics*, 1–12.

Maréchal, R., Mascherpa, J. M., & Stainer, F. (1978). Etude taxonomique d'un group complexe d'especes des genres *Phaseolus* et *Vigna* (Papillionaceae) sur la base de donnees morphologiques et polliniques traitees par l'analyse informatique. *Boissiera, 28*, 1–273.

Menendez, C. M., Hall, A. E., & Gepts, P. (1997). A genetic linkage map of cowpea (*Vigna unguiculata*) developed from a cross between two inbred, domesticated lines. *Theoretical and Applied Genetics, 95*, 1210–1217.

Micke, A. (1999). Mutations in plant breeding. In B. A. Siddiqui & S. Khan (Eds.), *Breeding in crop plants mutations and in vitro mutation breeding* (pp. 1–19). Kalyani Publishers.

Mignouna, H. D., Ng, Q., Ikea, J., & Thottapilly, G. (1998). Genetic diversity in cowpea as revealed by random amplified polymorphic DNA. *Journal of Genetics and Breeding, 52*, 151–159.

Mishili, F. J., Fulton, J., Shehu, M., Kushwaha, S., Marfo, K., Jamal, M., Kergna, A., & Lowenberg-DeBoer, J. (2009). Consumer preferences for quality characteristics along the cowpea value chain in Nigeria, Ghana, and Mali. *Agribusiness, 25*, 16–35.

Mitchell, D. C., Lawrence, F. R., Hartman, T. J., & Curran, J. M. (2009). Consumption of dry beans, peas, and lentils could improve diet quality in the US population. *Journal of the American Dietetic Association, 109*, 909–913.

Mohanasundaram, M., Thamburaj, S., & Natarajan, S. (2001). Observation on gamma ray induced viable mutations in vegetable cowpea. *Mutation Breeding Newsletter, 45*, 37–38.

Monti, L. M., Murdock, L. L., & Thottappilly, G. (1997). Opportunities for biotechnology in cowpea. In B. B. Singh, D. R. Mohan Raj, K. E. Dashiell, & J. Len (Eds.), *Advances in cowpea research. Copublication of IITA and Japan International Research Center for Agricultural Sciences (JIRCAS)* (pp. 341–351). IITA.

Moose, S. P., & Mumm, R. H. (2008). Molecular plant breeding as the foundation for 21st century crop improvement. *Plant Physiology, 147*, 969–977.

Muchero, W., Diop, N. N., Bhat, P. R., Fenton, R. D., Wanamaker, S., Pottorff, M., et al. (2009a). A consensus genetic map of cowpea [*Vigna unguiculata* (L.) Walp.] and synteny based on EST-derived SNPs. *Proceedings. National Academy of Sciences. United States of America, 106*, 18159–18164. https://doi.org/10.1073/pnas.0905886106

Muchero, W., Ehlers, J. D., Close, T. J., & Roberts, P. A. (2009b). Mapping QTL for drought stress-induced premature senescence and maturity in cowpea [*Vigna unguiculata* (L.) Walp.]. *Theoretical and Applied Genetics, 118*, 849–863. https://doi.org/10.1007/s00122-008-0944-7

Muchero, W., Ehlers, J. D., & Roberts, P. A. (2010). QTL analysis for resistance to foliar damage caused by Thrips tabaci and Frankliniella schultzei (Thysanoptera: Thripidae) feeding in cowpea [*Vigna unguiculata* (L.) Walp.]. *Molecular Breeding, 25*, 47–56. https://doi.org/10.1007/s11032-009-9307-6

Muchero, W., Ehlers, J. D., Close, T. J., & Roberts, P. A. (2011). Genic SNP markers and legume synteny reveal candidate genes underlying QTL for Macrophomina phaseolina resistance and maturity in cowpea [*Vigna unguiculata* (L) Walp.]. *BMC Genomics, 12*(1), 1–14.

Muchero, W., Roberts, P. A., Diop, N. N., Drabo, I., Cisse, N., Close, T. J., et al. (2013). Genetic architecture of delayed senescence, biomass, and grain yield under drought stress in cowpea. *PLoS One, 8*, e70041. https://doi.org/10.1371/journal.pone.0070041

Muller, H. J. (1927). Artificial transmutation of genes. *Science, 66*, 84–144.

Muñoz-Amatriaín, M., Lo, S., Herniter, I. A., Boukar, O., Fatokun, C., Carvalho, M., Castro, I., Guo, Y. N., Huynh, B. L., Roberts, P. A., & Carnide, V. (2021). The UCR Minicore: a resource for cowpea research and breeding. *Legume Science, 3*(3), e95.

Muthukumar, B., Mariamma, M., & Gnanam, A. (1995). Regeneration of plants from primary leaves of cowpea. *Plant Cell, Tissue and Organ Culture, 42*, 153–155.

Nagl, W., Ignacimuthu, S., & Becker, J. (1997). Genetic engineering and regeneration of *Phaseolus* and *Vigna*. State of the art and new attempts. *Journal of Plant Physiology, 150*, 625–644.

Nair, R. M., Craig, A., Auricht, G., Edwards, O. R., Robinson, S., Otterspoor, M., & Jones, J. A. (2003). Evaluating pasture legumes for resistance to aphids. *Animal Production Science, 43*, 1345–1349.

Nair, R., Mehta, A. K., Singh, K. P., & Sharma, S. K. (2014). Mutagenic effectiveness and efficiency in Cowpea. *Advances Applied Research, 6*, 78–85.

Nakagawa, H., Annai, T., Okabe, A., Takahshi, K., Hajika, M., & Takagi, Y. (2011). Mutation breeding of soybean in Japan. In S. Khan & M. I. Kozgar (Eds.), *Breeding of pulse crops* (pp. 55–84). Kalyani Publishers.

Nkongolo, K. K. (2003). Genetic characterization of Malawian cowpea [Vigna unguiculata (L.) Walp.] landraces: diversity and gene flow among accessions. *Euphytica, 129*, 219–228. https://doi.org/10.1023/A:1021935431873

Nielsen, S. S., Ohler, T. A. and Mitchell, C. A. (1997). Cowpea leaves for human consumption: production, utilization, and nutrient composition. *Advances in Cowpea Research, p.326.*

Ochatt, S., Atif, R. M., Patat-Ochatt, E., Jacas, L., & Conreux, C. (2010). Competence versus recalcitrance for in vitro regeneration. *Notulae Botanicae Horti Agrobotanica Cluj-Napoca, 38(2), 102–108.*

Odutayo, O. I., Akinrimisi, F. B., Ogunbosoye, I., & Oso, R. T. (2005). Multiple shoot induction from embryo derived callus cultures of cowpea (*Vigna unguiculata I.) Walp. African Journal of Biotechnology, 4, 1214–1216.*

Ogunkanmi, L. A., Ogundipe, O. T., Ng, N. Q., & Fatokun, C. A. (2008). Genetic diversity in wild relatives of cowpea (*Vigna unguiculata*) as revealed by simple sequence repeats (SSR) markers. *Journal of Food, Agriculture and Environment, 6*, 253–268.

Ohad, N., Margossian, L., Hsu, Y.-C., Williams, C., Repetti, P., & Fischer, R. L. (1996). A mutation that allows endosperm development without fertilization. *Proceedings of the National Academy of Sciences, 93*, 5319–5324.

Ojomo, O., & Cheda, A. (1975). Induced mutations in cowpea (*Vigna unguiculata* (L.)Walp.) mutation spectrum and rates. *Ghana Journal of Science, 15*, 155–158.

Oluwatosin, O. B. (1997). Genetic and environmental variation for seed yield. Protein and amino acid composition in cowpea (*Vigna unguiculata* L. Walp). *Journal of the Science of Food and Agriculture, 74*, I 07–I116.

Ombakho, G. A., Tyagi, A. P., & Pathak, R. S. (1987). Inheritance of resistance to the cowpea aphid in cowpea. *Theoretical and Applied Genetics, 74*(6), 817–819. https://doi.org/10.1007/BF00247562

Omo-Ikerodah, E. E., Fawole, I., & Fatokun, C. (2008). Genetic mapping of quantitative trait loci (QTLs) with effects on resistance to flower bud thrips (Megalurothrips sjostedti) in recombinant inbred lines of cowpea [*Vigna unguiculata* (L.)Walp.]. *African Journal of Biotechnology, 7*, 263–270.

Ouédraogo, J. T., Gowda, B. S., Jean, M., Close, T. J., Ehlers, J. D., Hall, A. E., et al. (2002a). An improved genetic linkage map for cowpea (*Vigna unguiculata* L.) combining AFLP, RFLP, RAPD, biochemical markers, and biological resistance traits. *Genome, 45*, 175–188. https://doi.org/10.1139/g01-102

Ouédraogo, J. T., Tignegre, J.-B., Timko, M. P., & Belzile, F. J. (2002b). AFLP markers linked to resistance against Striga gesnerioides race 1 in cowpea (*Vigna unguiculata*). *Genome, 45*, 787–793. https://doi.org/10.1139/g02-043

Ouédraogo, J. T., Maheshwari, V., Berner, D. K., St-Pierre, C. A., Belzile, F., & Timko, M. P. (2001). Identification of AFLP markers linked to resistance of cowpea (*Vigna unguiculata L.) to parasitism by Striga gesnerioides. Theoretical & Applied Genetics, 102.*

Padi, F. K., & Ehlers, J. D. (2008). Effectiveness of early generation selection in cowpea for grain yield and agronomic characteristics in semiarid West Africa. *Crop Science, 48*(2), 533–540.

Padulosi, S., & Ng, N. Q. (1997). Origin taxonomy, and morphology of *Vigna unguiculata.*

Pellegrineschi, A. (1997). In vitro plant regeneration via organogenesis of cowpea [*Vigna unguiculata (L.) Walp.]. Plant Cell Reports 17, 89–95.*

Panella, L., & Gepts, P. (1992). Genetic relationships within *Vigna unguiculata* (L.)Walp. based on isozyme analyses. *Genetic Resources and Crop Evolution, 39*, 71–88.

Pasquet, R. S. (1999). Genetic relationship among subspecies of *Vigna unguiculata* (L.)Walp. based on allozyme variation. *Theoretical and Applied Genetics, 98*, 1104–1119. https://doi.org/10.1007/s001220051174

Pasquet, R. S. (2000). Allozyme diversity of cultivated cowpea *Vigna unguiculata* (L.)Walp. *Theoretical and Applied Genetics, 101*, 211–219. https://doi.org/10.1007/s001220051471

Pathak, R. (1988). Genetics of resistance to aphid in cowpea. *Crop Science, 28*, 474–476.

Patil, R. B., & Shete, M. M. (1987). Heterosis in crosses of seven genotypes of cowpea. *Journal of the Maharashtra Agricultural University, 12*, 51–54.

Perry, J. A., Wang, T. L., Welham, T. J., Gardner, S., Pike, J. M., Yoshida, S., & Parniske, M. (2003). A TILLING reverse genetics tool and a web- accessible collection of mutants of the legume (*Lotus japonicas*.). *Plant Physiology, 131*, 866–871.

Popelka, J. C., Gollasch, S., Moore, A., et al. (2006). Genetic transformation of cowpea (*Vigna unguiculata* L.) and stable transmission of transgenes to progeny. *Plant Cell Reports, 25*, 304–312.

Pottorff, M., Ehlers, J. D., Fatokun, C., Roberts, P. A., & Close, T. J. (2012a). Leaf morphology in cowpea [*Vigna unguiculata* (L.) Walp.]: QTL analysis, physical mapping and identifying candidate gene using synteny with model legume species. *BMC Genomics, 13*, 234. https://doi.org/10.1186/1471-2164-13-234

Pottorff, M., Wanamaker, S., Ma, Y. Q., Ehlers, J. D., Roberts, P. A., & Close, T. J. (2012b). Genetic and physical mapping of candidate genes for resistance to Fusarium oxysporum f. sp. tracheiphilum race 3 in cowpea [*Vigna unguiculata* (L.)Walp]. *PLoS One, 7*, e41600. https://doi.org/10.1371/journal.pone.0041600

Pottorff, M., Roberts, P. A., Close, T. J., Lonardi, S., Wanamaker, S., & Ehlers, J. D. (2014a). Identification of candidate genes and molecular markers for heat-induced brown discoloration of seed coats in cowpea [*Vigna unguiculata* (L.) Walp]. *BMC Genomics, 15*, 328. https://doi.org/10.1186/1471-2164-15-328

Pottorff, M., Roberts, P. A., Close, T. J., Lonardi, S., Wanamaker, S., & Ehlers, J. D. (2014b). Identification of candidate genes and molecular markers for heat-induced brown discoloration of seed coats in cowpea [*Vigna unguiculata* (L.) Walp]. *BMC Genomics, 15*(1), 1–11.

Prem Anand, R., Ganapathi, A., Ramesh, A., Vengadesan, G., & Selvaraj, N. (2000). High frequency plant regeneration via somatic embryogenesis in cell suspension cultures of cowpea (*Vigna unguiculata* L. Walp). *In Vitro Cellular & Developmental Biology – Plant, 36*, 475–480.

Qin, J., Shi, A., Mou, B., Bhattarai, G., Yang, W., Weng, Y., & Motes, D. (2017). Association mapping of aphid resistance in USDA cowpea (*Vigna unguiculata* L. Walp.) core collection using SNPs. *Euphytica, 213*(2), 1–10.

Quass, C. F. (1995). *Guidelines for the production of cowpeas*. National Department of Agriculture.

Raina, A., & Khan, S. (2020). Increasing rice grain yield under biotic stresses: Mutagenesis, transgenics and genomics approaches. In C. Aryadeep (Ed.), *Rice research for quality improvement: Genomics and genetic engineering* (pp. 149–178). Springer. https://doi.org/10.1007/978-981-15-5337-0_8

Raina, A., Laskar, R. A., Khursheed, S., Amin, R., Tantray, Y. R., Parveen, K., & Khan, S. (2016). Role of mutation breeding in crop improvement-past, present and future. *Asian Research Journal of Agriculture, 2*(2), 1–13.

Raina, A., Laskar, R. A., Khursheed, S., Khan, S., Parveen, K., Amin, R., & Khan, S. (2017). Induce physical and chemical mutagenesis for improvement of yield attributing traits and their correlation analysis in chickpea. *International Letters of Natural Sciences, 61*, 14–22.

Raina, A., Laskar, R. A., Jahan, R., Khursheed, S., Amin, R., Wani, M. R., Nisa, T. N., & Khan, S. (2018a). Mutation breeding for crop improvement. In M. W. Ansari, S. Kumar, C. K. Babeeta, & R. K. Wattal (Eds.), *Introduction to challenges and strategies to improve crop productivity in changing environment* (pp. 303–317). Enriched Publications.

Raina, A., Khursheed, S., & Khan, S. (2018b). Optimisation of mutagen doses for gamma rays and sodium azide in cowpea genotypes. *Trends in Biosciences, 11*(13), 2386–2389.

Raina, A., Khan, S., Laskar, R. A., Wani, M. R., & Mushtaq, W. (2019). Chickpea (Cicer arietinum L.) cytogenetics, genetic diversity and breeding. In J. M. Al-Khayri et al. (Eds.), *Advances in plant breeding: Legumes* (pp. 53–112). Springer. https://doi.org/10.1007/978-3-030-23400-3_3

Raina, A., Laskar, R. A., Tantray, Y. R., Khursheed, S., & Khan, S. (2020a). Characterization of induced high yielding cowpea mutant lines using physiological, biochemical and molecular markers. *Scientific Reports, 10*, 3687.

Raina, A., Parmeshwar, K., & Khan, S. (2020b). Increasing rice grain yield under abiotic stresses: Mutagenesis, transgenics and genomics approaches. In C. Aryadeep (Ed.), *Rice research for quality improvement: Genomics and genetic engineering* (pp. 753–777). Springer. https://doi.org/10.1007/978-981-15-4120-9_31

Raina, A., Sahu, P. K., Laskar, R. A., Rajora, N., Sao, R., Khan, S., & Ganai, R. A. (2021). Mechanisms of genome maintenance in plants: Playing it safe with breaks and bumps. *Frontiers in Genetics, 12*, 675686. https://doi.org/10.3389/fgene.2021.675686

Raina, A., Laskar, R. A., Wani, M. R., Jan, B. L., Ali, S., & Khan, S. (2022a). Gamma rays and sodium azide induced genetic variability in high yielding and biofortified mutant lines in cowpea [*Vigna unguiculata* (L.) Walp.]. *Frontiers in Plant Science, 13*, 911049. https://doi.org/10.3389/fpls.2022.911049

Raina, A., Laskar, R. A., Wani, M. R., Jan, B. L., Ali, S., & Khan, S. (2022b). Comparative mutagenic effectiveness and efficiency of gamma rays and sodium azide in inducing chlorophyll and morphological mutants of cowpea. *Plants, 11*, 1322. https://doi.org/10.3390/plants11101322

Raina, A., Laskar, R. A., Wani, M. R., & Khan, S. (2022c). Chemical mutagenesis: Role in Breeding and Biofortification of lentil (*Lens culinaris* Medik) mutant lines. *Molecular Biology Reports, 49*(12), 11313–11325. https://doi.org/10.1007/s11033-022-07678-6

Raina, A., Laskar, R. A., Wani, M. R., & Khan, S. (2022d). Plant breeding strategies for abiotic stress tolerance in cereals. In *Omics approach to manage abiotic stress in cereals* (pp. 151–177). Springer. https://doi.org/10.1007/978-981-19-0140-9_8.

Ramakrishnan, K., Gnanam, R., Sivakumar, P., & Manickam, A. (2005). In vitro somatic embryogenesis from cell suspension cultures of cowpea [*Vigna unguiculata* (L.) Walp]. *Plant Cell Reports, 24*, 449–461.

Rasik, S., Raina, A., Laskar, R. A., Wani, M. R., Reshi, Z., & Khan, S. (2022). Lower doses of Sodium azide and Methyl methanesulphonate improved yield and pigment contents in vegetable cowpea [*Vigna unguiculata* (L.) Walp.]. *South African Journal of Botany, 148*, 727–736. https://doi.org/10.1016/j.sajb.2022.04.034

Ravelombola, W., Qin, J., Shi, A., Weng, Y., Bhattarai, G., Dong, L., & Morris, J. B. (2017). A SNP-based association analysis for plant growth habit in worldwide cowpea (*Vigna unguiculata* (L.) Walp) Germplasm. *Euphytica, 213*(12), 1–13.

Ravelombola, W., Shi, A., Weng, Y., Mou, B., Motes, D., Clark, J., Chen, P., Srivastava, V., Qin, J., Dong, L., & Yang, W. (2018). Association analysis of salt tolerance in cowpea (*Vigna unguiculata* (L.) Walp) at germination and seedling stages. *Theoretical and Applied Genetics, 131*(1), 79–91.

Sawadogo, M., Ouédraogo, J. T., Gowda, B. S., & Timko, M. P. (2010). Genetic diversity of cowpea (*Vigna unguiculata* L. Walp.) cultivars in Burkina Faso resistant to Striga gesnerioides. *African Journal of Biotechnology, 9*, 8146–8153. https://doi.org/10.5897/AJB10.1175

Schnapp, S. R., Shade, R. E., Kitch, L. W., Murdock, L. L., Bressan, R. A., & Hasegawa, P. M. (1990). Utilization of in vitro culture methods to facilitate introgression of insect resistance into cowpea *(Vigna unguiculata).* In *Abstracts, 7th International Congress on Plant Tissue and Cell* (p. 200).

Sellapillai, L., Dhanarajan, A., Raina, A., & Ganesan, A. (2022). Gamma ray induced positive alterations in morphogenetic and yield attributing traits of finger millet (*Eleusine coracana* (L.) Gaertn.) in M2 generation. *Plant Science Today, 9*(4), 939–949.

Sellapillaibanumathi, L., Dhanarajan, A., Raina, A., & Ganesan, A. (2022). Effects of gamma radiations on morphological and physiological traits of finger millet (*Eleusine coracana* (L.) Gaertn.). *Plant Science Today, 9*(1), 89–95.

Sherif, T. H. I., Omara, M. K., & Damarany, A. M. (1991). Genetic components for seed yield in cowpea under drought-stressed and non-stressed environments. *Assiut Journal of Agricultural Sciences, 25*, 259–281.

Shi, A., Buckley, B., Mou, B., Motes, D., Morris, J. B., Ma, J, Xiong, H., Qin, J., Yang, W., Chitwood, J., Weng, Y. (2016). Association analysis of cowpea bacterial blight resistance in USDA cowpea germplasm. *Euphytica 208(1), 143–155*

Shu, Q. Y., Forster, B. P., & Nakagawa, H. (2012). *Plant mutation breeding and biotechnology*. CAB International and FAO.

Simon, M. V., Benko-Iseppon, A. M., Resende, L. V., Winter, P., & Kahl, G. (2007). Genetic diversity and phylogenetic relationships in Vigna savi germplasm revealed by DNA amplification fingerprinting. *Genome, 50*, 538–547. https://doi.org/10.1139/G07-029

Singh, B. B. (2002). Recent genetic studies in cowpea. In C. A. Fatokun, S. A. Tarawali, B. B. Singh, P. M. Kormawa, & M. Tamo (Eds.), *Challenges and opportunities for enhancing sustainable cowpea production* (pp. 3–13). Intl Inst Tropical Agric.

Singh, B. B. (2005). Cowpea [*Vigna unguiculata* (L.) Walp]. In R. J. Singh & P. P. Jauhar (Eds.), *Genetic resources, chromosome engineering and crop improvement* (Vol. 1, pp. 117–162). CRC Press.

Singh, S. R., Jackai, L. E. N., Dos Santos, J. H. R., & Adalla, C. B. (1990). Insect pests of cowpea. In S. R. Singh (Ed.), *Insect pests of food legumes* (pp. 43–89). Wiley.

Singh, B. B., & Tarawali, S. A. (1997). Cowpea and its improvement: key to sustainable mixed crop/livestock farming systems in West Africa. Crop Residues in Sustainable Mixed Crop/Livestock Farming Systems. CAB in Association with ICRISAT and ILRI, Wallingford, UK, pp. 79–100.

Singh, B. B., Ehlers, J. D., Sharma, B., & Freire Filho, F. R. (2002). Recent progress in cowpea breeding. In C. A. Fatokun, S. A. Tarawali, B. B. Singh, P. M. Kormawa, & M. Tamo (Eds.), *Challenges and opportunities for enhancing sustainable cowpea production* (pp. 22–40). Intl Inst Tropical Agric.

Singh, D. P., Sharma, S. P., Lal, M., Ranwah, B. R., & Sharma, V. (2013). Induction of genetic variability for polygenic traits through physical and chemical mutagens in cowpea [*Vigna unguiculata* (L.) Walp]. *Legume Research: An International Journal, 36*(1).

Sneep, J. (1977). Selection for yield and early generation of self-fertilizing crops. *Euphytica, 26*, 27–30.

Spencer, M. M., Ndiaye, M. A., Gueye, M., Diouf, D., Ndiaye, M., & Gresshoff, P. M. (2000). DNA-based relatedness of cowpea [*Vigna unguiculata* (L.) Walp.] genotypes using DNA amplification fingerprinting. *Physiology and Molecular Biology of Plants, 6*, 81–88.

Stadler, L. J. (1928). Mutations in barley induced by X-rays and radium. *Science, 68*, 186–187.

Stalker, H. T. (1980). Utilization of wild species for crop improvement. *Advances in Agronomy, 33, 111–147.*

Tantray, A. Y., Raina, A., Khursheed, S., Amin, R. U., & Khan, S. A. (2017). Chemical mutagen affects pollination and locule formation in capsules of black cumin (Nigella sativa L.). *International Journal of Agricultural Sciences, 8*(1), 108–117.

Tarawali, S. A., Singh, B. B., Peters, M., & Blade, S. F. (1997). Cowpea haulms as fodder. In B. B. Singh, D. R. Mohan Raj, K. E. Dashiell, & L. E. N. Jackai (Eds.), *Advances in cowpea research* (pp. 313–325). Copublication Intl Inst Tropical Agric (IITA) and Japan Intl Res Center Agric Sci (JIRCAS).

Tarawali, S. A., Singh, B. B., Gupta, S. C., Tabo, R., Harris, F., et al. (2002). Cowpea as a key factor for a new approach to integrated crop–livestock systems research in the dry savannas of West Africa. In C. A. Fatokun, S. A. Tarawali, B. B. Singh, P. M. Kormawa, & M. Tamo (Eds.),

*Challenges and opportunities for enhancing sustainable cowpea production* (pp. 233–251). Intl Inst Tropical Agric.

Teofilo, E. M., da Silva, F. P., Alves, J. F., Paiva, J. B., & dos Santos, J. H. R. (1984). Analise genetica de urn cruzamento dialelico em caupi. *Pesquisa Agropecuaria Brasileira, 19*(7), 849–857.

Teyiou, B. J. B., Oumarou, S., Jean-Baptiste, T., Bao-Lam, H., Francis, K., Timothy, J. C., Philip, R., Eric, D., Kwadwo, O., & Tinga, J. O. (2018). Single nucleotide polymorphism (SNP)-based genetic diversity in a set of Burkina Faso cowpea germplasm. *African Journal of Agricultural Research, 13*(19), 978–987.

Timko, M. P., & Singh, B. B. (2008). Cowpea, a multifunctional legume. In P. H. Moore & R. Ming (Eds.), *Genomics of tropical crop plants* (pp. 227–258). Springer, LLC.

Timko, M. P., Ehlers, J. D., & Roberts, P. A. (2007a). Cowpea. In C. Kole (Ed.), *Pulses, sugar and tuber crops, genome mapping and molecular breeding in plants* (Vol. 3, pp. 49–67). Springer-Verlag.

Timko, M. P., Ehlers, J. D., & Roberts, P. A. (2007b). Cowpea. In C. Kole (Ed.), *Genome mapping and molecular breeding in plants, volume 3, pulses, sugar and tuber crops* (pp. 49–67). Springer Verlag.

Tollenaar, D. (1934). Untersuchungen ueber Mutation bei Tabak. I Entstehungsweise und Wesen kuenstlich erzeugter Gen-Mutanten. *Genetica, 16*(1–2), 111–152.

Ubi, B. E., Mignouna, H., & Thottappilly, G. (2000). Construction of a genetic linkage map and QTL analysis using a recombinant inbred population derived from an inter-subspecific cross of a cowpea [Vina unguiculata (L.) Walp.]. *Breeding Science, 50*, 161–173. https://doi.org/10.1270/jsbbs.50.161

Uma, M. S., Hittalamani, S., Murthy, B. C. K., & Viswanatha, K. P. (2009). Microsatellite DNA marker aided diversity analysis in cowpea [*Vigna unguiculata* (L.)Walp.]. *Indian Society of Genetics & Plant Breeding, 69*, 33–35.

Van Le, B. U. I., de Carvalho, M. H. C., Zuily-Fodil, Y., Thi, A. T. P. & Van, K. T. T. (2002). Direct whole plant regeneration of cowpea [*Vigna unguiculata (L.) Walp] from cotyledonary node thin cell layer explants. Journal of plant physiology, 159(11), 1255–1258.*

Vaillancourt, R. E., & Weeden, N. F. (1992). Chloroplast DNA polymorphism suggests Nigerian center of domestication for the cowpea, *Vigna unguiculata* (Leguminosae). *American Journal of Botany, 79*, 1194–1199. https://doi.org/10.2307/2445219

Verdcourt, B. (1970). Studies of the *Leguminosae-Papilionoideae* for 'Flora of Tropical East Africa': IV. *Kew Bulletin*, 507–569.

Wamalwa, E. N., Muoma, J. & Wekesa, C. (2016) Genetic Diversity of Cowpea (*Vigna unguiculata (L.) Walp.) Accession in Kenya Gene Bank Based on Simple Sequence Repeat Markers. International Journal of Genomics, 2016, 8956412.*

Wang, L. Z., Wang, L., Zhao, R. J., Pei, Y. L., Fu, Y. Q., Yan, Q. S., & Li, Q. (2003a). Combining radiation mutation techniques with biotechnology for soybean breeding. In *Improvement of new and traditional industrial crops by induced mutations and related biotechnology* (pp. 107–115). IAEA.

Wang, T. L., Domoney, C., Hedley, C. L., Casey, R., & Grusak, M. A. (2003b). Can we improve the nutritional quality of legume seeds? *Plant Physiology, 131*(3), 886–891.

Wani, M. R., Khan, S., Kozgar, M. I., & Goyal, S. (2011). Induction of morphological mutants in mungbean (*Vigna radiata* (L.) Wilczek) through chemical mutagens. *Nucleus, 48*(3), 243–247.

Wani, M. R., Kozgar, M. I., Khan, S., Ahanger, M. A., & Ahmad, P. (2014a). Induced mutagenesis for the improvement of pulse crops with special reference to mung bean: A review update. In *Improvement of crops in the era of climatic changes* (pp. 247–288). Springer.

Wani, M. R., Kozgar, M. I., Tomlekova, N. B., & Khan, S. (2014b). Selection for polygenic variability in early mutant generations of mungbean (*Vigna radiata* (L.) Wilczek). In N. B. Tomlekova, M. I. Kozgar, & M. R. Wani (Eds.), *Mutagenesis: exploring genetic diversity of crops* (pp. 213–232). Wageningen Academic Publishers.

Wani, M. R., Dar, A. R., Tak, A., Amin, I., Shah, N. H., Rehman, R., Baba, M. Y., Raina, A., Laskar, R., Kozgar, M. I., & Khan, S. (2017). Chemo-induced pod and seed mutants in mungbean (*Vigna radiata* L. Wilczek). SAARC. *Journal of Agriculture, 15*(2), 57–67.

Wani, M. R., Laskar, R. A., Raina, A., Khan, S., & Khan, T. U. (2021). Application of chemical mutagenesis for improvement of productivity traits in Lentil (Lens culinaris medik). *Annals of Biology, 37*(1), 69–75.

Weber, H., Borisjuk, L., & Wobus, U. (1996). Controlling seed development and seed size in *Vicia faba*: a role for seed coat-associated invertases and carbohydrate state. *The Plant Journal, 10*, 823–834.

Win, K. T., & Oo, A. Z. (2015). Genotypic difference in salinity tolerance during early vegetative growth of cowpea (*Vigna unguiculata* L. Walp.) from Myanmar. *Biocatalysis and Agricultural Biotechnology, 4*, 449–455.

Xavier, G. R., Martins, L. M. V., Rumjanek, N. G., & Filho, F. R. F. (2005). Variabilidade genética em acessos de caupi analisada por meio de marcadores RAPD. *Pesquisa Agropecuária Brasileira, 40*, 353–359. https://doi.org/10.1590/S0100-204X2005000400006

Xu, Y., & Crouch, J. H. (2008). Marker-assisted selection in plant breeding: from publications to practice. *Crop Science, 48*, 391–407.

Xu, P., Wu, X., Wang, B., Liu, Y., Quin, D., Ehlers, J. D., et al. (2010). Development and polymorphism of *Vigna unguiculata* ssp. unguiculata microsatellite markers used for phylogenetic analysis in asparagus bean [*Vigna unguiculata* ssp. sesquipedialis (L.) Verdc]. *Molecular Breeding, 25*, 675–684. https://doi.org/10.1007/s11032-009-9364-x

Xu, P., Wu, X., Muñoz-Amatriaín, M., Wang, B., Wu, X., Hu, Y., Huynh, B. L., Close, T. J., Roberts, P. A., & Zhou, W. (2017). Genomic regions, cellular components and gene regulatory basis underlying pod length variations in cowpea (*Vigna unguiculata* L. Walp). *Plant Biotechnology Journal, 15*, 547–557.

Zannou, A., Kossou, D. K., Ahanchédé, A., Zoundjihékpon, J., Agbicodo, E., Struik, P. C., et al. (2008). Genetic variability of cultivated cowpea in Benin assessed by random amplified polymorphic DNA. *African Journal of Biotechnology, 7*, 4407–4414. https://doi.org/10.5897/AJB08.856

Zaidi, M. A., Mohammadi, M., Postel, S., Masson, L., & Altosaar, I. (2005). The Bt gene cry2Aa2 driven by a tissue specific ST-LS1 promoter from potato effectively controls Heliothis virescens. *Transgenic Research, 14, 289–298.*

Zhou, Y., Zhang, X., Kang, X., Zhao, X., Zhang, X., & Ni, M. (2009). SHORT HYPOCOTYL UNDER BLUE1 associates with MINISEED3 and HAIKU2 promoters in vivo to regulate Arabidopsis seed development. *Plant Cell, 21*, 106–117.

# Germplasm Diversity and Breeding Approaches for Genetic Improvement of Mungbean


**Rafiul Amin Laskar, Bhaskar Dowarah, and Nilofer Sheikh**

**Abstract** In Asia, the mungbean [*Vigna radiata* (L.) R. Wilczek var. radiata] has been known to be an excellent source of nutritious food and income for the people. Mungbean growth in other locations, including Africa proper and South America, has been aided by the development of short-duration variants. Mungbean cultivation and production are limited by both biotic and abiotic causes. The main insect pests include aphids, bruchids, *Helicoverpa*, leafhopper, mirid, pod borers, stem fly, thrips, and whitefly. Halo blight, anthracnose, tan spot, yellow mosaic, and powdery mildew bacterial leaf spot and tan spot are the most common mungbean diseases. Drought, waterlogging, salt, and heat stress are among abiotic factors that impact mungbean productivity. Mungbean improvement through breeding techniques has indeed been crucial in generating resistant varieties against biotic and abiotic stressors. There are still numerous challenges to overcome, including the detection of consistent and reliable sources of resistance for specific features and qualities imparted by several genes. Understanding interactions of plants with the insect, pathogen, environment, and the essential factors conferring resistance to biotic and abiotic stressors might be greatly aided by the recent advancements in genetic improvement technologies. In this chapter, the present biotic and abiotic restrictions in cultivation and production of mungbean, as well as barriers to its genetic modification, and potential breeding approaches are examined.

**Keywords** Mungbean · Breeding · Environmental stresses · Insect-pests · Molecular approaches · Biotechnological tools

R. A. Laskar (✉)
Department of Botany, Pandit Deendayal Upadhyaya Adarsha Mahavidyalaya (PDUAM), Eraligool, Karimganj, Assam, India

B. Dowarah
Department of Botany, Bahona College, Jorhat, Assam, India

N. Sheikh
Department of Botany, Biswanath College, Biswanath, Assam, India



A. Raina et al. (eds.), *Advanced Crop Improvement, Volume 2*,
https://doi.org/10.1007/978-3-031-26669-0_7

# 1 Introduction

## *1.1 Taxonomic Classification and Geographic Distribution*

Mungbean, commonly called green gram or simply gram, is a dicotyledonous angiosperm belonging to the family Fabaceae. The cultivated mungbean was given the name *Phaseolus radiatus* L. by Carl Linnaeus (1753), and the wild mungbean was given the name *Phaseolus sublobatus* Roxb. by William Roxburgh (1832). Hara (1955) accepted the name for domesticated mungbean, but he called *P. radiatus* var. setulosus (Dalz.) Hara comb. nov. as a new combination to taxonomic biology aimed at the wild mungbean variety, keeping *P. sublobatus* Roxb. nom. nud. as synonym for the same in his publication. Ohwi and Ohashi (1969) designated *Vigna radiata* (L.) Wilczek var. setulosa (Dalz.) Ohwi et Ohashi comb. nov. by citing *P. sublobatus* in Roxburgh (1832) and *P. setulosus* Roxb. as its synonyms. Later, in 1970, Verdcourt described *V. radiata* (L.) Wilczek var. sublobata (Roxb.) Verdc. comb. & stat. nov. as a new combination with a new taxonomic rank based on *P. sublobatus* Roxb. This naming of Verdcourt was accepted by Takahashi et al. (2018). However, most taxonomists have recently had difficulties separating wild mungbean from *V. grandiflora* and/or *V. trinervia*, which Bairiganjan et al. (1985) considered being separate species. Therefore, Takahashi et al. (2018) in their description considered it appropriate to distinguish domesticated and wild mungbean as varieties. Because of these factors, *V. radiata* (L.) Wilczek var. radiata and *V. radiata* (L.) Wilczek var. sublobata (Roxb.) Verdc are the accepted nomenclature for domesticated and wild mungbean, respectively.

Mungbean is considered to have first evolved in India and has been developed from the variety sublobata, which grows wild in India and Burma (Purseglove, 1977). Afterwards, it is thought to have spread to various regions across Asia, Africa, the West Indies, and the USA. Mungbean is a type of low-altitude, short-term grain legume that typically thrives as a dryland crop at around 2000 meters above sea level (Akpapunam, 1996). Mungbean is cultivated across the globe, spanning over 7 million hectares, with a primary focus on Asia, though it's also grown in other regions (Nair et al. 2019). Its popularity stems from its ability to withstand drought conditions, its minimal prerequisites, and its fast-growing cycle. As a result, mungbean is widely cultivated across many Asian countries, as well as in dry parts of southern Europe and warmer regions of Canada and the United States (Hou et al., 2019).

## *1.2 History, Origin, and Domestication*

Archaeological evidence and domesticated mungbean diversity data are suggestive of the fact that the domestication of mungbean has started in its origin in India, approximately 3500 years ago (Fuller & Harvey, 2006). Crop domestication and

improvement, according to Dempewolf et al. (2017), is a process of multiple rounds of selection that leads to the separation of genetic diversity important to agriculture from progenitor wild species. During the early stages of domestication, the cultivation practice of mungbean migrated from its origin to other regions of Asia and gradually to the countries of African continent. The mungbean we cultivate today is the result of multiple rounds of domestication and have undergone many selections. The wild relative of the cultivated mungbean, i.e., *V. radiata* var. sublobata, is considered the putative progenitor. This putative progenitor is native to northern and eastern Australia's subtropical and tropical areas (Lawn & Cottrell, 1988). This weedy plant can be found in the wild. Luckily, the wild relatives of a domesticated plant are a source of beneficial genes, which is of no difference in the case of mungbean also. These useful genes get lost from the domesticated cultivars due to selection pressure and the domestication bottleneck effect. In recent decades, significant advancements have been achieved in integrating characteristics from wild plants into cultivated crops, primarily aimed at addressing biotic stress factors. Plant breeders have been successful in making use of the useful genes present in the wild relatives of domesticated mungbean in the breeding programs. The mungbean cultivar TC1966, for example, is entirely immune to two bruchid beetle species, *Callosobruchus chinensis* (adzuki bean weevil) and *Callosobruchus maculatus* (cowpea weevil), that otherwise prove to be detrimental to the mungbean in stores (Somta et al., 2007; Talekar, 1988). Plant breeders have taken advantage of this for developing mungbean varieties resistant towards bruchid (Tomooka et al., 1992). Apart from just breeding success, genetic linkage map construction using wild and domesticated mungbean accessions have provided valuable information regarding commercially important traits (Lambrides et al., 2000). So, one cannot deny the fact that the germplasm of the wild relatives of domesticated mungbean will be needed in the future to improve productivity.

## *1.3 Cytogenetics*

Mungbean is a diploid plant with $2n = 22$ somatic chromosomes. Bhatnagar (1974) devised the karyotype formula for mungbean as "4Lsm + 4 Msm + 3Mm" "[L = long (2.7–3.5 μm), M = medium (1.9–2.6 μm, sm = sub median centromere and m = median centromere)]."

## *1.4 Nutritional Values and Importance*

Many health organizations have suggested increasing plant-based food intake to enhance chronic disease prevention and general human health, leading to the inclusion of a range of plant-based foods in healthcare programs. Among such crops exhibiting tremendous health benefits is the mungbean. Studies of the biochemical

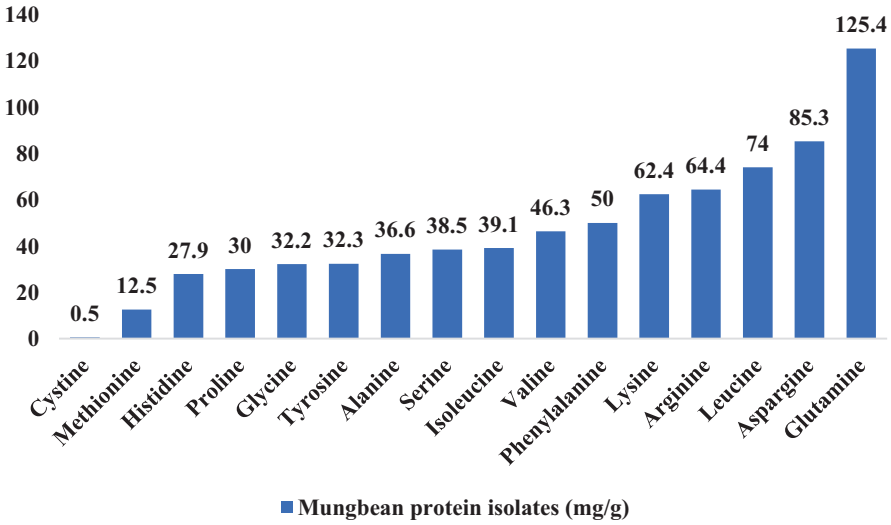



**Fig. 1** Amino acid compositions of mungbean seed protein isolates

composition of mungbean have shown that it is a plentiful source of protein, dietary fiber, vitamins, and various other nutrients. Due to its high nutrient-rich seeds, mungbean has been cultivated as an important food and feed crop for humans and animals for centuries. Compared to soy and kidney beans, mungbean seeds have a significantly higher protein content ranging from 20.97% to 31.32%, which is approximately twice as much as that found in maize, a cereal seed (Anwar et al., 2007). The proteins and peptides of mungbean have been shown to have antibacterial and angiotensin-converting enzyme (ACE)-inhibiting properties (Tang et al., 2014). According to FAO/WHO, mungbean is a decent protein and amino acid source except for sulfur-containing amino acids, methionine, and cysteine. But with the help of genetic engineering techniques, 8S globulin was being inserted with methionine and cysteine sequences (Yi-Shen et al., 2018). Proximate compositions of amino acids in mungbean protein isolates are given in Fig. 1. Total amino acid content of mungbean is 800.2 mg/g, where the total essential amino acids share is 348.2 mg/g, the total aromatic amino acid is 96.7 mg/g, and the total sulfur amino acids is 13 mg/g (Kudre et al., 2013). Apart from its nutritional value, mungbean improves the yield of other crops by minimizing the need for synthetic nitrogen fertilizers in the soil (Fernandez et al., 1988).

## *1.5 Adaptation and Cultivation*

Mungbean is an excellent food legume crop widely grown in South, East, and Southeast Asia, accounting for 90% of global output. Mungbean is a drought-tolerant, low-input crop that can offer both green manure and animal feed, making

it a popular choice among smallholder farmers. Mungbean thrives in a variety of agroclimatic environments. According to the World Vegetable Center, a warmer and humid climate with temperatures ranging from 250 °C to 350 °C and 400–550 mm of rainfall evenly dispersed throughout a growth period of 60 to 90 days is ideal for production. Mungbean exhibits drought tolerance to a reasonable extent but it is susceptible to waterlogging or overwater stress (Mehandi et al., 2019). Mungbean has the ability to be grown in different soil types, but it thrives the most in well-drained loamy to sandy loam soils. To ensure effective atmospheric nitrogen fixation by the bacteria living in the root nodules during the growing stage, proper drainage and adequate aeration in the field are necessary. Soil is readied for sowing by preparing ridges and furrows in the field. Pretreatment of the soil with well-decomposed farmyard manure enhances the quality of the soil. NPK fertilizers are applied as per soil nutrient status. Moreover, the application of the biofungicide *Trichoderma viride* along with farmyard manure before sowing can protect the mungbean plants from several fungal pathogens. Seeds can be pretreated with antifungal captan, thiram, and symbiotic diazotroph *Rhizobium*. Weed removal during the growing period is necessary for better grain yield. Mungbean cultivation needs attention for a wide range of diseases and pests such as seed and seedling rot, yellow mosaic, *Cercospora* leaf spot, powdery mildew, tobacco caterpillar, whitefly, bean pod borer, thrips, cowpea aphid, etc. When the pods are ripe and dried but not yet breaking, they are harvested using both manual and mechanized techniques.

## 2 Production Statistics

Mungbean is considerably an underused legume that is not individually classified by the Food and Agriculture Organization's (FAO) statistics database but is known as a "future smart food" for Asia (FAO, 2018). Mungbean is often used to make bean sprouts, translucent noodles, and mungbean paste in Eastern and Southeastern Asia, whereas in Eastern Africa, it is most typically served as a bean stew (Nair & Schreinemachers, 2020). Because there are no commercial hybrids and farmers can easily preserve their own seed, the private seed market is uninterested in the crop. As a result, the public sector is heavily involved in variety creation and scaling. The Asian mungbean research nations cultivated mungbean on around 10 lakh hectares, yielding roughly 0.77 megaton of dry grain, or around 16% of world mungbean production (Nair & Schreinemachers, 2020). Myanmar, India, Bangladesh, and Pakistan (Schreinemachers et al., 2019), which account for 66% of the world output, were the subjects of a previous research. According to secondary statistics, mungbean cultivation in Southeast Asia decreased by 100,000 hectares (18%) between 2008 and 2017. The majority of this drop was due to Indonesia, whose mungbean acreage declined by nearly 25% (Agriculture Mo, 2018). One possible cause is that mungbean yields are lower than those of other crops. In East Africa, on the other hand, the area under mungbean appears to be expanding, despite the fact that the available statistics indicate a large year-to-year variance. In Asia, the typical

mungbean farmer planted 0.5–1.0 ha, with Thailand (6.2 ha/farmer) having a greater average area and Vietnam (0.2 ha/farmer) having a smaller average area. The average area per producer in East Africa is 0.4–1.4 hectares.

Although mungbean has a yield potential of 2.5-3.0 t/ha, its actual average yield is significantly lower at 0.5 t/ha. This low production is attributed to various factors, including abiotic and biotic stresses, inadequate crop management techniques, and the absence of high-quality seeds of superior varieties (Chauhan et al., 2010; Pratap et al., 2019). Some of the most significant biotic factors affecting mungbean production include yellow mosaic, anthracnose, powdery mildew, Cercospora leaf spot (CLS), dry root rot, halo blight, and tan spot, as well as insect pests such as bruchids, whitefly, thrips, aphids, and pod borers (War et al., 2017; Pandey et al., 2018). Drought, waterlogging, heat, and salinity stress are all abiotic factors that impact mungbean productivity (HanumanthaRao et al., 2016). Owing to breeding attempts that were confined to only a handful of inbred lines, genetic diversity in cultivated mungbeans is limited, necessitating the broadening of the genetic basis of mungbeans under cultivations. Mungbean has been expanded to multiple intercropping systems with rice, wheat, and maize for production worldwide, including South America and Sub-Saharan Africa, thanks to the development of short-duration variants (Moghadam et al., 2011). To improve crop yield and stabilize agricultural output, it is important to develop varieties that can withstand both biotic and abiotic stress factors. Identifying the sources of tolerance traits displayed at the relevant growth stages requires crucial breeding information on stressors affecting mungbean, as well as the influence of environmental pressures on plant growth. The genetic foundation of symbioses with pests, pathogens, and the environment may be analyzed using advanced breeding approaches to build efficient crop improvement techniques.

## 3 Biotic and Abiotic Stress

In South Asia, Southeast Asia, and Sub-Saharan Africa, viral, bacterial, and fungal infections are economically significant (Mbeyagala et al., 2017; Pandey et al., 2018). Mungbean yellow mosaic disease (MYMD) is a serious viral mungbean disease (Noble et al., 2019). The whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) transmits numerous begomoviruses that cause MYMD (Nair et al., 2017). MYMD-related economic losses in India amount to an 85% drop in yield (Karthikeyan et al., 2014). In India and Pakistan, dry root rot caused output losses of 10–44% in mungbean production (Bashir & Malik, 1988). According to Singh et al., (2013), crop losses ranging from 33% to 44% were attributed to Rhizoctonia root rot in India. Additionally, Shukla et al., (2014) reported that anthracnose caused crop losses ranging from 30% to 70%. CLS caused 97% of yield losses in Pakistan and other Indian states (Bhat et al., 2014), whereas powdery mildew caused 40% of yield losses (Khajudparn et al., 2007). Fusarium wilt caused 20% production loss (Anderson, 1985), while *Alternaria* leaf spot caused 10% yield loss among minor

fungal infections (Maheshwari & Krishna, 2013). Between 2009 and 2014, a survey of mungbean farms across China found average output decreases of 30–50% caused by halo blight-led cropping disaster (Sun et al., 2017). Halo blight is a newly identified disease in China (Sun et al., 2017) and Australia (Noble et al., 2019). Pandey et al. (2018) investigated the influence of cultural practices on mungbean infections and assessed the efficacy of bactericides, fungicides, bio-fungicides, and botanicals for seed treatment and foliar spray. The most efficient and long-lasting technique for integrated disease control is to deploy genetically resistant cultivars.

Insect pests attack mungbean throughout the agricultural cycle, from seeding to storage, wreaking havoc on output. Some insect pests cause direct harm to crops, while others serve as disease carriers. Mungbean is susceptible to several pests, with the stem fly (bean fly), *Ophiomyia phaseoli*, being one of the most severe. Additionally, *Melanagromyza sojae* and *Ophiomyia centrosematis* are two other stem fly species that can attack mungbean crops (Talekar, 1990). The stem fly infests the crop within a week of germination, and under epidemic conditions, it can lead to complete crop loss (Chiang & Talekar, 1980). Another widespread mungbean pest is *B. tabaci*, which feeds on the plant's phloem sap, excreting honeydew or indirectly spreading MYMD, which causes black sooty mould on the plant. In addition to pests, abiotic stressors pose a significant threat to mungbean crops' growth and yield, resulting in significant agricultural losses worldwide (Ye et al., 2017). Crop production reduction owing to environmental variables has progressively grown throughout the decades (Boyer et al., 2013). Crops develop by using resources from their surrounding environment (light, water, carbon, and mineral nutrients). The growth and development of crops are influenced by both the microenvironment and the management practices used in cultivation. Due to climate change, the interactions between plants and their environment are becoming increasingly complex (Goyary, 2009). To understand how these factors impact crop growth and development, researchers use eco-physiological features and comprehensive phenotyping-based insights into crop physiology and external signals (Biswas et al., 2018). This information can help predict harvests and develop measures to control growth. When plants experience abiotic stress, such as changes in temperature or water availability, they often undergo molecular, biochemical, physiological, and morphological changes that affect their productivity (Ahmad & Prasad, 2012). Some crop production models predict a decrease in key agricultural crop yields due to changing climatic conditions, which can create unfavorable conditions for crop development due to abiotic factors (Rosenzweig et al., 2014). Such attempts in mungbean are uncommon and need extra care. Environmental pressures constitute a threat to global agriculture in the contemporary period and provide production consistency across geographies and crop seasons. New methods are being developed to better understand probable stress tolerance processes and to identify stress tolerance characteristics in order to promote sustainable agriculture (Fiorani & Schurr, 2013). The activation of several stress-regulated genes is required for basic tolerance mechanisms to be put into action, as they work together through coordinated cellular and molecular responses (Latif et al., 2016). Many factors that contribute to stress tolerance are neglected when breeding lines are phenotyped for plainly apparent

qualities such as growth and yield components. This might be owing to the ease with which these features can be measured precisely and quickly. As a result, modern plant phenotyping platforms include picture capture and automation in contemporary phenotyping technologies. These latest initiatives are projected to improve efforts to transform the fundamental physiology of agricultural plants for outputs with real-world standards to help breeding programs in severe settings (such as salinity, soil moisture, high temperatures, and so on).

## 4 Breeding Strategies and Constraints

It is crucial to identify sources of resistance for introducing resistance into cultivars through breeding. The primary gene pool is the initial choice for resistance sources, while the secondary and tertiary gene pools offer additional options for incorporating variation into the crop. To effectively breed for fungal stressors, easily accessible resistant germplasm and markers linked to QTL regions or critical genes are necessary for marker-assisted selection (MAS). In mungbean, molecular markers for *Cercospora* leaf spot and powdery mildew have been identified for use in breeding efforts. Both qualitative and quantitative inheritance routes have been observed for powdery mildew resistance (Kasettranan et al., 2009). Seeds can carry bacterial diseases that are capable of surviving in agricultural waste. Integrated disease management often involves varietal resistance, which has been recognized as a crucial element (Noble et al., 2019). However, little attention has been paid to the screening of mungbean genotypes for bacterial infections or the detection of genetic markers linked to bacterial illnesses. Identifying genetic markers/QTLs associated with resistance to bacterial leaf spot, halo blight, and tan spot in mungbean can accelerate the development of resistant commercial cultivars. Genome-wide association analysis of large and diverse mungbean mapping populations representative of global germplasm can be used to identify these markers (Noble et al., 2019). Additionally, the effectiveness of breeding programs that confer MYMD resistance has been improved by investigating genotypic diversity, identifying linked markers for the R gene, and constructing QTL maps using molecular markers (Sudha et al., 2013).

A marker related to resistance against yellow mosaic virus in mungbean, called "VMYR1," was identified by Basak et al. (2004). Linked marker-assisted genotyping can be used by plant breeders to perform repeat genotyping when disease incidence is absent during the growing season, as phenotyping for begomoviruses is challenging and requires significant labor. Interspecific sources have also been discovered as new MYMD resistance donors (Nair et al., 2017). Although various screening technologies have been developed, screening plants for insect resistance remains a particularly challenging task. This is due to the non-uniform insect infection patterns observed across seasons and locations for certain key pests, which also face difficulties in rearing and reproducing on feedstuffs. To achieve success in insect resistance breeding, it is essential to comprehend the nature of the pest, the infestation stage, and the bio-molecular aspects of the plant-insect relationship. It is

crucial to have the ideal population of insect pests at their most susceptible stage of the crop. This enables the identification of resistant genotypes against insects and prevents or eradicates escapes through uniform infestation during relevant phases of plant growth (Maxwell & Jennings, 1980). One of the most important strategies in insect resistance breeding involves identifying resistance coding genes from wild/cultivated species and transferring them into improved lines through recombination, hybridization, and selection. Conventional plant breeding, despite its limitations, has resulted in significant progress in mungbean output as well as disease and insect resistance (Fernandez & Shanmugasundaram, 1988). Physical and chemical mutagens have been utilized to develop insect and disease-resistant mungbean cultivars, as well as other desirable characteristics (Watanasit et al., 2001). Details of 39 mungbean varieties improved through induced mutagenesis are recorded in Table 1. One of the conditions for crop improvement is genetic heterogeneity (Laskar & Khan, 2017). There is a limited ability to select improved genotypes in mungbean due to insufficient diversity. To rapidly increase genetic diversity, induced mutagenesis has proven to be the most effective technique and has been utilized in several crops such as cowpea (Raina et al., 2018a, 2020a, 2022a, b; Rasik et al., 2022),

**Table 1** Details of mutant cultivars of mungbean released

| Variety name | Country | Registration year | Mutagen type | Mutant development type | Characters improved |
|---|---|---|---|---|---|
| AEM-96 | Pakistan | 1998 | Physical | Direct use of an induced mutant CV.6601 with 200 Gy | 1246–1298 kg/ha grain yield, short stature combined with short duration and synchrony in maturity |
| Binamoog-1 | Bangladesh | 1992 | – | – | Resistance to powdery mildew and suitable for rice fallows |
| Binamoog-2 | Bangladesh | 1994 | Physical | Crossing with one mutant Mutant MB-55(4) × D-2773 | Larger seed size, early and synchronous maturity (7–10 days earlier), high yield (16%), tolerant to leaf MYMV and *Cercospora* leaf spot |
| Binamoog-3 | Bangladesh | 1997 | Physical | Mutagenic treatment of breeding material (F1, F2, seeds, etc.) (mutant MB55-4 × AURDC line V1560D) with 200 Gy | Seed yield, synchronous pod maturity, tolerant to yellow mosaic virus and *Cercospora* leaf spot |

(continued)

**Table 1** (continued)

| Variety name | Country | Registration year | Mutagen type | Mutant development type | Characters improved |
|---|---|---|---|---|---|
| Binamoog-4 | Bangladesh | 1997 | Physical | Mutagenic treatment of breeding material (F1, F2, seeds, etc.) (mutant MB55-4 × AURDC line V1560D) with 200 Gy | Seed yield, synchronous pod maturity, early maturing, dwarf plant type, tolerant to yellow mosaic virus and *Cercospora* leaf spot |
| Binamoog-5 | Bangladesh | 1998 | Physical | Mutagenic treatment of breeding material (F1, F2, seeds, etc.) (mutant MB55-4 × AURDC line V1560D) with 200 Gy | Higher seed yield, synchronize pod maturity, tolerance to leaf MYMV and *Cercospora* leaf spot |
| Binamoog-6 | Bangladesh | 2005 | Physical | Direct use of an induced mutant VC-6173-10 with 400 Gy | Purple hypocotyl and stem, high number of pods and clusters, resistance to diseases |
| Binamoog-7 | Bangladesh | 2005 | Chemical | Direct use of an induced mutant Binamoog-2 with 0.75% EMS | Increased pod, reduced seed size, increased seed, tolerant to MYMV and *Cercospora* leaf spot |
| Binamoog-8 | Bangladesh | 2010 | Physical | Direct use of an induced mutant MB-149 with 400 Gy | Medium plant height (35–40 cm), early maturing (64–67 days), deep green leaf color, shiny green seed coat color, 22–23% protein content, average seed yield of 1.80 tons $ha^{-1}$, and tolerant to MYMV |

(continued)

**Table 1** (continued)

| Variety name | Country | Registration year | Mutagen type | Mutant development type | Characters improved |
|---|---|---|---|---|---|
| Binamoog-9 | Bangladesh | 2017 | Physical | Direct use of an induced mutant BARI Mung-6 with 400 Gy | The distinct features of the selected mutant MBM-07 (Binamoog-8) are medium plant height (35–40 cm), early maturing (64–67 days), deep green leaf color, shiny green seed coat color, 22–23% protein content, average seed yield of 1.74 t/ha and potential 1.95 t/ha, and tolerant to MYMV |
| BM 4 | India | 1992 | Chemical | Direct use of an induced mutant T-44 with 0.15% EMS | Resistant to *Macrophomina* blight and tolerant to MYMV |
| Camar | Indonesia | 1991 | Physical | Direct use of an induced mutant Manyar with 100 Gy | Resistance to *Cercospora* leaf spot, resistance to *Uromyces* sp., medium resistance to scrab diseases, high yield, and tolerance to salinity and acid soil |
| Chai Nat 72 | Thailand | 1999 | Physical | Direct use of an induced mutant Kamphangsaen 2 with 600Gy | High yield, larger grain size, and resistance to fungal diseases |
| Chai Nut 84-1 | Thailand | 2012 | Physical | Direct use of an induced mutant Chai Nut 36 with 500Gy | High yield and starch, large seeds |
| Co 4 | India | 1982 | Physical | Direct use of an induced mutant Co 1 with 200Gy | High yield, early maturity and resistance to drought |

(continued)

**Table 1** (continued)

| Variety name | Country | Registration year | Mutagen type | Mutant development type | Characters improved |
|---|---|---|---|---|---|
| Dhauli (TT9E) | India | 1979 | – | Crossing with one mutant T-51 × local type | High yield, early maturity with tolerance or resistance to MYMV |
| LGG 450 | India | 1993 | Physical | Direct use of an induced mutant Pant Mung-2 with 40 kR gamma rays | High yield, early maturity with tolerance or resistance to MYMV |
| LGG-407 | India | 1993 | Physical | Direct use of an induced mutant Pant Mung-2 with 40 kR gamma rays | High yield, early maturity with tolerance or resistance to MYMV |
| ML 26-10-3 | India | 1983 | Physical | Direct use of an induced mutant ML-26 with gamma rays | Resistance to MYMV and high yield |
| MUM-2 | India | 1992 | Chemical | Direct use of an induced mutant K-851 with 0.2% EMS | High yield and resistance to diseases |
| NIAB Mung 121-25 | Pakistan | 1985 | Physical | Direct use of an induced mutant RC 71-27 with 200 Gy | Early maturity (60–65 days), determinate type, high yield (44%), recommended as spring and summer crop |
| NIAB Mung 13-1 | Pakistan | 1986 | Physical | Direct use of an induced mutant 6601 with 100 Gy | Early maturity, shortness, more pods, harvest index (28%), TGW (40.5 g), and higher yield (44%) |
| NIAB Mung 19-19 | Pakistan | 1985 | Physical | Direct use of an induced mutant Pak 22 with 400 Gy | Early maturity (60–65 days), determinate type, high yield (35%), recommended as spring and summer crop, high tolerance to mungbean yellow mosaic virus |

(continued)

**Table 1** (continued)

| Variety name | Country | Registration year | Mutagen type | Mutant development type | Characters improved |
|---|---|---|---|---|---|
| NIAB MUNG 2006 | Pakistan | 2006 | – | Crossing with one mutant variety NIAB Mung 92 × VC-1560D | Yellow mosaic virus resistance, resistance to powdery mildew, *Rhizoctonia* root-rot disease resistance, early maturity, and large seeds |
| NIAB Mung 20-21 | Pakistan | 1986 | Physical | Direct use of an induced mutant Pak 22 with 400 Gy | Early maturity, shortness, harvest index (31%), high yield (65%), tolerance to yellow mosaic virus, resistance to *Cercospora* leaf spot, suitable as catch crop |
| NIAB Mung 51 | Pakistan | 1990 | Physical | Mutagenic treatment of breeding material (F1, F2, seeds, etc.) (6601x1973A) with 100 Gy | Early and synchronous maturity, non-shattering pods, profuse hairiness, tolerant to MYMV and CLS diseases, larger seed size, higher yield potential, crop vegetation: Summer (66 days) and spring (67 days) |
| NIAB Mung 54 | Pakistan | 1990 | Physical | Mutagenic treatment of breeding material (F1, F2, seeds, etc.) (6601x1973A) with 100 Gy | Early and synchronous maturity, non-shattering pods, tolerant to MYMV and CLS diseases, larger seed size, higher yield potential, crop vegetation: summer (71 days) and spring (73 days) |

(continued)

**Table 1** (continued)

| Variety name | Country | Registration year | Mutagen type | Mutant development type | Characters improved |
|---|---|---|---|---|---|
| NIAB Mung 92 | Pakistan | 1992 | – | Crossing with one mutant NIAB Mung 36 × VC 2768B | Resistance to MYMV, early maturity, resistance to grain shattering, and large seed size |
| NIAB Mung 98 | Pakistan | 1998 | Physical | Crossing with one mutant NIAB Mung 20–21 × VC 1482E | Resistance to diseases (yellow mosaic virus and *Cercospora* leaf spot), high yield, and medium seed size |
| NIAB Mung-28 | Pakistan | 1983 | Physical | Direct use of an induced mutant Pak 17 with 200 Gy | Early and uniform maturity and high yield |
| Pant Moong 2 | India | 1982 | Physical | Direct use of an induced mutant ML-26 with 100 Gy | Resistance to MYMV, more pods, and high yield |
| TAP-7 | India | 1983 | Physical | Direct use of an induced mutant S-8 with 30 kR gamma rays | Early maturity (5–7 days), resistance to mildew and leaf spot, higher yield (23%) |
| TARM-1 | India | 1997 | Physical | Direct use of an induced mutant RUM 5 with 30 kR gamma rays | High yield, resistance to powdery mildew disease, and medium maturity |
| TARM-18 | India | 1996 | Physical | Crossing with one mutant TARM-2 × PDM-54 | High yield and resistance to powdery mildew disease |
| TARM-2 | India | 1994 | Physical | Direct use of an induced mutant RUM 5 with 30 kR gamma rays | High yield, medium–late maturity, and resistance to powdery mildew disease |
| TJM-3 | India | 2007 | Physical | Crossing with one mutant TARM-1 × Kopargaon | Early maturity, large seeds, and resistance to powdery mildew, *Rhizoctonia* root-rot disease |

(continued)

**Table 1** (continued)

| Variety name | Country | Registration year | Mutagen type | Mutant development type | Characters improved |
|---|---|---|---|---|---|
| TM 2000-2 | India | 2010 | Physical | Crossing with one mutant TARM-1 × JL-781 | Higher seed yield and synchronous pod maturity, tolerance to leaf MYMV and *Cercospora* leaf spot |
| TM-96-2 | India | 2007 | Physical | Crossing with one mutant TARM-2 × Kopargaon | Resistance to powdery mildew and *Corynespora* leaf spot |
| TMB-37 | India | 2005 | Physical | Crossing with one mutant TARM-2 × Kopargaon | High yield, early maturity with tolerance or resistance to MYMV |

Source: The Joint FAO/IAEA Mutant Variety Database (https://mvd.iaea.org)

lentil (Laskar et al., 2018a, b, 2019; Wani et al., 2021), faba bean (Khursheed et al., 2015, 2016, 2018a, b, c, 2019), fenugreek (Hasan et al., 2018), mungbean (Wani et al., 2017), urdbean (Goyal et al., 2019a, b, 2020a, b, 2021a, b), chickpea (Laskar et al., 2015; Raina et al., 2017, 2019), black cumin (Tantray et al., 2017; Amin et al., 2020), and finger millet (Sellapillaibanumathi et al., 2022). Because natural mutations occur sporadically, artificial mutations are generated, and genetic gain is best achieved by using mutagens (Raina & Khan, 2020; Raina et al., 2016, 2018b, 2020b, 2021, 2022c). Auti (2012) stressed that mutation breeding or induced mutation has a lot of promise for improving mungbean. Traditional breeding methods for producing pest-resistant cultivars include pure line, mass, and recurrent selection (Burton & Widstorm, 2001). Insect resistance and enhanced agronomic features are being developed in mungbean using techniques such as pedigree, backcross, and bulk selection breeding.

Sehgal et al. (2018) reported on various successful projects related to mungbean, aimed at screening and developing cultivars that are resistant to high temperature, salt, waterlogging, and water stress. These projects considered the physiological, biochemical, and molecular aspects of the crop. To facilitate future crop development with specific traits, a panel of donor resources would consist of breeding lines that have been identified and chosen for the aforementioned circumstances. By selecting a few genotypes that are well-suited to the region in the initial stages of mungbean breeding, certain genotypes were identified as being particularly resistant to biotic stresses and high yield. Indirect selection was made for yield, plant type, and adaptation-related features, though no direct selection was done for abiotic stress tolerance. The selection of improved cultivars with increased resilience to

drought has been proven successful. Fernandez and Kuo (1993) used a stress tolerance measure to choose genotypes with high resilience to temperature and water shocks and yield in mungbean (STI). Singh (1997) reported mungbean plant types suitable for Kharif (rainy) and dry (spring/summer) seasons. Pratap et al. (2013) recommended the development of short-duration cultivars for Spring/Summer farming to minimize heat and drought stress toward the end of the growing season. Cultivars that are well adapted to the summer season have a crop cycle of 60–65 days, a determinate growth habit, a high harvest index, reduced photoperiod sensitivity, quick initial development, longer pods with more than 10 seeds per pod, and large seeds. In light of this, numerous early maturing mungbean lines have been selected and released as commercial cultivars.

Whenever wild resources are used as donors for disease or pest-resistant cultivars, linkage drag becomes a significant concern. In resistance breeding, the use of wild germplasm is a dominant contributor to resistance introgression into commercial cultivars, but unwanted hereditary linkages frequently hamper this process (Keneni et al., 2011). Undesirable traits such as leaf area index, seed structure, and color can be passed along with beneficial traits due to low dominance multigenic disease and insect resistance. To overcome linkage drag, crossing over between homologous chromosomes during meiosis is critical for transferring genes that govern desirable characteristics (Edwards & Singh, 2006). However, the inheritance of undesirable and desirable traits together can impact seed quality, germination, and other traits. Generating a high number of F2 populations is necessary to increase the recovery of novel recombinants due to crossing-over. The emergence and dissemination of whitefly-transmitted viruses are influenced by factors such as the evolution of viral strains, the creation of aggressive biotypes, and a rise in the whitefly population (Chiel et al., 2007). Insect biotypes reflect the genetic variety of a pest population, and although they may appear identical, their biological characteristics differ. Breeding for disease resistance is hindered by the creation of multiple strains by a pathogen, as well as biotypic variety in insect pests, as plant varieties resistant through one disease strain or pest biotype could be sensitive to a different pathogen or insect biotype of the same pathogen.

Although there were multiple ongoing efforts to develop plant cultivars for a particular biotic and abiotic stress on a wider level, achievements were limited due to the cumulative effect of many stresses and unforeseen increases in pest and pathogen episodes throughout the plant's growth stages, resulting in only a few calculable achievements in legumes. A comprehensive examination is necessary for various stages of the breeding process, including seed germination, early growth, vegetative phase, flowering, early pod development, as well as the reproductive and final maturity stages. With such a diverse range of developing phases, pinpointing a precise phase inducing a characteristic for breeding appears to be difficult; however, many approaches have focused on the flowering and reproductive phases in order to develop progenies that can sustain stress and result in better pod and seed yields.

## 5 Conclusion

The objective of high-yielding mungbean varieties is conceivable by utilizing a wider range of genetic diversity. Mungbean has typically been farmed in less productive vulnerable areas' minimal resources because of which the selection pressure has been focused on stress adaptability rather than yield. Thus, improving the genetics of such crops in order to increase output necessitates genetic restoration in order to generate diverse genotypes. Induced mutations can aid in the regeneration and restoration of diversity that has been vanished over time as a result of adaptation to various stressors. Although disease resistance genotypes were established for powdery mildew, yellow mosaic, and CLS, to accelerate the establishment of resistant breeding lines, molecular markers for anthracnose and dry root rot further required to be developed and identified markers must be employed in the breeding effort. Introduction of undesirable characteristics into the cultivars from insect-resistant origins for bruchids and whiteflies is challenging. To achieve stable resistance against diseases and insects in mungbeans, a combination of conventional breeding methods and molecular techniques is required. The identification of molecular markers has facilitated the evaluation of pest and disease resistance, minimizing our dependence on time-consuming phenotypic data, particularly in extensive trials. Insect resistance can also be transferred from related legumes like black gram to green gram using molecular markers. However, identifying and combining numerous resistance genes into the same cultivar are critical. In order to generate mungbean with disease and insect pest resistance while avoiding strain/biotype formation, breeders should focus on gene pyramiding. In order to understand the ways in which herbivores and pathogens function, it is important to explore the mechanisms of disease and insect resistance, as well as the specific signal molecules involved in these processes. In addition, RNAi technology could be employed to increase mungbean stress tolerance against biological factors. Though, Large-scale field experiments are necessary to prove the effectiveness of RNAi as a potential pest control method in plant breeding.

## References

Abdullah-Al-Rahad, M., Rahman, M. S., Akter, T., Akter, J., Rahman, M. A., & Aziz, S. M. S. (2018). Varietal Screening of Mungbean against Whitefly and Aphid. *Journal of Bioscience and Agriculture Research. 18*(01), 1478–1487.

Agriculture Mo. (2018). *Statistik Pertanian 2010, 2014, 2018*. Statistic Indonesia.

Ahmad, P., & Prasad, M. N. V. (2012). *Abiotic stress responses in plants: Metabolism, productivity and sustainability*. Springer. https://doi.org/10.1007/978-1-4614-0634

Akpapunam, M. (1996). Mung bean (Vigna radiata (L.) Wilczek). *Food and Feed from Legumes and Oilseeds*, 209–215. https://doi.org/10.1007/978-1-4613-0433-3_23

Amin, R., Wani, M. R., Raina, A., Khursheed, S., & Khan, S. (2020). Induced morphological and chromosomal diversity in the mutagenized population of black cumin (Nigella sativa L.) using

single and combination treatments of gamma rays and ethyl methane sulfonate. *Jordan Journal of Biological Sciences, 12*(1), 23–30.

Anderson, T. R. (1985). Root rot and wilt of mungbean in Ontario. *Canadian Plant Disease Survey, 65*, 3–6.

Anwar, F., Latif, S., Przybylski, R., Sultana, B., & Ashraf, M. (2007). Chemical composition and antioxidant activity of seeds of different cultivars of mung bean. *Journal of Food Science, 72*(7), S503–S510. https://doi.org/10.1111/j.1750-3841.2007.00462.x

Auti, S. G. (2012). Induced morphological and quantitative mutations in mungbean. *Bioremediation, Biodiversity and Bioavailability, 6*(1), 27–39.

AVRDC (1979). AVRDC Progress Report for (1978). Shanhua, Tainan: Asian Vegetable Research and Development Center, 173.

Bairiganjan, G. C., Panda, P. C., Choudhury, B. P., & Patnaik, S. N. (1985). Fabaceae in Orissa. *Journal of Economic and Taxonomic Botany, 7*(2), 249–276.

Basak, J., Kundagrami, S., Ghose, T. K., & Pal, A. (2004). Development of Yellow Mosaic Virus (YMV) resistance linked DNA marker in Vigna mungo from populations segregating for YMV-reaction. *Molecular Breeding, 14*, 375–383. https://doi.org/10.1007/s11032-004-0238-y

Bashir, M., & Malik, B. A. (1988). Diseases of major pulse crops in Pakistan—A review. *Tropical Pest Management, 34*, 309–314. https://doi.org/10.1080/09670878809371262

Bhat, F. A., Mohiddin, F. A., & Bhat, H. A. (2014). Reaction of green gram (Vigna radiata) to Cercospora canascens (ELL.) and Mart. *Indian Journal of Agricultural Research, 48*, 140–144. https://doi.org/10.5958/j.0976-058X.48.2.023

Bhaskar, V. (2017). Genotypes against major diseases in green gram and black gram under natural field conditions. *International Journal of Current Microbiology and Applied Sciences, 6*(6): 832–843.

Bhatnagar, C. P. (1974). Cytotaxonomic studies in the genus Phaseolus. *Indian Journal of Genetics, 34A*, 800.

Biswas, J. C., Kalra, N., Maniruzzaman, M., Choudhury, A. K., Jahan, M. A. H. S., Hossain, M. B., et al. (2018). Development of mungbean model (MungGro) and its application for climate change impact analysis in Bangladesh. *Ecological Modelling, 384*, 1–9. https://doi.org/10.1016/j.ecolmodel.2018.05.024

Boyer, J. S., Byrn, P., Cassman, K. G., Cooper, M., Delmer, D., & Greene, T. (2013). The U.S. drought of 2012 in perspective: A call to action. *Global Food Security, 2*, 139–143. https://doi.org/10.1016/j.gfs.2013.08.002

Burton, A., & Widstorm, N. W. (2001). Mass selection for agronomic performance and resistance to ear feeding insects in three corn populations. *Maydica, 46*, 207–212.

Chauhan, Y. S., Douglas, C., Rachaputi, R. C. N., Agius, P., Martin, W., King, K., et al. (2010). Physiology of mungbean and development of the mungbean crop model. In *Proceedings of the 1st Australian Summer Grains Conference Australia* (pp. 21–24). Gold Coast.

Chiang, H. S., & Talekar, N. S. (1980). Identification of sources of resistance to beanfly and two other agromyzid flies in soybean and mungbean. *Journal of Economic Entomology, 73*, 197–199. https://doi.org/10.1093/jee/73.2.197

Chiel, E., Gottlieb, Y., Zchori-Fein, E., Mozes-Daube, N., Katzir, N., Inbar, M., et al. (2007). Biotype-dependent secondary symbiont communities in sympatric populations of *Bemisia tabaci*. *Bulletin of Entomological Research, 97*, 407–413. https://doi.org/10.1017/S0007485307005159

Dempewolf, H., Baute, G., Anderson, J., Kilian, B., Smith, C., & Guarino, L. (2017). Past and future use of wild relatives in crop breeding. *Crop Science, 57*(3), 1070.

Edwards, O., & Singh, K. B. (2006). Resistance to insect-pests: What do legumes have to offer? *Euphytica, 147*, 273–285. https://doi.org/10.1007/s10681-006-3608-1

FAO. (2018). *Future smart food: Rediscovering hidden treasures of neglected and underutilized species for Zero Hunger in Asia, executive summary*. Food and Agriculture Organization of the United Nations.

Fernandez, G. C. J., & Kuo, C. G. (1993). Effective selection criteria for assessing plant stress tolerance. In C. G. Kuo (Ed.), *Proceedings of the international symposium on Adaptation of food crops to temperature and water stress*, August 13–18 (1992) Tainan, Taiwan (pp. 257–270).

Fernandez, G. C. J., & Shanmugasundaram, S. (1988). The AVRDC mungbean improvement program: The past, present and future. In S. Shanmugasundaram & B. T. McLean (Eds.), *Mungbean: Proceedings of the second international symposium held at Bangkok, Thailand* (pp. 58–70). Shanhua, Tainan: AVRDC. https://worldveg.tind.io/record/7061?ln=en

Fernandez, G., Shanmugasundaram, S., & Mclean, B. (1988). The AVRDC mungbean improvement program: The past, present and future. In *Mungbean: Proceedings of the second international symposium, Bangkok, Thailand* (pp. 58–70). Asian Vegetable Research and Development Center.

Fiorani, F., & Schurr, U. (2013). Future scenarios for plant phenotyping. *Annual Review of Plant Biology, 64*, 267–291. https://doi.org/10.1146/annurev-arplant-050312-120137

Fuller, D. Q., & Harvey, E. L. (2006). The archaeobotany of Indian pulses: Identification, processing and evidence for cultivation. *Environmental Archaeology, 11*, 241–268. https://doi.org/10.1179/174963106x123232

Goyal, S., Wani, M. R., Laskar, R. A., Raina, A., Amin, R., & Khan, S. (2019a). Induction of morphological mutations and mutant phenotyping in black gram [Vigna mungo (L.) Hepper] using gamma rays and EMS. *Vegetos, 32*(4), 464–472.

Goyal, S., Wani, M. R., Laskar, R. A., Aamir, R., & Samiullah, K. (2019b). Assessment on cytotoxic and mutagenic potency of gamma rays and EMS in Vigna mungo L. Hepper. *Biotecnología Vegetal, 19*(3), 193–204.

Goyal, S., Wani, M. R., Laskar, R. A., Raina, A., & Khan, S. (2020a). Mutagenic effectiveness and efficiency of individual and combination treatments of gamma rays and ethyl methanesulfonate in black gram [Vigna mungo (L.) Hepper]. *Advances in Zoology and Botany, 8*(3), 163–168.

Goyal, S., Wani, M. R., Laskar, R. A., Raina, A., & Khan, S. (2020b). Performance evaluation of induced mutant lines of black gram (Vigna mungo (L.) Hepper). *Acta Fytotechnica et Zootechnica, 23*(2), 70–77.

Goyal, S., Wani, M. R., Laskar, R. A., Raina, A., Amin, R., & Khan, S. (2021a). Quantitative assessments on induced high yielding mutant lines in urdbean [Vigna mungo (L.) hepper]. *Legume Science*. https://doi.org/10.1002/leg3.125

Goyal, S., Wani, M. R., Raina, A., Laskar, R. A., & Khan, S. (2021b). Phenotypic diversity in mutagenized population of urdbean (Vigna mungo (L.) Hepper). *Heliyon, 7*(5), e06356.

Goyary, D. (2009). Transgenic crops, and their scope for abiotic stress environment of high altitude: biochemical and physiological perspectives. *DRDO Science Spectrum*, 195–201.

HanumanthaRao, B., Nair, R. M., & Nayyar, H. (2016). Salinity and high temperature tolerance in mungbean [Vigna radiata (L.) Wilczwk] from a physiological perspective. *Frontiers in Plant Science, 7*, 1–20. https://doi.org/10.3389/fpls.2016.00957

Hara, H. (1955). Critical notes on some type specimens of East-Asiatic plants in foreign herbaria 3. *Journal of Japanese Botany, 30*, 138–142.

Hasan, N., Laskar, R. A., Raina, A., & Khan, S. (2018). Maleic hydrazide induced variability in fenugreek (Trigonella foenum-graecum L.) cultivars CO1 and Rmt-1. *Research & Reviews: Journal of Botanical Sciences, 7*(1), 19–28.

He, X., He, T., Xiong, Y., & Jiao, C. (1988). Research and use of mungbean germplasm resources in Hubei, China. In J. Fernandez & S. Shanmugsundaram (Eds.), Mungbean (pp. 35–41). Shanhua, Tainan: Asian Vegetable Research and Development Centre.

Hou, D., Yousaf, L., Xue, Y., et al. (2019). Mung Bean (Vigna radiata L.): Bioactive polyphenols, polysaccharides, peptides, and health benefits. *Nutrients, 11*(6), 1238. https://doi.org/10.3390/nu11061238. Published 2019 May 31.

Iqbal, S. M., Zubair, M., & Haqqani A. M. (2004). Resistant in Mungbean to Cercospora leaf spot disease. *International Journal of Agriculture and Biology, 6*, 792–793.

Iqbal, U., Iqbal, S. M., Afzal, R., Jamal, A., Farooq, M. A., & Zahid A. (2011). Screening of mungbean germplasm against Mungbean yellow mosaic virus (MYMV) under field conditions. *Pakistan Journal of Phytopathology, 23*, 48–51.

Karthikeyan, A., Shobhana, V. G., Sudha, M., Raveendran, M., Senthil, N., Pandiyan, M., et al. (2014). Mungbean yellow mosaic virus (MYMV): A threat to green gram (Vigna radiata) production in Asia. *International Journal of Pest Management, 60*, 314–324. https://doi.org/10.1080/09670874.2014.982230

Kasettranan, W., Somta, P., & Srinives, P. (2009). Genetics of the resistance to powdery mildew disease in mungbean (Vigna radiata (L.) Wilczek). *Journal of Crop Science and Biotechnology, 12*, 37–42. https://doi.org/10.1007/s12892-008-0074-4

Kaur, L., Singh, P., & Sirari, A. (2011). Biplot analysis for locating multiple disease resistant diversity in mungbean germplasm. *Plant Disease Research, 26*, 55–60.

Keneni, G., Bekele, E., Getu, E., Imtiaz, M., Damte, T., & Mulatu, B. (2011). Breeding food legumes for resistance to storage insect-pests: Potential and limitations. *Sustainability, 3*, 1399–1415. https://doi.org/10.3390/su3091399

Khajudparn, P., Wongkaew, S., & Thipyapong, P. (2007). Mungbean powdery resistant identification of genes for resistant to powdery mildew in mungbean. *African Crop Science Conference Proceedings, 8*, 743–745.

Khattak, G. S. S., Ashraf, M., & Khan, M. S. (2004). Assessment of genetic variation for yield and yield components in mungbean (Vigna radiata (L.) Wilczek) using generation mean analysis. *Pakistan Journal of Botany, 36*(3), 583–588.

Khattak, G. S. S., Saeed, I., & Muhammad, T. (2009). Flowers shedding under high temperature in mungbean (Vigna radiata (L.) Wilczek). *Pakistan Journal of Botany, 41*, 35–39.

Khursheed, S., Laskar, R. A., Raina, A., Amin, R., & Khan, S. (2015). Comparative analysis of cytological abnormalities induced in Vicia faba L. genotypes using physical and chemical mutagenesis. Chromosome. *Science, 18*(3–4), 47–51.

Khursheed, S., Raina, A., & Khan, S. (2016). Improvement of yield and mineral content in two cultivars of Vicia faba L. through physical and chemical mutagenesis and their character association analysis. *Archives of Current Research International, 4*(1), 1–7.

Khursheed, S., Raina, A., Laskar, R. A., & Khan, S. (2018a). Effect of gamma radiation and EMS on mutation rate: Their effectiveness and efficiency in faba bean (Vicia faba L.). *Caryologia: International Journal of Cytology, Cytosystematics and Cytogenetics, 71*(4), 397–404. https://doi.org/10.1080/00087114.2018.1485430

Khursheed, S., Raina, A., Amin, R., Wani, M. R., & Khan, S. (2018b). Quantitative analysis of genetic parameters in the mutagenized population of faba bean (Vicia faba L.). *Research on Crops, 19*(2), 276–284.

Khursheed, S., Raina, A., & Khan, S. (2018c). Physiological response of two cultivars of faba bean using physical and chemical mutagenesis. *International Journal of Advance Research in Science and Engineering, 7*(4), 897–905.

Khursheed, S., Raina, A., Parveen, K., & Khan, S. (2019). Induced phenotypic diversity in the mutagenized populations of faba bean using physical and chemical mutagenesis. *Journal of the Saudi Society of Agricultural Sciences, 18*(2), 113–119. https://doi.org/10.1016/j.jssas.2017.03.001

Kudre, T. G., Benjakul, S., & Kishimura, H. (2013). Comparative study on chemical compositions and properties of protein isolates from mung bean, black bean and bambara groundnut. *Journal of Science and Food Agriculture, 93*(10), 2429–2436. https://doi.org/10.1002/jsfa.6052

Lambrides, C. J., Lawn, R. J., Godwin, I. D., Manners, J., & Imrie, B. C. (2000). Two genetic linkage maps of mungbean using RFLP and RAPD markers. *Australian Journal of Agricultural Research, 51*, 415–425. https://doi.org/10.1071/AR99052

Lamichaney, A., Katiyar, P., Laxmi, V., & Pratap, A. (2017). Variation in pre-harvest sprouting tolerance and fresh seed germination in mungbean (Vigna radiata L.) genotypes. *Plant Genetic Resources: Characterization and Utilization, 16*, 437–445. https://doi.org/10.1017/S1479262117000296

Laskar, R. A., & Khan, S. (2017). Assessment on induced genetic variability and divergence in the mutagenized lentil populations of microsperma and macrosperma cultivars developed using

physical and chemical mutagenesis. *PLoS One, 12*(9), e0184598. https://doi.org/10.1371/journal.pone.0184598

Laskar, R. A., Khan, S., Khursheed, S., Raina, A., & Amin, R. (2015). Quantitative analysis of induced phenotypic diversity in chickpea using physical and chemical mutagenesis. *Journal of Agronomy, 14*(3), 102–111.

Laskar, R. A., Laskar, A. A., Raina, A., Khan, S., & Younus, H. (2018a). Induced mutation analysis with biochemical and molecular characterization of high yielding lentil mutant lines. *International Journal of Biological Macromolecules, 109*, 167–179.

Laskar, R. A., Wani, M. R., Raina, A., Amin, R., & Khan, S. (2018b). Morphological characterization of gamma rays induced multipodding mutant (mp) in lentil cultivar Pant L 406. *International Journal of Radiation Biology, 94*(11), 1049–1053.

Laskar, R. A., Khan, S., Deb, C. R., Tomlekova, N., Wani, M. R., Raina, A., & Amin, R. (2019). Lentil (Lens culinaris Medik.) diversity, cytogenetics and breeding. In J. M. Al-Khayri et al. (Eds.), *Advances in plant breeding: legumes* (pp. 319–369). Springer. https://doi.org/10.1007/978-3-030-23400-3_9

Latif, M., Akram, N. A., & Ashraf, M. (2016). Regulation of some biochemical attributes in drought-stressed cauliflower (Brassica oleracea L.) by seed pre-treatment with ascorbic acid. *The Journal of Horticultural Science and Biotechnology, 91*, 129–137. https://doi.org/10.1080/14620316.2015.1117226

Lawn, R. J., & Cottrell, A. (1988). Wild mungbean and its relatives in Australia. *Biologist, 35*, 267–273.

Lawn, R. J., Williams, R. W., & Imrie B. C. (1988). Potential of wild germplasm as a source of tolerance to environmental stresses in mungbean. In J. Fernandez & S. Shanmugsundaram (Eds.), *Mungbean* (pp. 136–145). Shanhua, Tainan: Asian Vegetable Research and Development Centre.

Linnaeus, C. (1753). *Species plantarum*. Impensis G. C. Nauk.

Mahalingam, A., Satya, V. K., Manivannan, N., Lakshmi Narayanan, S., & Sathya, P. (2018). Inheritance of Mungbean Yellow Mosaic Virus Disease Resistance in Greengram [Vigna radiata (L.) Wilczek]. *International Journal of Current Microbiology and Applied Sciences, 7*(1), 880–885.

Maheshwari, S. K., & Krishna, H. (2013). Field efficacy of fungicides and bioagents against Alternaria leaf spot of mungbean. *Annals of Plant Protection Sciences, 21*, 364–367.

Maxwell, F. G., & Jennings, P. R. (1980). *Breeding plants resistant to insects*. Wiley.

Mbeyagala, K. E., Amayo, R., Obuo, J. P., Pandey, A. K., War, A. R., & Nair, R. M. (2017). *A manual for mungbean (greengram) production in Uganda* (p. 32). National Agricultural Research Organization (NARO).

Mehandi, S., Quatadah, S. M., Mishra, S. P., Singh, I. P., Praveen, N., & Dwivedi, N. (2019). Mungbean (*Vigna radiata* L. Wilczek): Retrospect and Prospects. Legume Crops – Characterization and Breeding for Improved Food Security. https://doi.org/10.5772/intechopen.85657

Moghadam, M. B., Vazan, S., Darvishi, B., Golzardi, F., & Farahani, M. E. (2011). Effect of mungbean (Vigna radiate) living mulch on density and dry weight of weeds in corn (*Zea mays*) field. *Communications in Agricultural and Applied Biological Sciences, 76*, 555–559.

Nair, R., & Schreinemachers, P. (2020). Global status and economic importance of mungbean. In R. M. Nair, R. Schafleitner, & S.-H. Lee (Eds.), *The mungbean genome* (pp. 1–8). Springer International Publishing.

Nair, R. M., Götz, M., Winter, S., Giri, R. R., Boddepalli, V. N., Sirari, A., et al. (2017). Identification of mungbean lines with tolerance or resistance to yellow mosaic in fields in India where different begomovirus species and different Bemisia tabaci cryptic species predominate. *European Journal of Plant Pathology, 149*, 349–365. https://doi.org/10.1007/s10658-017-1187-8

Nair, R. M., Pandey, A. K., War, A. R., Hanumantharao, B., Shwe, T., Alam, A., Pratap, A., Malik, S. R., Karimi, R., Mbeyagala, E. K., Douglas, C. A., Rane, J., & Schafleitner, R. (2019, Ocotober 25). Biotic and abiotic constraints in mungbean production-progress in genetic improvement. *Frontiers in Plant Science, 10*, 1340. https://doi.org/10.3389/fpls.2019.01340. PMID: 31736995; PMCID: PMC6829579.

Noble, T., Young, A., Douglas, C., Williams, B., & Mundree, S. (2019). Diagnosis and management of halo blight in Australian mungbeans: a review. *Crop and Pasture Science, 70*, 195–203. https://doi.org/10.1071/CP18541

Ohwi, J., & Ohashi, H. (1969). Adzuki beans of Asia. *Journal of Japanese Botany, 44*, 29–31.

Pandey, A. K., Burlakoti, R. R., Kenyon, L., & Nair, R. M. (2018). Perspectives and challenges for sustainable management of fungal diseases of mungbean [Vigna radiata (L.) R. Wilczek var. radiata]: A review. *Frontiers in Environmental Science, 6*, 53. https://doi.org/10.3389/fenvs.2018.00053

Pratap, A., Gupta, D. S., Singh, B. B., & Kumar, S. (2013). Development of super early genotypes in greengram (Vigna radiata L. Wilczek). *Legume Research, 36*, 105–110.

Pratap, A., Gupta, S., Basu, S., Tomar, R., Dubey, S., Rathore, M., et al. (2019a). Towards development of climate-smart mungbean: challenges and opportunities. In C. Kole (Ed.), *Genomic designing of climate smart pulse crops*. Springer Nature. https://doi.org/10.1007/978-3-319-96,932-9_5

Pratap, A., Gupta, S., Nair, R. M., Gupta, S. K., Schafleitner, R., Basu, P. S., et al. (2019b). Using plant phenomics to exploit the gains of genomics. *Agronomy, 9*, 126. https://doi.org/10.3390/agronomy9030126

Purseglove, J. W. (1977). *Tropical crops: Dicotyledons, Vols 1 and 2*. The English Language Book Society and Longman Publishers. pp. 273–6, 290–4, 318–2l.

Raina, A., & Khan, S. (2020). Increasing rice grain yield under biotic stresses: Mutagenesis, transgenics and genomics approaches. In C. Aryadeep (Ed.), *Rice research for quality improvement: Genomics and genetic engineering* (pp. 149–178). Springer. https://doi.org/10.1007/978-981-15-5337-0_8

Raina, A., Laskar, R. A., Khursheed, S., Amin, R., Tantray, Y. R., Parveen, K., & Khan, S. (2016). Role of mutation breeding in crop improvement-past, present and future. *Asian Research Journal of Agriculture, 2*(2), 1–13.

Raina, A., Laskar, R. A., Khursheed, S., Khan, S., Parveen, K., & Amin, R. (2017). Induce physical and chemical mutagenesis for improvement of yield attributing traits and their correlation analysis in chickpea. *International Letters of Natural Sciences, 61*, 14–22.

Raina, A., Khursheed, S., & Khan, S. (2018a). Optimisation of mutagen doses for gamma rays and sodium azide in cowpea genotypes. *Trends in Biosciences, 11*(13), 2387–2389.

Raina, A., Laskar, R. A., Jahan, R., Khursheed, S., Amin, R., Wani, M. R., Nisa, T. N., & Khan, S. (2018b). Mutation breeding for crop improvement. In M. W. Ansari, S. Kumar, C. K. Babeeta, & R. K. Wattal (Eds.), *Introduction to challenges and strategies to improve crop productivity in changing environment* (pp. 303–317). Enriched Publications.

Raina, A., Khan, S., Laskar, R. A., Wani, M. R., & Mushtaq, W. (2019). Chickpea (Cicer arietinum L.) cytogenetics, genetic diversity and breeding. In J. M. Al-Khayri et al. (Eds.), *Advances in plant breeding: legumes* (pp. 53–112). Springer. https://doi.org/10.1007/978-3-030-23,400-3_3

Raina, A., Laskar, R. A., Tantray, Y. R., Khursheed, S., & Khan, S. (2020a). Characterization of induced high yielding cowpea mutant lines using physiological, biochemical and molecular markers. *Scientific Reports, 10*, 3687.

Raina, A., Parmeshwar, K., & Khan, S. (2020b). Increasing rice grain yield under abiotic stresses: Mutagenesis, transgenics and genomics approaches. In C. Aryadeep (Ed.), *Rice research for quality improvement: genomics and genetic engineering* (pp. 753–777). Springer. https://doi.org/10.1007/978-981-15-4120-9_31

Raina, A., Sahu, P. K., Laskar, R. A., Rajora, N., Sao, R., Khan, S., & Ganai, R. A. (2021). Mechanisms of genome maintenance in plants: Playing it safe with breaks and bumps. *Frontiers in Genetics, 12*, 675686. https://doi.org/10.3389/fgene.2021.675686

Raina, A., Laskar, R. A., Wani, M. R., Jan, B. L., Ali, S., & Khan, S. (2022a). Comparative mutagenic effectiveness and efficiency of gamma rays and sodium azide in inducing chlorophyll and morphological mutants of cowpea. *Plants, 11*, 1322. https://doi.org/10.3390/plants11101322

Raina, A., Laskar, R. A., Wani, M. R., Jan, B. L., Ali, S., & Khan, S. (2022b). Gamma rays and sodium azide induced genetic variability in high yielding and biofortified mutant lines in cowpea [Vigna unguiculata (L.) Walp.]. *Frontiers in Plant Sciences, 13*, 911049. https://doi.org/10.3389/fpls.2022.911049

Raina, A., Laskar, R. A., Wani, M. R., & Khan, S. (2022c). Plant breeding strategies for abiotic stress tolerance in cereals. In C. Aryadeep (Ed.), *Omics approach to manage abiotic stress in cereals* (pp. 151–177). Springer. https://doi.org/10.1007/978-981-19-0140-9_8

Rasik, S., Raina, A., Laskar, R. A., Wani, M. R., Reshi, Z., & Khan, S. (2022). Lower doses of Sodium azide and Methyl methanesulphonate improved yield and pigment contents in vegetable cowpea [*Vigna unguiculata* (L.) Walp.]. *South African Journal of Botany, 148*, 727–736. https://doi.org/10.1016/j.sajb.2022.04.034

Rosenzweig, C., Elliott, J., Deryng, D., Ruane, A. C., Müller, C., Arneth, A., et al. (2014). Assessing agricultural risks of climate change in the 21st century in a global gridded crop model intercomparison. *Proceedings of the National Academy of Sciences of the United States of America, 111*, 3268–3273. https://doi.org/10.1073/pnas.1222463110

Roxburgh W (1832) *Flora indica; or, descriptions of Indian plants* (Vol. III). W. Thacker and Co., Calcutta and Parbury, Allen and Co.

Salam, S. A., Patil, M. S., & Salimath, P. M. (2009). Evaluation of mungbean cultures against MYMV in Karnataka under natural conditions. *Legume Research, 32*, 286–289.

Schreinemachers, P., Sequeros, T., Rani, S., Rashid, M. A., Gowdru, N. V., Rahman, M. S., et al. (2019). Counting the beans: Quantifying the adoption of improved mungbean varieties in South Asia and Myanmar. *Food Security, 11*(3), 623–634.

Sehgal, A., Sita, A., Kadambot, H. M. S., Kumar, R., Sailaja, B., Varshney, R. K., et al. (2018). Drought or/and heat-stress effects on seed filling in food crops: Impacts on functional biochemistry, seed yields, and nutritional quality. *Frontiers in Plant Science, 9*, 1–19. https://doi.org/10.3389/fpls.2018.01705

Sekar, S., & Nalini, R. (2017). Varietal Screening of Mungbean Genotypes against Whitefly (Bemisia tabaci Genn.), Mungbean Yellow Mosaic Virus (MYMV) and Cercospora Leaf Spot. *International Journal of Current Microbiology and Applied Sciences, 6*(3), 1278–1285.

Sellapillaibanumathi, L., Dhanarajan, A., Raina, A., & Ganesan, A. (2022). Effects of gamma radiations on morphological and physiological traits of finger millet (Eleusine coracana (L.) Gaertn.). *Plant Science Today, 9*(1), 89–95.

Shakeel, S., & Mansoor, S. (2012). Salicylic acid prevents the damaging action of salt in mungbean [(Vigna radiata L.) Wilczek] seedlings. *Pakistan Journal of Botany, 44*, 559–562.

Sharma, L., Priya, M., Bindumadhava, H., Nair, R. M., & Nayyar, H. (2016). Influence of high temperature stress on growth, phenology and yield performance of mungbean (Vigna radiata (L.) Wilczek) under managed growth conditions. *Scientia Horticulturae, 213*, 379–391. https://doi.org/10.1016/j.scienta.2016.10.033

Shukla, V., Baghel, S., Maravi, K., & Singh, S. K. (2014). Yield loss assessment in mungbean [*Vigna radiata* (L.) Wilczek] caused by anthracnose [*Colletotrichum truncatum* (schw.) Andrus and moore]. *The Bioscan, 9*, 1233–1235.

Singh, D. P. (1997). Tailoring the plant type in pulse crops. *Plant Breed, 67*, 1213–1220.

Singh, J., Mishra, K. K., & Singh, A. K. (2013). Current status of web blight of mungbean. *Asian Journal of Soil Science, 8*, 495–504.

Somta, P., Ammaranan, C., Peter, A. C. O., & Srinives, P. (2007). Inheritance of seed resistance to bruchids in cultivated mungbean (Vigna radiata L. Wilczek). *Euphytica, 155*, 47–55. https://doi.org/10.1007/s10681-006-9299-9

Sudha, M., Karthikeyan, A., Anusuya, P., Ganesh, N. M., Pandiyan, M., Senthil, N., et al. (2013). Inheritance of resistance to mungbean yellow mosaic virus (MYMV) in inter and intra specific crosses of mungbean (Vigna radiata). *American Journal of Plant Sciences, 4*, 1924–1927. https://doi.org/10.4236/ajps.2013.410236

Sun, S., Zhi, Y., Zhu, Z., Jin, J., Duan, C., Wu, X., et al. (2017). An emerging disease caused by Pseudomonas syringae pv. phaseolicola Threatens mungbean production in China. *Plant Disease, 101*, 95–102. https://doi.org/10.1094/PDIS-04-16-0448-RE

Takahashi, Y., Muto, C., Iseki, K., Naito, K., Somta, P., Pandiyan, M., Natesan, S., & Tomooka, N. (2018). A new taxonomic treatment for some wild relatives of mungbean (Vigna radiata (L.) Wilczek) based on their molecular phylogenetic relationships and morphological variations. *Genetic Resources and Crop Evolution, 65*, 1109–1121.

Talekar, N. S. (1988). Biology, damage and control of bruchid pests of mungbean. In S. Shanmugasundaram & B. T. McLean (Eds.), *Mungbean: Proceedings of the second international symposium* (pp. 329–342). AVRDC.

Talekar, N. S. (1990). *Agromyzid flies of food legumes in the tropics* (p. 299). Wiley Eastern Limited.

Tang, D., Dong, Y., Ren, H., Li, L., He, C., et al. (2014). A review of phytochemistry, metabolite changes, and medicinal uses of the common food mung bean and its sprouts (Vigna radiata). *Chemistry Central Journal, 8*, 4.

Tantray, A. Y., Raina, A., Khursheed, S., Amin, R. U., & Khan, S. A. (2017). Chemical mutagen affects pollination and locule formation in capsules of black cumin (Nigella sativa L.). *International Journal of Agricultural Science, 8*(1), 108–117.

Tomooka, N., Lairungruang, C., Nakeeraks, P., Egawa, Y., & Thavarasook, C. (1992). Development of bruchid-resistant mungbean using wild mungbean germplasm in Thailand. *Plant Breed, 109*, 60–66. https://doi.org/10.1111/j.1439-0523.1992.tb00151.x

Verdcourt, B. (1970). Studies in the Leguminosae Papilionoideae for the flora of tropical east Africa IV. *Kew Bull, 24*, 507–569.

Wani, M. R., Dar, A. R., Tak, A., Amin, I., Shah, N. H., Rehman, R., Baba, M. Y., Raina, A., Laskar, R., Kozgar, M. I., & Khan, S. (2017). Chemo-induced pod and seed mutants in mungbean (Vigna radiata L. Wilczek). SAARC. *Journal of Agriculture, 15*(2), 57–67.

Wani, M. R., Laskar, R. A., Raina, A., Khan, S., & Khan, T. U. (2021). Application of chemical mutagenesis for improvement of productivity traits in lentil (Lens culinaris Medik). *Annals of Biology, 37*(1), 69–75.

War, A. R., Murugesan, S., Boddepalli, V. N., Srinivasan, R., & Nair, R. M. (2017). Mechanism of resistance in mungbean [Vigna radiata (L.) R. Wilczek var. radiata] to Bruchids, Callosobruchus spp. (Coleoptera: Bruchidae). *Frontiers in Plant Science, 8*, 1031. https://doi.org/10.3389/fpls.2017.01031

Watanasit, A., Ngampongsai, S., & Thanomsub, W. (2001). *The use of induced mutations for mungbean improvement*. Report of an FAO/IAEA seminar on mutation techniques and molecular genetics for tropical and subtropical plant improvement in Asia and the Pacific Region. October 11–15, 1999, in The Philippines, (pp. 11–12).

Wongpiyasatid, A., Chotechuen, S., Hormchan, P., & Srihattagum, M. (1999). Evaluation of yield and resistance to powdery mildew, cercospora leaf spot and cowpea weevil in mungbean mutant lines. *Kasetsart Journal (Natural Science), 33*, 204–215.

Yadav, G. S., & Dahiya, B. (2004). Performance of mungbean genotypes against whitefly and yellow mosaic. *Annales Biologiques, 20*, 57–59.

Yao, Y., Cheng, X., & Ren, G. (2015). A 90-day study of three bruchid-resistant mungbean cultivars in Sprague–Dawley rats. *Food and Chemical Toxicology, 76*, 80–85. https://doi.org/10.1016/j.fct.2014.11.024

Ye, H., Liu, S., Tang, B., Chen, J., Xie, Z., Nolan, T. M., et al. (2017). RD26 mediates crosstalk between drought and brassinosteroid signalling pathways. *Nature Communications, 8*(14), 573. https://doi.org/10.1038/ncomms14573

Yi-Shen, Z., Shuai, S., & FitzGerald, R. (2018). Mung bean proteins and peptides: nutritional, functional and bioactive properties. *Food & Nutrition Research, 62*. https://doi.org/10.29219/fnr.v62.1290. Published 2018 Feb 15.

# Mutation Breeding for Adaptation to Climate Change in Seed Propagated Crops


**J. G. Manjaya and S. K. Gupta**

**Abstract** The expected world population by 2050 will be 9.9 billion, and therefore, agricultural research efforts need to be concentrated on increasing agriculture productivity. Climate change is directly or indirectly responsible for many biotic and abiotic stresses. The biotic stresses include infestations of pests, diseases, and weeds, as well as abiotic stresses like drought, flooding, temperature variation, salinity, and metal toxicity, which limit crop growth and productivity. Developing new crop varieties with high yields coupled with improved plant architecture, a short maturity period, resistance to biotic and abiotic stresses, and better adaptability to climate change is the need of the hour. It means multi-objective breeding strategies need to be followed for developing crop varieties with better adaptability to climate change. The induced mutation experiments have contributed immensely to increasing crop productivity globally. The release of 3332 mutant cultivars in 228 crop species has played an important role in increasing cultivation area and thereby improving crop yields. All the mutant varieties are improved for increased yield and yield components, and resistance or tolerance to biotic and abiotic stress. A large majority of released mutant varieties have traits suitable to face the challenges of climate change and have contributed positively to sustaining crop yields, resulting in positive economic impacts.

**Keywords** Crop improvement · Climate change · Induced mutations · Mutant varieties · Biotic stress · Abiotic stress

J. G. Manjaya (✉) · S. K. Gupta
Homi Bhabha National Institute, Mumbai, India

Nuclear Agriculture and Biotechnology Division, Bhabha Atomic Research Centre, Mumbai, India
e-mail: manjaya@barc.gov.in



A. Raina et al. (eds.), *Advanced Crop Improvement, Volume 2*,
https://doi.org/10.1007/978-3-031-26669-0_8

## 1 Introduction

The improved productivity, stability, and sustainability of major cropping systems are responsible for sustained food and health security. The expected world population by 2050 will be 9.9 billion (UNFPA, 2012), and therefore agricultural research efforts need to be concentrated on increasing agriculture productivity (Conway, 1999). However, alteration in climate and weather conditions strongly affects agriculture, leading to heavy losses for farmers and the nation's economy. Temperature and rainfall play an important role in agriculture, and drastic changes in both components affect crop growth and productivity. The climatic variations include the total distribution of climate across the region, seasonal changes within the climate, and global climate changes, which have an impact on cropping systems throughout the globe (UNEP, 2002). Due to climatic variations, changes in cropping time and season, the maturity period, more infestations of diseases and pests, poor food and feed quality, and a tremendous reduction in agriculture productivity are being observed throughout the globe. Food production utilizes 70% of water and is also considered to be responsible for 30% of global greenhouse gas emissions causing climate change (Parry et al., 2005). The overuse of fossil fuels and deforestation are also responsible for the greenhouse effect and increase in global warming arising as a result of the emission of dangerous gases, especially $CO_2$ (Vaughan et al., 2018). Gas emissions, UV-B, and various factors are responsible for abiotic stresses. Plant growth and crop productivity are highly affected by abiotic stresses like heat, cold, drought, floods, and salinity (Ronald, 2011; Tester & Langridge, 2010). The changes in temperature and rainfall also enhance the growth, and survival of crop pathogens (Rosenzweig et al., 2001). The menace of weeds spreading and the evolution of herbicide-resistant weeds arising due to climate change also pose detrimental effects on agriculture productivity (Matzrafi et al., 2016). The crop varieties developed for a particular region will lose their identity because of decreased productivity due to shifts in rainfall patterns, rising temperatures, and increased infestations of diseases and pests. The development of new crop varieties having high yields coupled with improved plant architecture, a shorter maturity period, and resistance to biotic and abiotic stresses with better adaptability to climate change is the need of the hour. It means multi-objective breeding strategies need to be followed for developing crop varieties with better adaptability to climate change (Akdemir et al., 2019). The approach of Climate-Smart Agriculture (CSA) needs to be implemented to increase productivity, enhance resilience, and reduce emissions to overcome problems arising because of climate change (Lipper et al., 2018). Different cultural approaches, like changes in agronomic practices (crop rotation, irrigation, planting dates, fertilizer application), cultivation of short duration crops, and biotic and abiotic tolerant varieties, have been adapted to combat climate change (Raza et al., 2019). However, the best approach to overcoming the complexity of climate change is to generate diversity by using different plant breeding methods (Ceccarelli & Grando, 2020). In the past, different plant breeding methods have helped to increase the diversity of crop germplasm by developing genetically better varieties for cultivation. However, to meet the global food demand, existing germplasm resources may not be adequate

(Tester & Langridge, 2010; Shiferaw et al., 2013). Genetic improvement of crop plants is a continuous endeavor, and the success of a crop improvement program depends on the availability of large genetic variability, which a plant breeder can combine to generate new varieties (Holme et al., 2019). There are large numbers of instances in the past, where naturally occurring mutations served an important role in crop improvement. The introduction of spontaneous or natural mutants into breeding programs was the success behind the Green Revolution. However, spontaneous mutations are not adequate for their use in intensive breeding programs, and the same can be enhanced severalfolds by using ionizing radiation or chemical mutagens (Muller, 1928; Stadler, 1928). After the discovery of the mutagenic effects of X-rays on plants by Stadler, mutation breeding techniques gained popularity among plant breeders throughout the world. The role of induced mutations is well proven in creating genetic variability for a specific genetic trait that is not immediately available for breeding programs (Jankowicz-Cieslak et al., 2017; Roychowdhury & Tah, 2013; Khursheed et al., 2019; Raina et al., 2020). The widespread use of mutation breeding techniques in 228 crop species has resulted in the development and release of more than 3332 mutant cultivars around the globe [Mutant Variety Database (MVD) (http://mvd.iaea.org)]. Induced mutagenesis is the most efficient technique to greatly increase genetic variation in a short period of time and has been employed in various crops such as cowpea (Rasik et al., 2022), lentil (Laskar et al., 2018a; Wani et al., 2021), faba bean (Khursheed et al., 2016, 2017), fenugreek (Hasan et al., 2018), mungbean (Wani et al., 2017), urdbean (Goyal et al., 2019a, b), chickpea (Laskar et al., 2015; Raina et al., 2019), black cumin (Tantray et al., 2017, Amin et al., 2019), and finger millet (Sellapillai et al., 2022; Sellapillaibanumathi et al., 2022). Because natural mutations occur sporadically, artificial mutations are generated, and genetic gain is best achieved by using mutagens (Raina & Khan, 2020). The mutant varieties are known for improved plant architecture, better quality, increased yield, and resistance/tolerance to biotic and abiotic stresses (Raina et al., 2018a, b). The detailed review of the contribution and global impact of mutation-derived varieties is well documented (Ahloowalia et al., 2004; Kharkwal & Shu, 2009; Mba et al., 2012; Raina et al., 2016; Sarsu et al., 2020). Most of the mutant varieties are well adapted to climate change and have helped to improve the socioeconomic status of the farmers and the national economy by generating additional revenue (Sarsu et al., 2020). In this review, the worldwide achievements and contributions of induced mutations for adaptation to climate change in seed propagated crops for sustainable agriculture and food security are presented.

## 2 Impact of Climate Change on the Productivity of Seed Propagated Crops

Changes in weather conditions and increases in the occurrence of extreme events are being felt more often. The Earth's climate continues to warm, and all the simulation models predict a global trend toward warmer temperatures (Lean & Rind, 2009).

A change in climate poses significant risks to future crop productivity due to global change in atmospheric $CO_2$ or ozone level, variations in average temperature, heat waves, annual rainfall, fluctuations in sea level, and alterations in pests and weeds. The excessive utilization of fossil fuels and deforestation resulted in an increase in $CO_2$ in the atmosphere, and a two-fold increase is predicted by the end of this century. The increase in $CO_2$ concentration in the atmosphere is the main factor responsible for the greenhouse effect and increased global temperatures (Vaughan et al., 2018). The factors responsible for abiotic stresses are gas emissions ($CO_2$, $O_3$, and $CH_4$), light intensities, UV-B, which cause drought, water logging, extreme heat and cold, and salinity, which greatly influences plant growth and yield (Ashraf et al., 2018). The moisture stress caused by low rainfall or a delay for a longer period of time may lead to a drought situation, whereas high rainfall will cause flooding. The different kinds of crop cultivation and methods of farming generally depend upon the climatic conditions and the availability of inputs prevailing in that particular region. Climate change is directly or indirectly responsible for many abiotic stresses like drought, flooding, temperature variation, salinity, and metal toxicity. These abiotic stresses are the major limiting factors for crop growth and productivity. The rate of photosynthesis increases because of the higher concentrations of $CO_2$ in $C_3$ plants such as wheat, rice, and soybean (Deryng et al., 2016). However, the additive effect of the increased $CO_2$ concentration will have a negative impact on plant growth and yield (Senapati et al., 2019). The increase in highly reactive oxidants $O_3$ in the climate affects crop yield by causing decreased photosynthesis and accelerated senescence and cell death (Vandermeiren et al., 2009). All areas of cropland are projected to experience some degree of warming, but the largest change in warming is projected in the northern hemisphere as compared to the southern hemisphere (Foster & Rahmstorf, 2011). Low and high temperatures both affect crop growth by disturbing various physiological and biochemical processes in plants (Paulsen, 1994).

The crop productivity is expected to increase for cereals and temperate crops at mid- and high altitudes (Olesen et al., 2007). Higher temperatures reduce the photosynthesis and maturity period, leading to reduced biomass and grain yield. Increased temperatures also cause a higher rate of evapotranspiration, thereby reducing the moisture content of the soil (Liu et al., 2019). In cereals like rice, wheat, and sorghum, increased temperatures cause a reduction in the number of spikes, grain per spike, and seed size (Fahad et al., 2017). High temperatures also affect the quality of starch in cereals, oil in oilseeds, and protein in pulse crops. The crops respond differently to the temperature fluctuations, and a correlation is observed between climate change and yield losses. Global warming has resulted in annual combined losses of 40 million tonnes or US$5 billion since 1981 (Lobell & Field, 2007). Drought and high temperatures are key stress factors with a high impact on yields, and in a warmer climate, the demand for water by plants is more due to excess water loss by evapotranspiration, creating an overall soil water deficit and reducing water absorption (Heckathorn et al., 2013). Climate change is also responsible for the excess and sporadic events of flooding in the last few years. The instances of floods have increased by 50% in the last ten years, as reported by European Academies' Science Advisory Council (EASAC). Excess floods resulted

in rising sea levels, which will ultimately lead to a decline in agricultural lands in coastal regions and also cause salinity of soils and decreased respiration, photosynthesis, and transpiration in plants. Drought and salinity cause cellular dehydration, resulting in poor crop yields (Tester & Bacic, 2005). In seed propagated crops, approximately 59% of the crop production generally results from organs that are meant for sexual reproduction. The moisture stress at flowering causes a decrease in photosynthesis per plant and affects the grain yield and production. A cereal crop like rice is highly sensitive to water stress at flowering. Reduction in yield of up to 2–3 times as compared to average yield is reported in maize because of water stress at the flowering stage. Grain legumes are also sensitive to moisture stress at flowering (soybean) and early reproductive stages (common bean). Flooding is another abiotic stress that causes anoxia or hypoxia, $CO_2$ deficiency, and the accumulation of toxins in soils. In irrigated soils, salinity is another major limitation for crop growth and productivity, and many crops are susceptible to salinity. High rainfall during the winter season causes more infection of diseases in rapeseeds (Sharif et al., 2017). In China and Bangladesh, high rainfall during the harvest period causes crop damage and economic losses to the farmers. Flooding increases soil moisture content and ultimately disturbs the sowing season (Xu et al., 2013). Another important abiotic stress is heavy metal toxicity, which inhibits crop growth and productivity. All these stresses affect plant growth in different ways but are interrelated, and their severity is directly or indirectly controlled by climate change. Pests, diseases, and vectors of crops are likely to be altered by climate change, and it can also affect the distribution, population size, and impacts of pests and diseases on food production (Pangga et al., 2011) by changing the biology of pests and diseases (Latham et al., 2015). Climate change and increased $CO_2$ concentration also indirectly impact crops through the effects of biotic stresses. The severity of diseases may also be affected by a changing climate. An elevated level of $CO_2$ also causes an increase in the population of aphids (Newman, 2003) and weevil larvae (Staley & Johnson, 2008). The migration patterns of locusts in sub-Saharan Africa were supposed to be influenced by rainfall patterns (Cheke & Tratalos, 2007). To tackle the menace of these stresses, crop management is one of the solutions, which requires proper knowledge and guidance. The best way is to develop plants' resistance to biotic and abiotic stresses.

## 3 Mutation Breeding for Adaptation to Climate Change

### *3.1 Mutations for Quantitative Traits*

The fluctuations in global temperature, low and high rainfall, insect pest infestations, salinity (Dhankher & Foyer, 2018), and heavy metal toxicity will affect plant growth and production. The foremost objective of any crop improvement program is to increase crop yields, which ultimately depend on other quantitative

characteristics like plant height, number of branches, number of pods per plant, number of seeds per pod, 100/1000 seed weight, and harvest index. The induced mutation experiments have contributed immensely to increasing crop productivity globally (Khursheed et al., 2018a, b, c; Laskar et al., 2019; Raina et al., 2022a, b, c). The release of 3332 mutant cultivars in 228 crop species has played an important role in increasing cultivation area and thereby improving crop yields. These mutant varieties are improved for different traits such as agronomic and botanic traits (2981), quality and nutrition traits (1173), increased yield and yield components (1029), resistance to biotic stress (557), and tolerance to abiotic stress (248) (Sarsu et al., 2020). The mutation breeding technique is a powerful tool in the hands of the plant breeder to generate variability and has created variability for traits like improved plant architecture, increased yield, improved quality, and resistance to biotic and abiotic stress (Table 1). The mutant varieties are well adapted to the drastic effects of climate change and have immensely helped to increase productivity and the economy worldwide (Goyal et al., 2021a, b; Khursheed et al., 2015; Laskar et al., 2018b; Raina et al., 2017).

### 3.1.1 Yield and Yield Components

Asia

Climate change has also threatened agriculture and food security in Asia. Agricultural production in China is highly influenced by extreme weather conditions. To overcome the problem of climate change, many mutant varieties are released in Asia, which account for more than 60% of induced mutations and mutation-derived varieties. The Asian countries that are pioneers in commercializing a maximum number of mutant varieties are China, Japan, India, Bangladesh, Pakistan, Vietnam, Republic of Korea, and Indonesia (Sarsu et al., 2020). All cropping regions in China are highly affected by different types of abiotic stresses due to their wide latitude. In China, changes in the phenology and productivity of winter wheat (Tester & Langridge, 2010) and spring cotton (Paulsen, 1994) were observed due to climate change. The days of reproductive growth and maturity in spring and winter wheat increased in Northwest China (Pangga et al., 2011). However, an increase in area and yield was observed in rice in spite of warming (1.43 °C) in the last century (Latham et al., 2015). In China, the wheat mutant varieties Luyuan 502, Taikong 5, Zhengmai 3596, Fumai 2008, Yufeng 11, Zhengpinmai 8, Yutong 843, and Fumai 2008 are known for their wide adaptability, high productivity, and stable yield. In Japan, 479 mutant varieties have been developed and registered and some of the rice mutant varieties Akihikari, Reimei, Kinuhikari, Haenuki, and Tsugaru-roman, are known for their high yield and are widely cultivated (Nakagawa, 2018). Indian agriculture is solely dependent on the climate, and drastic changes in the climate will have a negative impact on crop production. The effects of climate change, like warmer temperatures, may have an advantage for some crops, but the disadvantages will likely be greater. It is expected that by 2080, agriculture productivity in India

**Table 1** Popular mutant varieties of seed propagated crops suitable for climate change

| Country | Crop | Mutants | Characters | References |
|---|---|---|---|---|
| Asia | | | | |
| China | Rice | Yuanfengzao | Early maturity | Wang (1991) |
| | | Zhefu 802 | Resistance to rice blast and tolerance to cold | Christov et al. (2014) |
| | Wheat | H6756 | Salt tolerance | Liu et al. (2019) |
| | | Luyuan 502 | Sprouting-resistant, lodging-resistant, more tolerant of drought and main diseases | Liu (2021) |
| Japan | Rice | Reimei | Semidwarf | Nakagawa (2021) |
| | | Kinuhikari | Semidwarf | |
| | Soybean | Ryuhou | Early maturing | |
| India | Rice | PNR-381 and PNR-102 | Early maturing | Chakrabarti (1995) |
| | | CRM 2007–1 | Semidwarf | Patnaik et al. (2006) |
| | Wheat | Sharbati | Early maturity | Chopra (2005) |
| | | HW 1095 | Non-lodging habit and non shattering resistance to black (stem), yellow, and brown (leaf) rusts | Nirmalakumari et al. (2010) |
| | Groundnut | TAG 24 | Semidwarf, small, dark green thick leaves, earliness, high harvest index, high partitioning %, wider adaptability | Badigannavar et al. (2020a, b) |
| Vietnam | Rice | TNDB100 | Early maturity | Do (2009) |
| | Soybean | DT84 | Resistance to rust, tolerance to high and winter low temperature | Le & Pham (2021) |
| | | DT90 | Tolerance to canopy, resistance to powdery winter mildew, lodging | |
| | | DT99 | Resistance to rust, tolerance to high and winter low temperature | |
| | | DT2008 | Drought tolerance and resistance to rust, downy mildew, powdery mildew, and bacterial pustule | |
| Malaysia | Rice | MR219-4 | Blast and drought resistant | Ibrahim (2018) |
| | | MR219-9) | Blast and drought resistant | |
| | Groundnut | KARISMA sweet | Resistant to *Cercospora* leaf spot disease | |
| | | KARISMA serene | Resistant to *Cercospora* leaf spot disease | |

(continued)

**Table 1** (continued)

| Country | Crop | Mutants | Characters | References |
|---|---|---|---|---|
| Thailand | Rice | RD 6 | Early maturity | Ahloowalia et al. (2004) |
| | | RD 15 | Early maturity | |
| Myanmar | Rice | Shwe war Tun | Rainfed lowland area | Khin (2006) |
| Sri Lanka | Rice | MI 273 | Drought tolerant | Parasuraman and Weerasinghe (2021) |
| | | BW 372 | Moderately tolerant to blast, bacterial leaf blight, brown planthopper, gall midge, and iron toxicity | |
| | Sesame | Malee | Resistant to Phytophthora blight | |
| Indonesia | Sorghum | Pahat | Early maturing, semidwarf resistant to lodging in a strong wind | Human & Indriatama (2020) |
| Europe | | | | |
| Czechoslovakia | Barley | Diamant | Short stature, lodging resistance | Kharkwal and Shu (2009) |
| France | Barley | Betina | Mildew resistance | Ahloowalia et al. (2004) |
| Bulgaria | Maize | Kneja 509 | Drought tolerant | Christov et al. (2014) |
| Brazil | Rice | SCS118 marques | Herbicide resistant | Livore et al. (2018) |
| Italy | Wheat | Creso | Resistance to lodging and resistance to leaf rust, | Ahloowalia et al. (2004) |
| USA | Rice | Calrose 76 | Semidwarf | Van Harten (1998) |
| | Barley | Luther | Short stature, lodging resistance | |
| | Wheat | Stadler | Early maturity, resistant to loose smut and leaf rust | |
| Latin America | | | | |
| Bulgaria | Maize | Kneja 509 | Drought tolerant | Sarsu et al. (2020) |
| Peru | Amaranth | Centenario | Cultivated at high altitude (3000 m above sea level) | Ahloowalia et al. (2004) |
| | Barley | UNA La Molina 95 | Early maturing, suitable for high altitude (5000 m above sea level) | Gomez-Pando et al. (2009) |
| | | Centenario | Cultivated at high altitude (5000 m above sea level) | Ahloowalia et al. (2004) |
| Cuba | Rice | LP7 | Salt tolerant | Croughan et al. (1996) |
| Africa | | | | |
| Egypt | Rice | Giza 176 | Semidwarf | Badawi (2001) |
| | Rice | Sakha 101 | Semidwarf | |
| Sudan | Groundnut | Tafra-1 | Drought tolerant | Abdalla et al. (2018) |

will be reduced by 40% (IPCC, 2007). The rise of 2.5 °C to 4.9 °C in temperature will reduce yield by 32%–40% in rice and 41%–52% in wheat (GOI 2011). The warmer climate also affects the rainfall pattern, causing drought situations if precipitation is less or causing floods if rainfall is in excess. India has developed about 386 mutant varieties in 62 crops through induced mutagenesis. In cereals, the mutant rice varieties of the PNR series, IIT-48, IIT-60, K-84, Jagannath, Keshari, Sattari (Chakrabarti, 1995), TCDM-1, TKR Kolam, Vikram-TCR (Trombay Chhattisgarh), and CG Jawaphool Trombay are popular among the farmers for their high yield. A high-yielding dicoccum wheat mutant variety, 'HW 1095' having semidwarf stature, being disease resistant, and being nutritionally rich is becoming popular among the farmer's community (Nirmalakumari et al., 2010). In legumes, popular high-yielding mutant varieties are of mungbean (TAP-7, TARM-1, 2, 18, TMB-37, TJM-3, TM-96-2, TM-2000-2), black gram (TAU-1, TAU-2, TPU-4, TU94-2, and TU-40), pigeonpea (TT-6, TAT-10, TT-401, TJT-501, PKV-TARA), cowpea (Khalleshwari (TRC-77-4), TC 901), and groundnut (TG 1, 3, 17, 22, 26, 37A, 38, 47, 51, TGS 1, TAG 24, TKG 19A, TPG 41, TLG 45, TBG 39), are widely grown in different states of India (Badigannavar et al., 2020a, b). The black gram variety TAU-1 and groundnut variety TAG-24 is extensively cultivated and command a major share of the national breeder seed indent. The maturity periods of spring and autumn maize were increased as a result of climate change in Pakistan (Abbas et al., 2017). In Pakistan, 59 high-yielding mutant varieties were found in different crops, and the cotton mutant variety "NIAB 78" covered 80% of the cotton area in the Punjab and Sindh provinces (Haq, 2009). In Bangladesh, 40% of cultivable land is prone to abiotic stresses like salinity, drought, flooding, and temperature fluctuations, and 40% of yield losses is reported due to drought. The rice crop, which is the staple food of Bangladesh, is also susceptible to various biotic stresses (Islam et al., 2021). Bangladesh has released more than 60 plant mutant varieties and mutant varieties of rice, wheat, lentils, chickpeas, peanuts, mustard, sesame, soybean, jute, and tomato, which account for about 8% of its total crop area. The high-yielding rice mutant varieties Binasail, Iratom-24, Binadhan-6, and Binadhan-7 are extensively grown in the country (Azad & Imtiaz, 2012; Jawerth, 2017). The other Asian countries are also under threat from food security due to climate change. In Vietnam, mutant varieties of rice (DT10, VND95–20, TNDB-100, VND95–19, VND99–3, VN212, VN214, OM2717) are known for higher yields, lodging resistance, tolerance to acid soils, salinity, tolerance to biotic stress, short duration, and better nutritional quality (Vinh et al., 2009; Do, 2009; Ham & Xuan, 2018; Khanh et al., 2021). The popular soybean mutant varieties known for higher yield are DT84, DT90, and DT2008 (Le & Pham, 2021). The mutation breeding program in Malaysia resulted in the development of 53 mutant varieties, which include 19 varieties of rice and 2 varieties of groundnuts. The high-yielding mutant varieties of rice (MR219-4 and MR2199, MRQ74) and groundnut (KARISMA Sweet and KARISMA Serene) are grown in a large area by the farmers (Ibrahim, 2018). In Thailand, two aromatic indica glutinous rice varieties, RD6 and non-glutinous early maturing variety RD15, are grown extensively in the north and northeastern regions of Thailand. (Ahloowalia et al., 2004; Kharkwal & Shu, 2009). In Myanmar, four mutant rice varieties

ShweThwe Tun, Shwe War Tun, Thukayin, and Yezin Lone Thwe are known for higher yield stability. In Sri Lanka, mutant varieties of rice (MI 273, BW 372), groundnut (Tissa), and sesame (Malee) are popular among the farmers because of their high yield (Parasuraman & Weerasinghe, 2021). In Indonesia, three high-yielding sorghum mutant varieties Pahat, Samurai-1, and Samurai-2, are extensively cultivated on an estimated area of about 800,000 ha (Human & Indriatama, 2020).

## Europe

The rise in greenhouse gas emissions may enhance the crop yield in North-Western Europe and decrease the crop yield in the Mediterranean area (Olesen & Bindi, 2002). The crop productivity will decrease in southern Europe and the Pannonia zone, which includes Hungary, Serbia, Bulgaria, and Romania, because of low rainfall and extreme temperatures (Olesen et al., 2011). In Europe, the drastic changes in climatic conditions resulted in a reduction of wheat and barley yields by 2.5% and 3.8%, respectively (Moore & Lobell, 2015). The increase in average rainfall was recorded in cooler regions of the UK and Ireland due to warming. The impact of climate changes on yields of cereals like barley and wheat was also observed in Italy, Greece (Mavromatis, 2015), the Czech Republic (Potopova et al., 2017), and Hungary (Pinke & Lovei, 2017)**.** In Europe, the mutation breeding technique has become one of the most important breeding methods since its initiation in the year 1920. The entire 959 mutant varieties released in Europe, especially wheat and barley, are well adapted to climate change (Saxena et al., 2016). To tackle the effect of climate change on crop production, the farmer community of Bolivia modified agronomical practices (Iizumi & Ramankutty, 2016). In maize and soybeans, yield variability has been observed due to climatic changes (Ketiem et al., 2017). In Europe, extensive use of the mutation breeding technique resulted in the release of 959 mutant varieties, particularly in wheat and barley. In Bulgaria, 76 mutant cultivars in different crops like maize, durum wheat, tomato, barley, wheat, soybean, lentil, pepper, sunflower, bean, tobacco, chickpea, vetch, cotton, and pea are released for commercial cultivation. The most widely cultivated maize mutant hybrid varieties are Kneja 509 and Kneja 683A. The high protein mutant hybrids Kneja 556, Kneja HP 633, Kneja HP 556, and Kneja HP 556 occupies about 40%–50% of the maize growing area in Bulgaria and are well-known for silage making. High-yielding durum mutant varieties have significantly increased productivity in Bulgaria. In Bulgarian agriculture, all the mutant varieties have attained a special status because of their high productivity, resistance to biotic stresses, and better quality (Tomlekova, 2010). Italy is well-known for durum wheat research and production among the European Union (EU) countries and has released 22 mutant varieties (Xynias et al., 2020). One of the mutant varieties, Creso, was cultivated in one-third of the total area of durum wheat cultivation in Italy. The barley mutants "Diamant" and "Golden Promise" released in Czechoslovakia have made a major impact on the brewing industry in Europe and were used as parents in the crossing program, where more than 150 cultivars were developed (Kharkwal & Shu, 2009).

### North America

In the USA, the average yields of crops like corn, soybeans, wheat, rice, sorghum, cotton, and oats have decreased due to temperature fluctuations. On the other side, because of the warmer climate, the yields of crops such as wheat and barley are expected to increase as a result of increased rainfall and C fertilization (NSAC, 2019). In the USA, 139 mutant varieties have been developed, and the first semidwarf rice mutant variety, Calrose, was used in the crossbreeding program, and 25 semidwarf varieties were developed in California, Australia, and Egypt. A high-yielding wheat mutant Stadler having early maturity, resistant to loose smut and leaf rust, was cultivated on two million acres annually in the USA. The high-yielding barley mutant varieties, Luther and Pennard, were released in Pennsylvania and was grown in a larger area (Ahloowalia et al., 2004).

### Latin America

The mutation breeding program in 18 Latin American countries developed 53 mutant varieties in different crops for cultivation (Sarsu et al., 2020). In Argentina, the high-yielding rice mutant variety Puita INTA-CL was also successfully grown in other Latin American countries, such as Uruguay, Colombia, Chile, Costa Rica, Panama, the Dominican Republic, Nicaragua, and Honduras. In Brazil, the high-yielding herbicide-resistant rice mutant varieties, SCS118 Marques and Clearfield rice, were successfully grown in a large area (Livore et al., 2018).

### Africa

In total, 82 mutant varieties are released in different countries in Africa (Sarsu et al., 2020). In Egypt, semidwarf mutant rice varieties, Giza 176 and Sakha 101, were released during the 1990s, which increased yield levels from 3.8 t $ha^{-1}$ to 8.9 t $ha^{-1}$. The mutant variety Giza 176 is cultivated as the most popular and promising variety, having a yield of 10 t $ha^{-1}$. Five sesame varieties and two safflower varieties were developed with high yield and good quality, contributing to higher income for farmers in Egypt (Badwai, 2001). Application of induced mutation techniques toward crop improvement in Ghana for the last two decades has helped develop a cassava mutant variety with high dry matter content (40%), Tekbankye. This mutant variety is tolerant of the Africa Cassava Mosaic Virus (ACMV), and it is used to prepare fufu, the nation's most popular cassava-based food (Kharkwal & Shu, 2009). The Ministry of Agriculture, Water and Forestry of Namibia developed four sorghum and seven cowpea varieties with 10–20% higher yields than local cultivars under drought conditions and pre-released them to farmers (Abdalla et al., 2018). In Sudan, successful breeding programs using nuclear techniques and plant biotechnologies were started to enhance the productivity of cereal crops, bananas, tomatoes, and groundnuts in stressful environments to ensure sustainable food security.

The research resulted in the release of the banana variety "Albeely," which has a high yield and is widely cultivated by farmers in banana production areas along the Blue Nile in the south of Wad Medani. A drought-tolerant peanut mutant variety, "Tafra-1," was released for Sudanese farmers in drought-prone areas, which improved their livelihoods and led to an increase in the country's exports (Sarsu et al., 2020; Abdalla et al., 2018).

### 3.1.2 Mutations for Early Maturity

Drought can affect the vegetative, pre-anthesis, and post-anthesis stages of plant growth with varying degrees of severity. The development of varieties with an early flowering time and early maturity traits can escape drought conditions at the terminal stage by completing their life cycle faster (Dolferus, 2014). Several early maturing mutants have been identified and released as direct varieties or used in a breeding program. The high-yielding, early-maturing mutant rice variety, Zhefu 802, was developed by the Institute of Nuclear Agricultural Sciences at Zhejiang Agricultural University in 1981. It was widely cultivated in an area of about 10.6 million ha. In Japan, the early maturity soybean mutant variety Raikou was developed by gamma-ray irradiation in 1960. It was the third ruling variety in Japan and was cultivated on 10,548 ha. The early maturing, aromatic mutant rice varieties, PNR-381 and PNR-102 developed in India were very popular among farmers. The mutant variety Sharbati, because of its earliness coupled with the desirable grain color is reported to have been cultivated on large acreage in India (Chopra, 2005). The mutant variety TNDB-100 from Vietnam was developed for improved characteristics like early maturity, reduced height, resistance to pests and diseases, and good cooking quality. The variety was grown on a larger scale and was most preferred by the farmers since they could take three crops because of its early maturity. In Thailand, the gamma-ray-induced early maturing mutant variety, RD-15, was extensively cultivated.

### 3.1.3 Mutations for Improved Plant Architecture

Crop cultivars with high yield potential are also affected by climate change, resulting in decreased crop productivity. One of the approaches to tackling the negative effects of climate vagaries is the modification of plant architecture for increasing crop productivity (Goyal et al., 2020a, b; Raina et al., 2021). For example, tall genotypes are susceptible to lodging, which results in crop damage and a loss of crop yield. The development of reduced plant height varieties by incorporating a recessive gene (SD-1) for reduced height has resulted in an increase in crop production in rice. These semidwarf genotypes possess morphological traits that are responsible for proper light interception and assimilate portioning resulting in higher yield (Donald, 1968). Numerous mutant varieties of seed propagated crops like cereals, pulses, oilseeds, and industrial crops registered globally have modified plant architecture and are well adapted to the challenges of climate change (Table 1). The first

gamma ray-induced semidwarf rice mutant variety, Reimei, developed in Japan, recorded the highest yield (Futsuhara, 1968). The contribution of the semidwarf rice mutant variety Reimei to increasing rice production in Japan is significant. More than 99 mutant rice varieties have been developed using the mutant variety Reimei, and these varieties occupied 12.4% of total rice cultivation in Japan. In India, recently released thigh-yielding mutant rice varieties, TCDM-1, TKR Kolam, and Vikram-TCR, were released for cultivation and are having dwarf plant stature and tolerance to lodging. The other important semidwarf rice mutant variety, CRM 2007-1, is early and recorded a high yield of up to 6.2 t/ha (Chakrabarti, 1995). The groundnut variety TAG 24 from India has wider adaptability due to its semidwarf habit, high harvest index, tolerance to late leaf spots and bud necrosis (Patil et al., 1995), acid soils (Basu, 1997), and better water-use efficiency (AC1AR 1995). The sorghum mutant variety Pahat from Indonesia is semidwarf (148 cm) and resistant to lodging in strong winds and can be used to overcome the adverse effects of climate change. The mutant variety Giza 176 from Egypt has high yield potential, a short stature, and a medium growth duration best suited for cultivation to avoid lodging. The spring barley gamma-ray mutant Diamant possesses reduced stalk length, higher tillering, and high yield, and was developed in Czechoslovakia. It was used as a parent in the crossing program due to its yield stability and short stature, and more than 113 cultivars were developed in different parts of the world. In the USA, the first semidwarf gamma-ray induced mutant rice variety, Calrose 76, was developed in 1976 and was used in crossbreeding, and a number of varieties were developed in California, Australia, and Egypt. The dwarf gene was integrated into the tall Japonica types, and the resulting varieties gave 14% more yield than the tall varieties. The first USA barley mutant, Luther, a six-row, reduced height, and lodging resistant winter barley, was developed from chemical mutagenesis (Diethyl sulfate), which gave a 20% higher yield.

## *3.2 Mutations for Abiotic Stresses*

Climate change is directly or indirectly responsible for many abiotic stresses like drought, flooding, temperature variation, salinity, and metal toxicity. All these abiotic stresses affect plant growth in different ways but are interrelated to one another, and their severity is directly or indirectly controlled by climate change. To tackle the menace of these abiotic stresses, crop management is one of the solutions, which requires proper knowledge and guidance. The best way is to develop the plant's resistance to abiotic stresses. The mutation technique was successfully used to develop several mutant varieties resistant to various abiotic stresses (Table 1). The cold-tolerant mutant varieties of rice, Zhefu 802 (China), soybean DT 84, and DT 99 (Vietnam) were released for commercial cultivation. The salt-tolerant mutant (H6756) variety of wheat was developed in China. Several drought-tolerant mutant varieties of wheat, maize, and groundnut have been released globally. The barley mutant variety Centenario is grown at altitudes of about 5000 m above sea level

(Gomez-Pando et al., 2009), and similarly, the amaranth mutant variety Centenario is also cultivated at higher altitudes (Gomez-Pando et al., 2009).

In addition to the above popular mutant varieties, the identification of several new mutant lines resistant or tolerant to various abiotic stresses like drought, heat, and cold, salt, cadmium, aluminum, arsenic, and herbicide have been reported (Table 2). These mutants were developed using various physical (gamma rays, nitrogen ion beams, fast neutrons, heavy ion beams) and chemical (EMS, MNH, ENU, SA) mutagens. All these mutant lines can be valuable genetic stock for the improvement of seed propagated crops to overcome the situation arising because of climate change.

Zahra et al. (2021) reported the identification of five drought-resistant wheat mutants after treating the cultivated wheat variety NN-Gandum-1 by EMS. The selected mutants under drought stress increased the accumulation of proline content, total soluble sugars, and total free amino acids, while decreasing total chlorophyll content, carotenoids, and total soluble protein. The identification of moisture stress-tolerant wheat mutant RYNO3936 derived after treating a red hard winter wheat cultivar, Tugela DN, by chemical mutagen sodium azide was reported by Le Roux et al. (2020). Another mutant BIG8–1 was also isolated from the parental line (WT) BIG8 by chemical mutagenesis (EMS) (Le Roux et al., 2021). Heat stress at the flowering and grain-filling stages seriously affects spikelet fertility and grain quality in rice. Heat-tolerant mutants M9962, M3181, and M7988 were isolated from the variety Jao Hom Nil (JHN) after irradiating with 33 Gy fast neutrons. The mutants had high spikelet fertility of 70%–78% under heat stress (Cheabu et al., 2019). Drought stress also negatively affects the yield of the maize crops. Zhang et al. (2020) isolated the drought-tolerant maize mutant C7–2t by irradiating the seeds of the maize inbred line ChangC7–2 with gamma rays. The mutant showed higher drought tolerance, delayed wilting, and high water-holding capacity under both controlled and field conditions. In sorghum, ten putative mutant lines were isolated from the cultivar Durra and treated with gamma rays. Out of the ten lines, mutant B-68, B-72, B-95, and B-100 were tolerant to drought and gave yields (4.0 ton/ha) significantly higher than the parent Durra and check varieties (Human & Sihono, 2010). In cowpea, drought-resistant mutant lines 447, 217, MA 2, and 346 were identified after irradiating line IT93K129-4 by gamma rays. The yield performance of the mutant lines 447 and 217 proved to be outstanding under well-watered conditions, whereas lines 447, 217, and 346 performed well under drought stress conditions (Ronde & Spreeth, 2007). Three cowpea mutant lines (MoussaM51-4P10 and MoussaM43-20P14) tolerant to water stress were identified. These gamma-ray-induced mutants showed better stress tolerance and produced a higher yield under water stress conditions (Gnankambary et al., 2020). Ronde and Spreeth (2007) isolated six gamma-ray-induced mutant lines of cowpea tolerant to drought; one of the mutant lines, 217, performed very well in terms of relative water content, free proline concentration, and yield. Mutant lines 447, 217, and 346 performed well under drought stress conditions. In groundnut cultivars from Sodari and Barberton, lCGV 89,104, lCGV 86,744, lCGV 8674 and lCG 221 were irradiated with different doses of gamma rays, and nine promising mutants showing tolerance to terminal drought

**Table 2** Mutant lines developed for abiotic stress tolerance in seed propagated crops

| Abiotic stress | Crop | Parent | Mutant | Mutagen | Reference |
|---|---|---|---|---|---|
| Water stress | | | | | |
| Drought tolerance | Rice | IAPAR9 | *idr1–1* | Gamma | Zu et al. (2021) |
| | Rice | Khao Dawk Mali 105 | HyKOS22 | Nitrogen ion beam | Khitka et al. (2021) |
| | Rice | Nagina 22 | N22-PDT-17 and N22-PDT-64 | EMS | Manonmani et al. (2020) |
| | Wheat | NN Gandum-1 | – | EMS | Zahra et al. (2021) |
| | Wheat | Pasa | KM14 | Gamma | Naju et al. (2005) |
| | Wheat | BIG8 | BIG8-1 | EMS | Le Roux et al. (2021) |
| | Sorghum | Durra | B-68, B-72, B-94, and B-100 | Gamma | Human and Sihono (2010) |
| | Maize | Chang C7-2 | C7-2t | Gamma | Zhang et al. (2020) |
| | Maize | DR18 | DR18.8 and DR18.5 | Gamma | Ruswandi et al. (2014) |
| | Soybean | Dering 1 | – | EMS | Savitri and Fauziah (2018) |
| | Cowpea | Moussa Local | MoussaM51-4P10 and MoussaM43-20P14 | Gamma | Gnankambary et al. (2020) |
| Temperature stress | | | | | |
| Heat tolerance | Rice | Nagina 22 | NH219 | EMS | Poli et al. (2013) |
| | Rice | Jao Hom nil | M9962 | Fast neutrons | Cheabu et al. (2019) |
| | Wheat | Guardian | Tht | EMS | Mullarkey & Jones (2000) |
| Cold tolerance | Rice | ZY66 | *ltt1* | SA | Xu et al. (2020) |
| Salinity stress | | | | | |
| Salt tolerance | Rice | Wuyunjing7 | *Sdbc* | Heavy-ion beam | Ye et al. (2021) |
| | Rice | TH899 | M89 | Heavy-ion beam | Zhang et al. (2022) |
| | Rice | Bahia | *SaT20*, *SaS62*,and *SaT58* | Gamma and fast neutron | Domingo et al. (2016) |
| | Rice | Nagina 22 | – | EMS | Mohapatra et al. (2014) |
| | Rice | Dongan | ST-495 and ST-532 | Gamma | Kim et al. (2010) |
| | Rice | Hitomebore | *hst1* | EMS | Takagi et al. (2015) |
| | Wheat | BARI Gom 25 | OA42 and OA70 | EMS | Lethin et al. (2020) |

(continued)

**Table 2** (continued)

| Abiotic stress | Crop | Parent | Mutant | Mutagen | Reference |
|---|---|---|---|---|---|
| Metal toxicity | | | | | |
| Cadmium Tolerance | Rice | Zhongjiazao 17 | *cadt1* | EMS | Chen et al. (2020) |
| | Brasica rapa | R-o-18 | BraA.hma4a-3 | EMS | Navarro-León et al. (2019) |
| | Brasica rapa | R-o-18 | BraA.cax1a-12 | EMS | Navarro-León et al. (2020) |
| | Pea | SGE | SGECd$^t$ | EMS | Tsyganov et al. (2007) |
| Aluminium Tolerance | Soybean | Muria | H218 | Gamma | Yuliasti and Sudarsono (2011) |
| | Wheat | IAC-24 and IAC-60 | – | Gamma | Neto et al. (2001) |
| | Barley | Roland | RL819/2 and RL820/6 | MNH and SA | Nawrot et al. (2001) |
| Arsenic tolerance | Rice | Nipponbare | *phf1* | EMS | Chen et al. (2011) |
| Herbicide resistance | | | | | |
| Imidazolinone tolerance | Chickpea | WT F01 | M2033 | EMS | Galili et al. (2021) |
| | Rice | 9311 | JD164 | EMS | Piao et al. (2018) |
| | Wheat | Fidel | FS4 | SA | Newhouse et al. (1992) |
| Imazethapyr tolerance | Rice | Nagina 22 | HTM-N22 | EMS | Shoba et al. (2017) |
| Sulfonylurea tolerance | Soybean | Williams | 1–184A | ENU | Sebastian and Chaleff (1987). |

stress were identified (Sundra, 2006). Four rice mutants with dark green leaves, N22-H-dgl56, N22-H-dgl101, N22-H-dgl219, and N22-H-dgl162, induced by EMS in Nagina22 (N22), maintained higher chlorophyll and carotenoid contents under prolonged drought and heat conditions in the field. It was also observed that these mutants accumulated fewer reactive oxygen species and maintained a higher chlorophyll content than their parents (Panigrahy et al., 2011). In Thailand, rice mutants tolerant to submergence were isolated from the cultivar RD31 after irradiation of seeds with a 0.44 kGy electron beam (Promnart et al., 2017). In Malaysia, three gamma-ray-induced mutant rice lines tolerant to submergence were identified (Ahmad et al., 2020).

Among the abiotic stresses, acid soil and associated aluminum toxicity (Al) affect crop production. In Poland, thirteen barley mutants showing tolerance to Al toxicity were selected after treating the seeds of four barley varieties with N-methyl-N-nitroso urea (MNH) and sodium azide (Nawrot et al., 2001). Similarly, in soybean, AL tolerant mutant lines have been identified (Yuliasti and Sudarsono, 2011).

The heavy metal (HM) cadmium (Cd) is one of the abiotic stresses present in the soil, toxic to plants. It inhibits root and shoot growth, water and nutrient uptake, and it affects photosynthesis. In the pea, a mutant line, SGECdt, with increased Cd tolerance and accumulation, was isolated and characterized after treating seeds of the pea line SGE by EMS. The roots and shoots of the mutant plant showed accumulation of increased Cd concentrations and normal plant growth. The inheritance studies indicated that tolerance to Cd is controlled by the monogenic recessive gene (Tsyganov et al., 2007). Similarly, in soybean (Hirata et al., 2019) and Brassica rapa (Navarro-León et al., 2020), mutant lines tolerant to Cd have been identified.

Andrade et al. (2018) reported the development of rice mutants tolerant to aryloxyphenoxypropionate (APP) herbicides. The mutants were isolated from the gamma rays irradiated population of the cultivar Sabbore and showed normal plant growth. In soybean, four mutant lines were selected as being tolerant to sulfonylurea (SU) herbicides from the population treated with ethylnitrosourea (ENU) (Sebastian & Chaleff, 1987). In wheat, mutants tolerant to imidazolinone are derived from the population through seed mutagenesis of the variety with sodium azide. The mutants showed normal plant growth and yield (Newhouse et al., 1992). Shoba et al. (2017) reported the identification of Imazethapyr herbicide-tolerant rice mutant HTM-N22 (HTM) after treating seeds of an upland rice variety, Nagina 22 (N22) by EMS. In chickpea, mutant line M2033, resistant to imidazolinone herbicides, was obtained from the EMS mutagenized population (Galili et al., 2021).

Kim et al. (2010) reported the identification of two salt-tolerant rice mutant lines, ST-495 and ST-532, from the variety Dongan irradiated with gamma rays. Under salt stress, the mutants had lower malonaldehyde (MDA) contents and normal chlorophyll and carotenoid contents. Two salt-tolerant rice mutants, ST-87 and ST-301, induced by gamma-irradiation were also selected in rice (Song et al., 2012). In wheat, 70 lines were identified showing tolerance to salt stress after treating the seed of BARI Gom-25 by EMS. The selected lines showed a 70% germination rate in 200 mM NaCl and also showed a better salt tolerance phenotype than both BARI Gom-25 and other local wheat varieties.

### *3.3 Mutations for Biotic Stresses*

Climate change can affect the distribution, population size, and impacts of pests and diseases on crops (Paulsen, 1994). Interactions between climate change, crop development, and biotic stresses (Heeb et al., 2019) are well-known. The elevated level of $CO_2$ increases the susceptibility of soybean (Zavala et al., 2008), rice, maize, and wheat (Deutsch et al., 2018) to insect attack. Climate change also affects the adaptation and spread of weeds (Ziska & Dukes, 2010). Development of crop varieties' resistance to pests and diseases and sustainable integrated pest management systems can help to overcome the negative effect of climate change on crop production. A large number of mutant lines resistant to various biotic stresses have been developed in different seed propagated crops (Table 1). In Vietnam, a series of soybean

mutant varieties were developed that had resistance to various diseases. Some of these soybean mutant varieties, like DT84 (resistant to rust), DT90 (resistant to powdery mildew), DT2008 (resistant to rust, downy mildew, and bacterial blight), and DT2008DB (tolerant to rust and downy mildew), have wider adaptability and cover 70% of the cultivated area in different climatic zones of Vietnam. The *Phytophthora* blight disease-resistant mutant variety of sesame was released in Sri Lanka for commercial cultivation and is grown on around a 268-ha area there. The mutant rice variety BW 372, having resistance to blast, bacterial leaf blight, brown planthopper, and gall midge, is widely cultivated in Sri Lanka. The other advanced, high yielding mutant lines, BW 03-1198 and BW 12-574, having resistance to blast and gall midge and moderate resistance to brown planthopper are some of the important lines ready to combat the menace of various pests and diseases arising due to climate change. In Bangladesh, a new chickpea mutant variety, Binasola-9, tolerant to root rot, and Botrytis gray mold, and pod borer, was released for commercial cultivation. It matures in 115 days, gives a yield up to 1.7 t/ha, and is well adapted for Bangladesh's drought-affected areas. In India, several mutant crop varieties resistant to biotic stresses have been released for commercial cultivation (Table 3) (Kharkwal et al., 2004; Jagadeesan & Ganapathi, 2021). In cereals, rice mutant varieties are resistant to diseases like *Helminthosporium,* Bacterial Leaf Blight (BLB), Bacterial Leaf Streak (BLS), blast, sheath blight, neck blast, and insects like the brown planthopper (BPH) and stem borer, wheat mutant varieties are resistant to leaf and stem rust diseases; sorghum mutant varieties are resistant to charcoal rot, Downey mildew, head moulds, and stem borer; and barley mutant varieties are resistant to yellow rust, smut, Molya disease, and Cereal Cyst Nematode (CCN), were released for commercial cultivation. In pulse crops, resistant mutant lines in mungbean (Yellow mosaic virus, powdery mildew, *Rhizoctonia* root rot diseases, *Corynespora* leaf spot; black gram (Yellow mosaic virus, powdery mildew); pigeon pea (*Phytophthora* blight, *Fusarium* wilt disease); cowpea (cowpea mosaic virus) were released. In wheat, stripe rust, also known as yellow rust, is a serious disease detrimental to wheat production. Mutant lines resistant to yellow rust were isolated from the wheat cultivars, PBW343 and HD2967, using gamma ray and electron beam irradiation (Bakshi et al., 2021). The downy mildew disease is one of the diseases that affects the grain yield of the Quinoa crop in Peru. Mutant lines tolerant to downy mildew disease were isolated from the gamma rays irradiated population of the cultivar Amarilla Marangani (Gomez-Pando et al., 2021). In Bulgaria, mutation breeding using chemical mutagenesis (EMS) was undertaken to identify mutants in beans resistant to bacterial blight and halo blight diseases. From two parents, 50 putative mutant lines showing resistance to the above diseases were identified. In Bangladesh, Chickpea mutant, CPM-Kabuli, tolerant to root rot and Botrytis grey mould disease, and greater tolerance to pod borer insect-pest infestation, were isolated from gamma rays irradiated chickpea variety Desi Binasola-2 (Begum et al., 2021). The other important biotic stress is weeds, which compete with crops for space, water, sunlight, and nutrients, ultimately compromising the crop yields. It also chokes up irrigation and drainage channels and acts as a host for various insects and diseases. Manual hand weeding and spraying of herbicide are

**Table 3** Indian mutant crop varieties resistant to biotic and abiotic stresses

| Mutant variety | Mutagen | Important traits |
|---|---|---|
| Cereals | | |
| Rice (*Oryza saliva* L.) | | |
| A U -1 | Gamma rays | Tolerant to alkalinity and salinity |
| Biraj | X-rays | Tolerant to submergence and moderately resistant to *Helminthosporium* |
| CNM-20 | X-rays | Resistant to Bacterial Leaf Blight (BLB), bacterial leaf streak (BLS), and brown planthopper (BPH) |
| CNM-25 | X-rays | Resistant to thrips and early maturity |
| CNM-31 | X-rays | Resistant to BLB, BLS, BPH, brown spot, and early maturity |
| CRM 49 | $NaN_3$ | Resistant to blast |
| CRM 51 | $NaN_3$ | Resistant to blast |
| CRM 53 | EMS | Resistant to blast |
| Hari | Fast neutrons/mutant derivative | Tolerant to bacterial leaf blight and blast |
| HM 95 | Gamma rays/ mutant derivative | Photo and thermo insensitive, early maturity, and dwarf |
| IIT 48 | Ethylene oxide (EO) | Earliness |
| IIT 60 | EMS | Earliness |
| Indira | EMS | Tolerant to blast, BLB, and stem borer |
| Jagannath | X-rays | Earliness |
| Lakshmi | X-rays | Drought tolerant and early maturity |
| Mohan | Gamma rays | Resistant to lodging, salinity, and semidwarf |
| PNR-162 | Mutant derivative | Early maturity and semidwarf |
| Prabhavati | EMS | Resistant to lodging and tolerant to iron chlorosis |
| Rasmi | Gamma rays | Tolerant to salinity |
| Savitri | Mutant derivative | Tolerant to blast and sheath blight |
| TCDM-1 | Gamma rays | Tolerant to bacterial leaf blight, neck blast and stem borer, and semidwarf |
| TKR Kolam | Gamma rays/ mutant derivative | Resistant to lodging |
| Vikram-TCR | Gamma rays | Lodging resistant, drought tolerant, and early maturing |
| Wheat | | |
| NP 836 | X-rays | Resistant to rust |
| HW 1095 | Gamma rays | Resistant to leaf and stem rust diseases |
| Sorghum | | |
| SPV-80 | X-rays | Tolerant to Striga |
| SPV-126 | Mutant derivative | Resistant to charcoal rot, Downey mildew, head moulds, and stem borer |
| Barley | | |
| BH-75 | Mutant derivative | Resistant to yellow rust and cereal cyst nematode (CCN) |

(continued)

**Table 3** (continued)

| Mutant variety | Mutagen | Important traits |
|---|---|---|
| DL-253 | Gamma rays +EMS | Resistant to smut and yellow rust diseases |
| Karan-3 | Mutant derivative | Resistant to lodging and dwarf |
| Karan-4 | Mutant derivative | Resistant to lodging and semidwarf |
| Karan-265 | Mutant derivative | Resistant to lodging and dwarf |
| RD-2035 | RD-137 x PL-IOI | Resistant to CNN and early maturity |
| Rajkiran | Mutant derivative | Resistant to Molya disease |
| Pulses | | |
| French bean | | |
| Pusa Parvati | X-rays, wax podded | Early and resistant to mosaic and powdery mildew |
| Mungbean | | |
| TJM-3 | Gamma rays/ mutant derivative | Resistant to yellow mosaic virus, powdery mildew, and *Rhizoctonia* root rot diseases |
| TM-96-2 | Gamma rays/ mutant derivative | Resistant to *Corynespora* leaf spot, powdery mildew |
| TM-2000-2 | Gamma rays/ mutant derivative | Resistant to powdery mildew |
| Urdbean or blackgram | | |
| TU 94-2 | Gamma rays/ mutant derivative | Resistant to yellow mosaic virus |
| TU-40 | Gamma rays/ mutant derivative | Resistance to powdery mildew |
| Pigeonpea | | |
| TT-401 | Fast neutrons/ mutant derivative | Tolerant to pod borer and pod fly damage |
| TJT-501 | Fast neutrons/ mutant derivative | Tolerant to *Phytophthora* blight |
| PKV-TARA | Fast neutrons/ mutant derivative | Resistant to *fusarium* wilt disease |
| Cowpea | | |
| TC 901 | Gamma rays | Resistant to cowpea mosaic virus |
| Oilseeds | | |
| Groundnut | | |
| TAG 24 | Gamma rays, x rays/ / mutant derivative | Salinity and drought tolerance |
| TG-26 | Gamma rays/ mutant derivative | Iron chlorosis tolerance, salinity tolerance |
| TG 37A | Gamma rays/ mutant derivative | Drought tolerance |
| Soybean | | |
| TAMS 98–21 | Gamma rays | Resistant to *Myrothecium* leaf spot, bacterial pustules, and soybean mosaic virus diseases |
| Mustard | | |
| TPM 1 | Beta | Tolerant to powdery mildew |
| Linseed | | |
| TL 99 | Mutant derivative | Moderately resistant to powdery mildew, Alternaria blight, and resistant to rust |

the methods used to control weeds but are not profitable because of labor charges, which increase production costs. Secondly, the use of herbicides causes soil, air, and water pollution. The development of herbicide-tolerant crops will help to overcome the problems of labor shortage and cost, as well as the negative effect of herbicides on the ecosystem. Several mutant lines tolerant to imazethapyr herbicide (rice and lentil), sulfonylurea herbicides (soybean, wheat), imidazolinines, and sulfonylurea (sunflower) were developed using chemical mutagens (Tan & Bowe, 2012).

## 4 Strategies to Overcome the Negative Impact of Climate Change

Climate change hampers agricultural productivity by disturbing the agro-ecological environment. The rise in global temperatures, variation in rainfall patterns, and increased pressure from biotic and abiotic stresses pose significant risks to agriculture. The various stresses encountered by plants in the field affect crop production. The various biochemical and physiological processes in plant cells are disturbed by stress. The negative impact of climate change can only be managed by climate-smart agriculture. Approaches to tackle situations arising due to climate will have a long-lasting effect on agriculture and food security. Genetic improvement for tolerance of temperature and drought stress is an important component in stabilizing food crop production in the tropics. This will require a combination of agronomy and plant breeding solutions, like altering the time of planting, seedbed preparation, mulching, and irrigation, the development of new ideotypes and early maturing plants, and development of efficient screening techniques. Crops grown in saline and heavy metal toxic soils should be able to use all the available water in the soil and have high water use efficiency. Therefore, the development of genotypes requires high early vigor, a large leaf area canopy to prevent excess evaporation from the soil surface, a deep root system, and good salt excluders. In the present situation of climate change, plant roots play an important role in protecting plant growth through self-modifications. During drought situations, there is inhibition of lateral root growth and an increase in primary and secondary root growth is observed. The characteristics, like deep rooting or root angle, help to improve vegetative growth and subsequent increase in plant yield (El Hassouni et al., 2018). The role of roots in enhancing crop yield is well-known (Jia et al., 2019), and therefore more emphasis should be given to improving root architecture (Koevoets et al., 2016).

The strategies adopted by the farmers to tackle the situation arising from climate change include modified cultural practices like growing early maturing crops, altering sowing and harvesting dates, and crop rotations. The development and cultivation of varieties tolerant to various biotic and abiotic stresses are one of the best policies to sustain productivity in these unavoidable circumstances arising due to climate change. The best solution is to develop tolerant varieties resistant to biotic and abiotic stresses by conventional breeding methods like introduction, selection,

germplasm collection, and hybridization. Each environment poses a different set of problems, and in marginal environments where the climate is highly variable, it is often difficult to define precisely the causes of crop failure or yield reduction. Therefore, the adverse effect of climate change challenges the breeder to set the breeding objective for each component of biotic and abiotic stresses. Therefore, multi-trait breeding schemes such as tandem selection, independent culling, and index selection (Falconer, 1960) need to be chalked out.

Molecular marker technology offers another powerful tool to combat complex genetic systems controlling abiotic stress resistance in plants. The other new biotechnology methods like Genome-wide association studies (GWAS), Genomic Selection (GS) with high throughput phenotyping and genotyping for identifying the different genes, Genetic Engineering and Genome Editing (CRISPR/Cas9) strategies can also be applied to tackle the problem of climate change (Karavolias et al., 2021). Genetic modification through biotechnology is another modern tool for developing transgenic plants to tackle climate change. Genome editing (GE) is one of the latest biotechnology methods to modify the plant genome by means of sequence-specific nucleases. To find out novel variation patterns and determine if the genes have functions in significant ecological traits, molecular marker studies are carried out in the population genomics (Keurentjes et al., 2008). Modern biotechnology methods are currently available to deal with the consequences of climate change; however, each method has its own limitations. However, a large number of breeding traits are complex quantitative traits, and therefore, gene editing or molecular breeding techniques based on one or a few genes are not ideal for the improvement of quantitative traits (Holme et al., 2019).

In the past, different plant breeding methods have helped to increase the diversity of crop germplasm by developing genetically better varieties for cultivation. Step by step improvements in traditional plant breeding techniques like distant hybridization, mutagenesis, tissue culture-based approaches, and molecular breeding have been applied to increase crop production. Development of crop varieties tolerant to rapidly changing environmental conditions using genetic resources will be an important part of agricultural adaptation to climate change. The genetic resources need to be built up by creating new genetic variability in seed propagated crop plants. Genetic improvement of crop plants is a continuous endeavor, and the success of a crop improvement program depends on the availability of large genetic variability, which a plant breeder can combine to generate new varieties (Oladosu et al., 2016). While some plant species of cultivated crops have rich genetic diversity, others have very limited genetic variation. There are large numbers of instances in the past where naturally occurring mutations served an important role in crop improvement. Plant breeders depend on genetic diversity to develop new and improved cultivars with desirable traits by means of hybridization, recombination, and mutation (spontaneous and induced). Therefore, conventional breeding methods, including mutation breeding, are the answer for combating the negative effects of climate change. The main objective of induced mutations is to create genetic variability and rectify the lacunae in the well-adapted variety. Mutation breeding has been universally accepted as one of the plant breeding methods, and a large

amount of genetic variability has been induced by various mutagens (Sarsu et al., 2020). Crop improvement through mutation breeding has resulted in the development of improved varieties that are directly used for commercial cultivation or in recombination breeding. Genetic variability for almost all the quantitative and qualitative characters has been created using induced mutations. In the future, the objective of plant breeding needs to be focused on creating mutants for improved plant architecture, early maturity, better nodulation, photo insensitiveness, and resistance to biotic and abiotic stresses.

## 5 Conclusion

Climate variability increases the susceptibility of seed propagated crops to various types of stresses. The crop yield and productivity are hampered because of different biotic and abiotic stresses. Rapid changes in environmental conditions increase the chances of disease epidemics. Drought, flooding, salinity, and heavy metal toxicity are the abiotic stresses that inhibit crop growth and productivity. In the future, the objectives of plant breeding should give more emphasis to improving the root system since it manages the plant system during various stresses. Development of varieties with improved plant architecture, early maturity, better nodulation, photo insensitiveness, and resistance to biotic and abiotic stresses will help in times of drastic climate change and sustain crop productivity. The use of induced mutations over the past five decades has played a major role in the development of superior plant varieties having resistance to biotic and abiotic stresses in the entire world, and the majority are seed propagated crops. Most of the existing mutant varieties of seed propagated crops are well adapted to adverse climatic conditions. In the current era of modern biotechnology, mutation breeding will still play an important role in the development of elite mutant varieties for the drastic climate change.

## References

Abbas, G., Ahmad, S., Ahmad, A., Nasim, W., Fatima, Z., Hussain, S., Rehman, M. H. u., Khan, M. A., Hasanuzzaman, M., Fahad, S., Boote, K. J., & Hoogenboom, G. (2017). Quantification the impacts of climate change and crop management on the phenology of maize-based cropping system in Punjab, Pakistan. *Agricultural and Forest Meteorology, 247*, 42–55.

Abdalla, E., Bakhit, O., & Elsheikh, S. (2018). *Groundnut mutants with end-of-season drought tolerance for the marginal dry lands of North Kordofan state, Sudan*. FAO/IAEA, International Symposium on Plant Mutation Breeding and Biotechnology.

Ahloowalia, B. S., Maluszynski, M., & Nichterlein, K. (2004). Global impact of mutation derived varieties. *Euphytica, 135*, 187–204.

Ahmad, F., Hisham, S. N., Hassan, N. A., Shamsudin, N. A., Sukiran, N. L., Simoli, J. M. A., Hussein, S., & Harun, A. R. (2020). Evaluation of selected rice mutant genotypes tolerant to submergence at germination and seedling stage. *International Journal of Agriculture, Forestry and Plantation, 10*, 108–112.

Akdemir, D., Beavis, W., Fritsche-Neto, R., Singh, A. K., & Isidro-Sanchez, J. (2019). Multi-objective optimized genomic breeding strategies for sustainable food improvement. *Heredity, 122*, 672–683.

Amin, R., Wani, M. R., Raina, A., Khursheed, S., & Khan, S. (2019). Induced morphological and chromosomal diversity in the mutagenized population of black cumin (Nigella sativa L.) using single and combination treatments of gamma rays and ethyl methane sulfonate. Jordan. *Journal of Biological Sciences, 12*(1), 23–30.

Andrade, A., et al. (2018). Development of rice (Oryza sativa) lines resistant to aryloxyphenoxy-propionate herbicides through induced mutation with gamma rays. *Plant Breeding, 137*, 364–369.

Ashraf, M. A., Akbar, A., Askari, S. H., Iqbal, M., Rasheed, R., & Hussain, I. (2018). *Recent advances in abiotic stress tolerance of plants through chemical priming: An overview* (pp. 51–79). In Advances in Seed Priming; Springer.

Azad, M. A. K., & Imtiaz, U. M. (2012). Achievements in rice research at BINA through induced mutation. *Bioremediation, Biodiversity and Bioavailability, 6*(Special Issue 1), 53–57.

Badawi, A. T. (2001). Yield gap and productivity decline in rice production in Egypt. In *Yield gap and productivity decline in rice production* (pp. 429–441). International Rice Commission, FAO.

Badigannavar, A. M., Jambhulkar, S. J., Manjaya, J. G., Souframanien, J., Das, B. K., Badigannavar, A. M., Ganapathi, T. R., & Suprasanna, P. (2020a). Radiation technology for genetic enhancement of crop plants. In A. K. Tyagi & A. K. Mohanty (Eds.), *Non-power applications of nuclear technologies. scientific information resource division* (pp. 33–49). Bhabha Atomic Research Centre, Mumbai.

Badigannavar, A. M., Mondal, S., & Bhad, P. G. (2020b). Radiation based induced mutagenesis in Trombay groundnuts: Developments and accomplishments. *IANCAS Bulletin, XV*(1), 45–49.

Bakshi, S., Singh, J., & Jambhulkar, S. J. (2021). Isolation and characterization of yellow rust resistant mutants in wheat. In S. Sivasankar, N. Ellis, L. Jankuloski, & I. Ingelbrecht (Eds.), *Application of mutation Breeding techniques in the development of green crop varieties in Sri Lanka: The way forward* (pp. 103–110). Mutation breeding, genetic diversity and crop adaptation to climate change CABI.

Basu, M. S. (1997). *Rabi/summer groundnut research under the All India Coordinated Project: Summary-cum-Project Coordinator's Report, page II in Progress Report 1996–97*. Rabi/summer Groundnut Workshop. 6–7 Oct 1997, National Research Centre for Groundnut (N R CG), Junagadh, India: Project Coordinating Unit, Groundnut (ICAR), NRCG.

Begum, S. N., Islam, M. M., & Gupta, R. (2021). In S. Sivasankar, N. Ellis, L. Jankuloski, & I. Ingelbrecht (Eds.), *Development of the first Kabuli type chickpea mutant variety in Bangladesh* (pp. 203–208). Mutation Breeding, Genetic Diversity and Crop Adaptation to Climate Change CABI.

Ceccarelli, S., & Grando, S. (2020). Evolutionary plant breeding as a response to the complexity of climate change. *Science, 23*(12), 101815.

Chakrabarti, S. N. (1995). Mutation breeding in India with particular reference to PNR rice varieties. *Journal of Nuclear Agriculture and Biology, 24*, 73–82.

Chen J, Liu Y, Ni J, Wang Y, Bai Y, Shi J, Gan J, Wu Z, Wu P. (2011). OsPHF1 regulates the plasma membrane localization of low- and high-affinity inorganic phosphate transporters and determines inorganic phosphate uptake and translocation in rice. *Plant Physiology 157*: 269–278.

Chen, J, Huang, X. Y., Salt, D. E., & Zhao F. J. (2020). Mutation in OsCADT1 enhances cadmium tolerance and enriches selenium in rice grain. *New Phytol. 226*(3): 838–850.

Cheabu, S., Panichawong, N., Rattanametta, P., Wasuri, B., Kasemsap, P., Arikit, S., Vanavichit, A., & Malumpong, C. (2019). Screening for spikelet fertility and validation of heat tolerance in a large rice mutant population. *Rice Science, 26*(4), 229–238.

Cheke, R. A., & Tratalos, J. (2007). Migration, patchiness, and population processes illustrated by two migrant pests. *Bioscience, 57*(2), 145–154.

Chopra, V. L. (2005). Mutagenesis: Investigating the process and processing the outcome for crop improvement. *Current Science, 89*(2), 353–359.

Christov, N. K., et al. (2014). Chemical mutagenesis, mutation breeding and quantitative genetic analyses of maize mutants: From theory to practice. In N. B. Tomlekova, M. I. Kozgar, & M. R. Wani (Eds.), *Mutagenesis: Exploring genetic diversity of crops* (pp. 169–196). Wageningen Academic Publishers.

Conway, G. (1999). *The doubly green revolution: Food for all in the twenty-first century*. Cornell University Press.

Croughan, T., Utomo, H., Sanders, D., & Braverman, M. (1996). Herbicide-resistant rice offers potential solution to red rice problem. *Louisiana agriculture, 46*, 24–26.

Deryng, D., Elliott, J., Folberth, C., Müller, C., Pugh, T. A. M., Boote, K. J., et al. (2016). Regional disparities in the beneficial effects of rising $CO_2$ concentrations on crop water productivity. *Nature Climate Change, 6*, 786–790.

Deutsch, C. A., Tewksbury, J. J., Tigchelaar, M., Battisti, D. S., Merrill, S. C., Huey, R. B., & Naylor, R. L. (2018). Increase in crop losses to insect pests in a warming climate. *Science, 361*, 916–919.

Dhankher, O. P., & Foyer, C. H. (2018). Climate resilient crops for improving global food security and safety. *Plant, Cell & Environment, 41*, 877–884.

Do, K. T. (2009). Socio-economic impacts of mutant rice varieties in southern Vietnam. In Q. Y. Shu (Ed.), *Induced plant mutation in the genomics era. In join FAO/IAEA Program, Nuclear Techniques in Food and Agriculture* (pp. 62–65). Food and Agriculture Organization of the United Nation.

Dolferus, R. (2014). To grow or not to grow: A stressful decision for plants. *Plant Science, 229*, 247–261.

Donald, C. M. T. (1968). The Breeding of crop Ideotypes. *Euphytica, 17*, 385–403.

Domingo C, Lalanne E, Catalá MM, Pla E, Reig-Valiente JL and Talón M (2016) Physiological basis and transcriptional profiling of three salt-tolerant mutant lines of Rice. Front. *Plant Sci. 7*, 1462.

El Hassouni, K., Alahmad, S., Belkadi, B., Filali-Maltouf, A., Hickey, L. T., & Bassi, F. M. (2018). Root system architecture and its association with yield under different water regimes in durum wheat. *Crop Science, 58*, 2331–2346.

Fahad, S., Bajwa, A. A., Nazir, U., Anjum, S. A., Farooq, A., & Zohaib, A. (2017). Crop production under drought and heat stress: Plant responses and management options. *Frontiers in Plant Science, 8*, 1147.

Falconer, D. S. (1960). *Introduction to quantitative genetics* (pp. 1–140). Oliver and Boyd Ltd..

Foster, G., & Rahmstorf, S. (2011). Global temperature evolution 1979–2010. *Environmental Research Letters, 6*, 044022.

Futsuhara, Y. (1968). Breeding of a new rice variety Reimei by gamma-ray irradiation. *Gamma Field Symposia, 6*, 87–109.

Galili, S., Hershenhorn, J., Edelman, M., Sobolev, V., Smirnov, E., Amir-Segev, O., Bellalou, A., & Dor, E. (2021). Novel mutation in the acetohydroxyacid synthase (AHAS), gene confers Imidazolinone resistance in chickpea Cicer arietinum L. plants. *Plants, 10*, 2791.

Gnankambary, K., Sawadogo, N., Di'eni, Z., Batieno, T. B. J., Tignegr'e, J. B. D. S., Sawadogo, M., & Ou'edraogo, T. J. (2020). Assessment of cowpea (*Vigna unguiculata* (L.) Walp.) mutant lines for drought tolerance. *International Journal of Agronomy, 9*. Article ID 8823498.

Gomez-Pando, L., Eguiluz, A., Jimenez, J., Falconi, J., & Aguilar, E. A. (2009). In Q. Y. Shu (Ed.), *Barley (Hordeun vulgare) and Kiwicha (Amaranthus caudatus) improvement by mutation induction in Peru*. Induced Plant Mutations in the Genomics Era.

Gomez-Pando, L., Bernardo-Rojas, J., Deza-Montoya, D., Ibañez-Tremolada, M., & Aguilar-Castellanos, E. (2021). In S. Sivasankar, N. Ellis, L. Jankuloski, & I. Ingelbrecht (Eds.), *Mutation induction to improve quinoa (Chenopodium quinoa) resistance to downy mildew (Peronospora variabilis)* (pp. 194–202). Mutation Breeding, Genetic Diversity and Crop Adaptation to Climate Change CABI.

Goyal, S., Wani, M. R., Laskar, R. A., Raina, A., Amin, R., & Khan, S. (2019a). Induction of morphological mutations and mutant phenotyping in black gram [Vigna mungo (L.) Hepper] using gamma rays and EMS. *Vegetos, 32*(4), 464–472.

Goyal, S., Wani, M. R., Laskar, R. A., Aamir, R., & Samiullah, K. (2019b). Assessment on cytotoxic and mutagenic potency of gamma rays and EMS in Vigna mungo L. Hepper. *Biotecnología Vegetal, 19*(3), 193–204.

Goyal, S., Wani, M. R., Laskar, R. A., Raina, A., & Khan, S. (2020a). Mutagenic effectiveness and efficiency of individual and combination treatments of gamma rays and ethyl Methanesulfonate in black gram [Vigna mungo (L.) Hepper]. *Advances in Zoology and Botany, 8*(3), 163–168.

Goyal, S., Wani, M. R., Laskar, R. A., Raina, A., & Khan, S. (2020b). Performance evaluation of induced mutant lines of black gram (Vigna mungo (L.) Hepper). *Acta fytotechn zootechn, 23*(2), 70–77.

Goyal, S., Wani, M. R., Laskar, R. A., Raina, A., Amin, R., & Khan, S. (2021a). Quantitative assessments on induced high yielding mutant lines in urdbean [Vigna mungo (L.) hepper]. *Legume Science*. https://doi.org/10.1002/leg3.125

Goyal, S., Wani, M. R., Raina, A., Laskar, R. A., & Khan, S. (2021b). Phenotypic diversity in mutagenized population of urdbean (Vigna mungo (L.) Hepper). *Heliyon, 7*(5), 06356.

Ham, L. H., & Xuan, H. P. (2018). *Impact of mutation breeding to food security in Vietnam*. FAO/IAEA International Symposium on Plant Mutation Breeding and Biotechnology. 27–31 August 2018.

Haq, M. A. (2009). In Q. Y. Shu (Ed.), *Development of mutant varieties of crop plants at NIAB and the impact on agricultural production in Pakistan proceedings*. Induced Plant Mutations in the Genomics Era.

Hasan, N., Laskar, R. A., Raina, A., & Khan, S. (2018). Maleic hydrazide induced variability in fenugreek (Trigonella foenum-graecum L.) cultivars CO1 and Rmt-1. *Research & Reviews: Journal of Botany Science, 7*(1), 19–28.

Heckathorn, S. A., Giri, A., Mishra, S., & Bista, D. (2013). Heat stress and roots. In N. Tuteja & S. S. Gill (Eds.), *Climate change and plant abiotic stress tolerance* (pp. 109–136). Wiley-VCH Verlag GmbH & Co.

Heeb, L., Jenner, E., & Cock, M. J. W. (2019). Climate-smart pest management: Building resilience of farms and landscapes to changing pest threats. *Journal of Pest Science, 92*, 951–969.

Hirata, K., Takagi, K., Yamada, T., Sayama, T., Anai, T., Kikuchi, A., & Ishimoto, M. (2019). Isolation and characterization of induced mutants in the gene associated with seed cadmium accumulation in soybean. *Breeding Science, 69*(2), 345–351.

Holme, I. B., Gregersen, P. L., & Pedersen, H. B. (2019). Induced genetic variation in crop plants by random or targeted mutagenesis: Convergence and differences. *Frontiers in Plant Science, 10*, 1468.

Human, S., & Indriatama, W. (2020). Sorghum improvement program by using mutation breeding in Indonesia IOP Conf. Ser. *Earth and Environmental Science, 484*(1), 012003.

Human, S., & Sihono, S. (2010). Sorghum breeding for improved drought tolerance using induced mutation with gamma irradiation. *Journal Agronomi Indonesia, 38*(2).

Ibrahim, R. (2018). *Impact of mutant varieties in Malaysia: Challenges and future perspective of mutation breeding*. FAO/IAEA International Symposium on Plant Mutation Breeding and Biotechnology. 27–31 August.

Iizumi, T., & Ramankutty, N. (2016). Changes in yield variability of major crops for 1981–2010 explained by climate change. *Environmental Research Letters, 11*, 34003.

IPCC. (2007). In M. L. Parry, O. F. Canziani, J. P. Palutikof, P. J. van der Linden, & C. E. Hanson (Eds.), *Climate change 2007: Impacts, adaptation and vulnerability. Contribution of Working Group II to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge University Press. 976pp.

Islam, M. A., Sarkar, M. A. R., Rahman, M. C., & Rahaman, M. S. (2021). Assessing the level of comparative advantage for the Boro Rice production in Bangladesh: A time series analysis. *Journal of Bangladesh Agricultural University, 19*(3), 361–370.

Jagadeesan, S., & Ganapathi, T. R. (2021). Mutation breeding in India: Accomplishments and socio-economic impact. *Plant Breeding & Genetics Newsletter, 46*, 23.

Jankowicz-Cieslak, J., Mba, C., & Till, B. J. (2017). Biotechnologies for plant mutation breeding: Protocols. In J. Jankowicz-Cieslak, T. H. Tai, J. Kumlehn, & B. J. Till (Eds.), *Mutagenesis for crop Breeding and functional genomics* (pp. 3–18). Springer International Publishing.

Jawerth, N. (2017). Bangladesh triples rice production with help of nuclear science. *IAEA Bulletin, 58*(2), 14–15.

Jia, Z., Liu, Y., Gruber, B. D., Neumann, K., Kilian, B., Graner, A., et al. (2019). Genetic dissection of root system architectural traits in spring barley. *Frontiers in Plant Science, 10*, 400.

Karavolias N. G., Horner W., Abugu M. N., & Evanega SN (2021). Application of Gene Editing for Climate Change in Agriculture. Front. Sustain. Food Syst. 5, 685801. https://doi.org/10.3389/fsufs.2021.685801

Ketiem, P., Makeni, P. M., Maranga, E. K., & Omondi, P. A. (2017). Integration of climate change information into drylands crop production practices for enhanced food security: A case study of lower Tana Basin in Kenya. *African Journal of Agricultural Research, 12*, 1763–1771.

Keurentjes, J. J. B., Koornneef, M., Vreugdenhil, D. (2008). Quantitative genetics in the age of omics. *Current Opinion in Plant Biology, 11*(2): 123–128.

Khanh, T. D., Duong, V. X., Nguyen, P. C., Xuan, T. D., Trung, N. T., Trung, K. H., Gioi, D. H., Hoang, N. H., Tran, H.-D., Trung, D. M., et al. (2021). Rice breeding in Vietnam: Retrospects. *Challenges and Prospects. Agriculture, 11*, 397.

Kharkwal, M. C., & Shu, Q. Y. (2009). The role of induced mutations in world food security. In Q. Y. Shu (Ed.), *Induced plant mutations in the genomics era* (pp. 33–38). Food and Agriculture Organization of the United Nations.

Kharkwal, M. C., Pandey, R. M., & Pawar, S. E. (2004). Mutation breeding in crop improvement. In H. K. Jain & M. C. Kharkwal (Eds.), *Plant breeding – Mendelian to molecular approaches* (pp. 601–645). Narosa Publishing House.

Khin, T. N. (2006). *Rice mutation breeding for varietal improvement in Myanmar (INIS-XA-965)*. International Atomic Energy Agency (IAEA): IAEA.

Khitka, B., Phanchaisri, B., Sutipatanasomboon, A., Nuangmek, W., Yu, L. D., Techarang, J. (2021). Low-energy heavy-ion-beaminduced mutation of novel high-yielding drought-tolerant Thai Jasmine rice. *Nuclear Instruments and Methods in Physics Research, Section B: Beam Interactions with Materials and Atoms, 492*, 34–42.

Khursheed, S., Laskar, R. A., Raina, A., Amin, R., & Khan, S. (2015). Comparative analysis of cytological abnormalities induced in Vicia faba L. genotypes using physical and chemical mutagenesis. Chromosome. *Science, 18*(3–4), 47–51.

Khursheed, S., Raina, A., & Khan, S. (2016). Improvement of yield and mineral content in two cultivars of Vicia faba L. through physical and chemical mutagenesis and their character association analysis. *Archives of Current Research International, 4*(1), 1–7.

Khursheed, S., Raina, A., Parveen, K., & Khan, S. (2017). Induced phenotypic diversity in the mutagenized populations of faba bean using physical and chemical mutagenesis. *Journal of the Saudi Society of Agricultural Sciences, 18*, 113–119. https://doi.org/10.1016/j.jssas.2017.03.001

Khursheed, S., Raina, A., Laskar, R. A., & Khan, S. (2018a). Effect of gamma radiation and EMS on mutation rate: Their effectiveness and efficiency in faba bean (Vicia faba L.). *Caryologia: International Journal of Cytology, Cytosystematics and Cytogenetics, 71*(4), 397–404. https://doi.org/10.1080/00087114.2018.1485430

Khursheed, S., Raina, A., & Khan, S. (2018b). Physiological response of two cultivars of faba bean using physical and chemical mutagenesis. *International Journal of Advance Research in Science and Engineering, 7*(4), 897–905.

Khursheed, S., Raina, A., Amin, R., Wani, M. R., & Khan, S. (2018c). Quantitative analysis of genetic parameters in the mutagenized population of faba bean (Vicia faba L.). research on. *Crops, 19*(2), 276–284.

Khursheed S, Aamir Raina, Kouser Parveen, Samiullah Khan. (2019). Induced phenotypic diversity in the mutagenized populations of faba bean using physical and chemical mutagenesis. *Journal of the Saudi Society of Agricultural Sciences. 18*(2019) 113–119.

Kim, D. S., Song, J. Y., Chun, J., Lee, K. J., Kim, J.-B., Kim, S. H., Yun, S. J., & Kang, S.-Y. (2010). Selection of gamma-ray induced salt tolerant rice mutants by in vitro mutagenesis. *Journal of Radiation Industry, 4*(2), 179–184.

Koevoets, I. T., Venema, J. H., Elzenga, J. T. M., & Testerink, C. (2016). Roots withstanding their environment: Exploiting root system architecture responses to abiotic stress to improve crop tolerance. *Frontiers in Plant Science, 7*, 1335.

Laskar, R. A., Khan, S., Khursheed, S., Raina, A., & Amin, R. (2015). Quantitative analysis of induced phenotypic diversity in chickpea using physical and chemical mutagenesis. *Journal of Agronomy, 14*(3), 102–111.

Laskar, R. A., Laskar, A. A., Raina, A., Khan, S., & Younus, H. (2018a). Induced mutation analysis with biochemical and molecular characterization of high yielding lentil mutant lines. *International Journal of Biological Macromolecules, 109*, 167–179.

Laskar, R. A., Wani, M. R., Raina, A., Amin, R., & Khan, S. (2018b). Morphological characterization of gamma rays induced multipodding mutant (mp) in lentil cultivar pant L 406. *International Journal of Radiation Biology, 94*(11), 1049–1053.

Laskar, R. A., Khan, S., Deb, C. R., Tomlekova, N., Wani, M. R., Raina, A., & Amin, R. (2019). Lentil (Lens culinaris Medik.) diversity, cytogenetics and breeding. In J. M. Al-Khayri et al. (Eds.), *Advances in plant breeding: Legumes* (pp. 319–369). Springer. https://doi.org/10.1007/978-3-030-23400-3_9

Latham, A. D. M., Latham, M. C., Cieraad, E., Tompkins, D. M., & Warburton, B. (2015). Climate change turns up the heat on vertebrate pest control. *Biological Invasions, 17*, 2821–2829.

Lethin, J., Shakil, S.S.M., Hassan, S. et al. (2020). Development and characterization of an EMS-mutagenized wheat population and identification of salt-tolerant wheat lines. *BMC Plant Biol 20*,18.

Le, T. D., & Pham, C. T. (2021). In S. Sivasankar, N. Ellis, L. Jankuloski, & I. Ingelbrecht (Eds.), *Soybean breeding through induced mutation in Vietnam* (pp. 47–52). Mutation Breeding, Genetic Diversity and Crop Adaptation to Climate Change CABI.

Le Roux, M.-S., Burger, N.F.V., Vlok, M., Kunert, K.J., Cullis, C.A., Botha, A.-M. (2020). Wheat line "RYNO3936" is associated with delayed water stress-induced leaf senescence and rapid water-deficit stress recovery. *Front. Plant Sci., 11*, 1053.

le Roux, M.-S. L., Burger, N. F. V., Vlok, M., Kunert, K. J., Cullis, C. A., & Botha, A.-M. (2021). EMS derived wheat mutant BIG8-1 (*Triticum aestivum* L.) – A new drought tolerant mutant wheat line. *International Journal of Molecular Sciences, 22*(10), 5314.

Lean J. L., & Rind D. H. (2009). How will Earth's surface temperature change in future decades? Geophys. *Research Letters, 36*, L15708, https://doi.org/10.1029/2009GL038932.

Lipper, L., McCarthy, N., Zilberman, D., Asfaw, S., & Branca, G. (Eds.). (2018). *Climate smart agriculture: Building resilience to climate change* (p. 630). Springer International Publishing.

Liu, L. (2021). *New breakthroughs in plant mutation breeding prominent among the top ten scientific research advances in China in 2019 (INIS-XA--21K0015)*. International Atomic Energy Agency (IAEA).

Liu, B., Martre, P., Ewert, F., Porter, J. R., Challinor, A. J., et al., (2019). Global wheat production with 1.5 and 2.0°C above preindustrial warming. *Global Change Biology, 25*(4), 1428–1444. https://doi.org/10.1111/gcb.14542.

Livore, A. B., Landau, A., & Prina, A. R. (2018). *The success of IMI tolerant rice varieties in Latin America*. FAO/IAEA International Symposium on Plant Mutation Breeding and Biotechnology.

Lobell, D. B., & Field, C. B. (2007). Global-scale climate-crop yield relationships and the impacts of recent warming. *Environmental Research Letters, 2*, 14002.

Matzrafi, M., Seiwert, B., Reemtsma, T., Rubin, B., & Peleg, Z. (2016). Climate change increases the risk of herbicide-resistant weeds due to enhanced detoxification. *Planta, 244*, 1217–1227.

Manonmani, K, Jeyaprakash, P., Manonmani, S., Raveendran, M., & Jeyakumar, P. (2020). Assessment of drought tolerance in Nagina 22 mutants of rice using Poly Ethylene Glycol (PEG). *Electronic Journal of Plant Breeding, 11*(2), 479–486.

Mavromatis, T. (2015). Crop–climate relationships of cereals in Greece and the impacts of recent climate trends. *Theoretical and Applied Climatology, 120*, 417–432.

Mba, C., Afza, R., & Shu, Q. Y. (2012). Mutagenic radiations: X-rays, ionizing particles and ultraviolet. In Q. Shu, B. P. Forster, & H. Nakagawa (Eds.), *Plant mutation breeding and biotechnology* (pp. 83–90). CABI.

Mohapatra, Trilochan & Robin, Shahidul & Neelamraju, Sarla & Sheshasayee, M. & Singh, Ashok & Singh, Kuldeep & Singh, Nagendra & Sevanthi, Amitha Mithra & Sharma, R. P. (2014). EMS Induced Mutants of Upland Rice Variety Nagina22: Generation and Characterization. *Proceedings of the Indian National Science Academy. 80*. 163. https://doi.org/10.16943/ptinsa/2014/v80i1/55094.

Moore F. C., & Lobell D. B. (2015). The fingerprint of climate trends on European crop yields. *Proc Natl Acad Sci U S A. 112*(9), 2670–2675. https://doi.org/10.1073/pnas.1409606112. Epub 2015 Feb 17. PMID: 25691735; PMCID: PMC4352830.

Mullarkey, M., & Jones, P. (2000). Isolation and analysis of thermotolerant mutants of wheat. *Journal of Experimental Botany, 51*(342)*, 139–146*.

Muller, H. J. (1928). The measurement of gene mutation rate in drosophila, its high variability, and its dependence upon temperature. *Genetics, 13*, 279–357.

Naju, P. N., Kinyua, M. G., Kimurto, P. K., Okwaro, H. K., & Maluszynski, M. (2005). Drought tolerant wheat varieties developed through mutation breeding technique. *Journal of Agriculture, Science and Technology, 7*(1), 18–29.

Nakagawa, H. (2018). *Induced mutations in plant breeding and biological researches in Japan history of mutation breeding and molecular research using induced mutations in Japan*. FAO/IAEA International Symposium on Plant Mutation Breeding and Biotechnology.

Nakagawa, H. (2021). In S. Sivasankar, N. Ellis, L. Jankuloski, & I. Ingelbrecht (Eds.), *History of mutation breeding and molecular research using induced mutations in Japan* (pp. 24–39). Mutation Breeding, Genetic Diversity and Crop Adaptation to Climate Change CABI.

Navarro-León E, Oviedo-Silva J, Ruiz JM, Blasco B. (2019). Possible role of HMA4a TILLING mutants of Brassica rapa in cadmium phytoremediation programs. *Ecotoxicol Environ Saf. 180*: 88–94.

Navarro-León, E., Ruiz, J. M., Albacete, A., & Blasco, B. (2020). Tolerance to cadmium toxicity and phytoremediation potential of three Brassica rapa CAX1a TILLING mutants. *Ecotoxicology and Environmental Safety, 189*, 109961.

Nawrot M, Szarejko I, Maluszynski M (2001). Barley mutants with increased tolerance to aluminium toxicity. *Euphytica. 120*: 345–356.

Neto A.T., Alves M.C., Camargo C.E.de.O., Castro J.L. de, Filho W.P.F. (2001) New wheat genotypes tolerant to aluminum toxicity obtained by mutation induction. Pesq. agropec. bras.. Vol. 36(1):61–70.

Newman, J. A. (2003). Climate change and cereal aphids: The relative effects of increasing $CO_2$ and temperature on aphid population dynamics. *Global Change Biology, 10*(1), 5–15.

Newhouse KE, Smith WA, Starrett M.A., Schaefer T.J., Singh B.K. (1992). Tolerance to Imidazolinone herbicides in wheat. *Plant Physiology, 100*(2): 882–886.

Nirmalakumari, A., Senthil, N., Veerabadhiran, P., Thiyagarajan, K., Sivasamy, M., Nayeem, K. A., Kumar, J., Vinod, T. S. M. S., Vikas, V. K., & Jayaprakash. (2010). A high yielding samba wheat (Triticum dicoccum) variety TNAU wheat COW2 for Tamil Nadu. *The Madras Agricultural Journal, 97*(10–12), 353–355.

NSAC National Sustainable Agriculture Coalition. (2019). Agriculture and climate change: Policy imperatives and opportunities to help producers meet the challenge.

Oladosu, Y., Rafii, M. Y., Abdullah, N., Hussin, G., Ramli, A., Rahim, H. A., Miah, G., & Usman, M. (2016). Principle and application of plant mutagenesis in crop improvement: A review. *Biotechnology & Biotechnological Equipment, 30*(1), 1–6.

Olesen, J. E., & Bindi, M. (2002). Consequences of climate change for European agricultural productivity, land use and policy. *European Journal of Agronomy, 16*, 239–262.

Olesen, J. M., Bascompte, J., Dupont, Y. L., & Jordano, P. (2007). The modularity of pollination networks. *Proceedings of the National Academy of Sciences, 104*(50), 19891–19896.

Olesen, J. E., Trnka, M., Kersebaum, K. C., Skjelvåg, A., Seguin, B., Peltonen-Sainio, P., Rossi, F., Kozyra, J., & Micale, F. (2011). Impacts and adaptation of European crop production systems to climate change. *European Journal of Agronomy, 34*, 96–112.

Pangga, I. B., Hanan, J., & Chakraborty, S. (2011). Pathogen dynamics in a crop canopy and their evolution under changing climate. *Plant Pathology, 60*, 70–81. https://doi.org/10.1111/j.1365-3059.2010.02408.x

Panigrahy, M., Neelamraju, S., Rao, D. N., & Ramanan, R. (2011). Heat tolerance in rice mutants is associated with reduced accumulation of reactive oxygen species. *Biologia Plantarum, 55*, 721–724.

Parasuraman, M., & Weerasinghe, P. (2021). In S. Sivasankar, N. Ellis, L. Jankuloski, & I. Ingelbrecht (Eds.), *Application of mutation Breeding techniques in the development of green crop varieties in Sri Lanka: The way forward* (pp. 76–82). Mutation Breeding, Genetic Diversity and Crop Adaptation to Climate Change CABI.

Parry, M. A. J., Flexas, J., & Medrano, H. (2005). Prospects for crop production under drought: Research priorities and future directions. *Annals of Applied Biology, 147*, 211–226.

Patil, S. H., Kale, D. M., Deshmukh, S. N., Fulzele, G. R., & Weginwar, B. G. (1995). Semi-dwarf, early maturing and high yielding new groundnut variety, TAG-2 4. *Journal of Oilseeds Research, 12*, 254–225.

Patnaik, D., Chaudhary, D., & Rao, G.J.N. (2006). *Genetic improvement of long grain aromatic Rices through mutation approach* (INIS-XA--965). Plant Mutation Reports, Vol. 1(1) International Atomic Energy Agency (IAEA): IAEA.

Paulsen, G. M. (1994). High-temperature responses of crop plants. In K. J. Boote, J. M. Bennett, T. R. Sinclair, & G. M. Paulsen (Eds.), *Physiology and determination of crop yield*. https://doi.org/10.2134/1994.physiologyanddetermination.c25

Piao Z, Wang W, Wei Y, Zonta F, Wan C, Bai J, Wu S, Wang X, Fang J. (2018). Characterization of an acetohydroxy acid synthase mutant conferring tolerance to imidazolinone herbicides in rice (Oryza sativa). *Planta. 247*(3): 693–703.

Pinke, Z., & Lovei, G. L. (2017). Increasing temperature cuts back crop yields in Hungary over the last 90 years. *Global Change Biology, 23*, 5426–5435.

Poli, Y., Basava, R. K., Panigrahy, M. et al. (2013). Characterization of a Nagina22 rice mutant for heat tolerance and mapping of yield traits. *Rice, 6*, 36.

Potopova, V., Zahradníček, P., Štěpanek, P., Turkott, L., Farda, A., & Soukup, J. (2017). The impacts of key adverse weather events on the field-grown vegetable yield variability in The Czech Republic from 1961 to 2014. *International Journal of Climatology, 37*, 1648–1664.

Promnart, U., Puripunyavanich, V., Boonsirichai, K., Doungsoongnern, P., Kaewchuenchai, R., Chamotri, S., Klakhaeng, K., & Sarsu, F. (2017). Breeding Thai rice for flood tolerance through electron beam-induced mutations. *International Journal of Genetic Engineering, 5*(1), 1–10.

Raina, A., Laskar, R. A., Khursheed, S., Amin, R., Tantray, Y. R., Parveen, K., & Khan, S. (2016). Role of mutation breeding in crop improvement- past, present and future Asian research. *Journal of Agriculture, 2*(2), 1–13.

Raina, A., Laskar, R. A., Khursheed, S., Khan, S., Parveen, K., & Amin, R. (2017). Induce physical and chemical mutagenesis for improvement of yield attributing traits and their correlation analysis in chickpea. *International Letters of Natural Sciences, 61*, 14–22.

Raina, A., Laskar, R. A., Jahan, R., Khursheed, S., Amin, R., Wani, M. R., Nisa, T. N., & Khan, S. (2018a). Mutation breeding for crop improvement. In M. W. Ansari, S. Kumar, C. K. Babeeta, & R. K. Wattal (Eds.), *Introduction to challenges and strategies to improve crop productivity in changing environment* (pp. 303–317). Enriched Publications.

Raina, A., Khursheed, S., & Khan, S. (2018b). Optimisation of mutagen doses for gamma rays and sodium Azide in cowpea genotypes. *Trends in Biosciences, 11*(13), 2387–2389.

Raina, A., Khan, S., Laskar, R. A., Wani, M. R., & Mushtaq, W. (2019). Chickpea (Cicer arietinum L.) cytogenetics, genetic diversity and breeding. In J. M. Al-Khayri et al. (Eds.), *Advances in plant breeding: Legumes* (pp. 53–112). Springer. https://doi.org/10.1007/978-3-030-23400-3_3

Raina, A., & Khan, S. (2020). Increasing rice grain yield under biotic stresses: Mutagenesis, Transgenics and genomics approaches. In C. Aryadeep (Ed.), *Rice research for quality improvement: Genomics and genetic engineering* (pp. 149–178). Springer. https://doi.org/10.1007/978-981-15-5337-0_8

Raina, A., Laskar, R. A., Tantray, Y. R., Khursheed, S., & Khan, S. (2020). Characterization of induced high yielding cowpea mutant lines using physiological, biochemical and molecular markers. *Scientific Reports, 10*, 3687.

Raina, A., Sahu, P. K., Laskar, R. A., Rajora, N., Sao, R., Khan, S., & Ganai, R. A. (2021). Mechanisms of genome maintenance in plants: Playing it safe with breaks and bumps. *Frontiers in Genetics, 12*, 675686. https://doi.org/10.3389/fgene.2021.675686

Raina, A., Laskar, R. A., Wani, M. R., Jan, B. L., Ali, S., & Khan, S. (2022a). Comparative mutagenic effectiveness and efficiency of gamma rays and sodium azide in inducing chlorophyll and morphological mutants of cowpea. *Plants, 11*, 1322. https://doi.org/10.3390/plants11101322

Raina, A., Laskar, R. A., Wani, M. R., Jan, B. L., Ali, S., & Khan, S. (2022b). Gamma rays and sodium azide induced genetic variability in high yielding and biofortified mutant lines in cowpea [Vigna unguiculata (L.) Walp.]. *Frontiers in Plant Science, 13*, 911049. https://doi.org/10.3389/fpls.2022.911049

Raina, A., Laskar, R. A., Wani, M. R., & Khan, S. (2022c). Plant Breeding strategies for abiotic stress tolerance in cereals. In C. Aryadeep (Ed.), *Omics approach to manage abiotic stress in cereals* (pp. 151–177). Springer. https://doi.org/10.1007/978-981-19-0140-9_8

Rasik, S., Raina, A., Laskar, R. A., Wani, M. R., Reshi, Z., & Khan, S. (2022). Lower doses of sodium azide and methyl methanesulphonate improved yield and pigment contents in vegetable cowpea [Vigna unguiculata (L.) Walp.]. *South African Journal of Botany, 148*, 727–736. https://doi.org/10.1016/j.sajb.2022.04.034

Raza, A., Razzaq, A., Mehmood, S. S., Zou, X., Xuekun Zhang, X., Lv, Y., & Xu, J. (2019). Impact of climate change on crops adaptation and strategies to tackle its outcome: A review. *Plants, 8*, 34.

Ronald, P. (2011). Plant genetics, sustainable agriculture and global food security. *Genetics 188*(1), 11–20.

Ronde, J. D., & Spreeth, M. H. (2007). Development and evaluation of drought resistant mutant germplasm of *Vigna unguiculata*. *Water SA, 33*, 381–386.

Rosenzweig, C., Iglesias, A., Yang, X. B., Epstein, P. R., & Chivian, E. (2001). Climate change and extreme weather events: Implications for food production, plant diseases, and pests. *Global Change Human Health, 2*, 90–104.

Roychowdhury, R., & Tah, J. (2013). Mutagenesis-A Potential Approach for Crop Improvement. In: K. Hakeem, P. Ahmad, M. Ozturk, (Eds), *Crop Improvement* (pp. 149–187). Springer, Boston, MA. https://doi.org/10.1007/978-1-4614-7028-1_4

Ruswandi, D., Agustian, E. P., Anggia, A. O., Canama, H., Marta, S. R., & Suryadi, E. (2014). Mutation breeding of maize for anticipating global climate change in Indonesia. *Asian Journal of Agricultural Research, 8*, 234–247.

Sarsu, F., Bimpong, I. K., & Jankuloski, L. (2020). Contribution of induced mutation in crops to global food security. *ACI Advances, 12*(22), 2–11.

Savitri, E. S., & Fauziah, S. M. (2018). *Characterization of drought tolerance of GmDREB2 soybean mutants (Glycine max (L.) Merr) by ethyl methane sulfonate induction*. AIP Conference Proceedings 2019, 020017.

Saxena, A. K., Fuentes, X. C., Herbas, R. G., & Humphries, D. L. (2016). Indigenous food systems and climate change: Impacts of climatic shifts on the production and processing of native and traditional crops in the Bolivian Andes. *Frontiers in Public Health, 4*, 20.

Sebastian, S. A., & Chaleff, R. S. (1987). Soybean mutants with increased tolerance for sulfonylurea herbicides. *Crop Science, 27*, 948–952.

Sellapillai, L., Dhanarajan, A., Raina, A., & Ganesan, A. (2022). Gamma ray induced positive alterations in morphogenetic and yield attributing traits of finger millet (*Eleusine coracana* (L.) Gaertn.) in M2 generation. *Plant Science Today, 9*(4), 939–949.

Sellapillaibanumathi, L., Dhanarajan, A., Raina, A., & Ganesan, A. (2022). Effects of gamma radiations on morphological and physiological traits of finger millet (Eleusine coracana (L.) Gaertn.). *Plant Science Today, 9*(1), 89–95.

Senapati, N., Brown, H. E., & Semenov, M. A. (2019). Raising genetic yield potential in high productive countries: Designing wheat ideotypes under climate change. *Agricultural and Forest Meteorology, 271*, 33–45.

Sharif, B., Makowski, D., Plauborg, F., & Olesen, J. E. (2017). Comparison of regression techniques to predict response of oilseed rape yield to variation in climatic conditions in Denmark. *European Journal of Agronomy, 82*, 11–20.

Shiferaw, B., Smale, M., Braun, H.-J., Duveiller, E., Reynolds, M., & Muricho, G. (2013). Crops that feed the world 10. Past successes and future challenges to the role played by wheat in global food security. *Food Security, 5*, 291–317.

Shoba, D., Raveendran, M., Manonmani, S., Utharasu, S., Dhivyapriya, D., Subhasini, G., Ramchandar, S., Valarmathi, R., Grover, N., Krishnan, S. G., Singh, A. K., Jayaswal, P., Kale, P., Ramkumar, M. K., Mithra, S. V. A., Mohapatra, T., Singh, K., Singh, N. K., Sarla, N., Sheshshayee, M. S., Kar, M. K., Robin, S., & Sharma, R. P. (2017). Development and genetic characterization of a novel herbicide (Imazethapyr) tolerant mutant in Rice (*Oryza sativa* L.). *Rice, 10*(1), 10.

Song J, Kim D Sub, Lee M.-C, Lee K J, Kim J.-B, Kim S-H, Ha B-K, Yuan SJ, Kang S-Y. (2012). Physiological characterization of gamma-ray induced salt tolerant rice mutants. *Australian Journal of Crop Science. 6*: 421–429.

Stadler, L. J. (1928). Mutations in barley induced by x-rays and radium. *Science, 68*, 186–187.

Staley, J. T., & Johnson, S. N. (2008). Climate change impacts on root herbivores. In S. N. Johnson & P. J. Murray (Eds.), *Root feeders – An ecosystem perspective* (pp. 192–213). CABI.

Sundra, E. A. (2006). *Development of drought tolerant groundnut mutants for the rainfed areas of Western Sudan*. AGRIS.

Takagi, H., Tamiru, M., Abe, A., et al. (2015). Mut map accelerates breeding of a salt-tolerant rice cultivar. *Nature Biotechnology, 33*, 445–449.

Tan, S., & Bowe, S. J. (2012). Herbicide – Tolerant crops developed from mutations. In Q. Shu, B. P. Forster, & H. Nakagawa (Eds.), *Plant mutation breeding and biotechnology* (pp. 423–436). CABI.

Tantray, A. Y., Raina, A., Khursheed, S., Amin, R. U., & Khan, S. A. (2017). Chemical mutagen affects pollination and locule formation in capsules of black cumin (Nigella sativa L.). *International Journal of Agricultural Science, 8*(1), 108–117.

Tester, M., & Bacic, M. (2005). Abiotic stress tolerance in grasses. From model plants to crop plants. *Plant Physiology, 137*, 791–793.

Tester, M., & Langridge, P. (2010). Breeding technologies to increase crop production in a changing world. *Science, 327*, 818–822.

Tomlekova, N. B. (2010). Induced mutagenesis for crop improvement in Bulgaria. *Plant Mutation Reports, 2*(2).

Tsyganov, V. E., Belimov, A. A., Borisov, A. Y., Safronova, V. I., Georgi, M., Dietz, K. J., & Tikhonovich, I. A. (2007). A chemically induced new pea (Pisum sativum) mutant SGECdt with increased tolerance to, and accumulation of, cadmium. *Annals of Botany, 99*(2), 227–237.

UNEP. (2002). *Global environment outlook*. United Nations.

UNFPA. (2012). *The state of the world population report. By choice, not by chance: Family planning, Human rights and development*. United Nations Population Fund.

Van Harten, A. M. (1998). *Mutation breeding: Theory and practical applications*. Cambridge University Press.

Vandermeiren, K., Harmens, H., Mills, G., & De Temmerman, L. (2009). Impacts of ground-level ozone on crop production in a changing climate. In S. N. Singh (Ed.), *Climate change and crops. environmental science and engineering* (pp. 213–243). Springer.

Vaughan, M. M., Block, A., Christensen, S. A., Allen, L. H., & Schmelz, E. A. (2018). The effects of climate change associated abiotic stresses on maize phytochemical defenses. *Phytochemistry Reviews, 17*, 37–49.

Vinh, M. Q., Thinh, D. K., Bang, D. T., At, D. H., & Ham, L. H. (2009). Current status and research directions of induced mutation application to seed crops improvement in Vietnam. In Q. Y. Shu (Ed.), *Induced plant mutation in the genomic era* (pp. 341–345). FAO.

Wang, L. Q. (1991). *Induced mutation for crop improvement in China* (pp. 9–32). Proc. lAEA Symp. on Plant Mutation Breeding for Crop Improvement. IAEA.

Wani, M. R., Dar, A. R., Tak, A., Amin, I., Shah, N. H., Rehman, R., Baba, M. Y., Raina, A., Laskar, R., Kozgar, M. I., & Khan, S. (2017). Chemo-induced pod and seed mutants in mungbean (Vigna radiata L. Wilczek). SAARC. *Journal of Agriculture, 15*(2), 57–67.

Wani, M. R., Laskar, R. A., Raina, A., Khan, S., & Khan, T. U. (2021). Application of chemical mutagenesis for improvement of productivity traits in lentil (Lens culinaris Medik). *Annals of Biology, 37*(1), 69–75.

Xu, L., Zhang, Q., Zhou, A.-L., & Huo, R. (2013). Assessment of flood catastrophe risk for grain production at the provincial scale in China based on the BMM method. *Journal of Integrative Agriculture, 12*, 2310–2320. https://doi.org/10.1016/S2095-3119(13)60587-0

Xu, Y., Wang, R., Wang, Y., Zhang, L., Yao, S. (2020). A point mutation in LTT1 enhances cold tolerance at the booting stage in rice. *Plant Cell Environment, 43*(4), 992–1007.

Xynias, I. N., Mylonas, I., Korpetis, E. G., Ninou, E., Tsaballa, A., Avdikos, I. D., & Mavromatis, A. G. (2020). *Durum wheat breeding in the Mediterranean region* (Vol. 10, p. 432). Current Status and Future Prospects Agronomy.

Ye, Y., Wang, S., Wu, K., Ren, Y., Jiang, H., Chen, J., Tao, L., Fu, X., Liu, B., & Wu, Y. (2021). A Semi-Dominant mutation in OsCESA9 improves salt tolerance and favors field straw decay traits by altering cell wall properties in Rice. *Rice, 14*(1), 19.

Yuliasti and Sudarsono. (2011). Responses of soybean mutant lines to aluminium under *in vitro* and *in vivo* condition. *Atom Indonesia, 37*(3), 126–132.

Zahra, S., Hussain, M., Zulfiqar, S., et al. (2021). EMS-based mutants are useful for enhancing drought tolerance in spring wheat. *Cereal Research Communications*. https://doi.org/10.1007/s42976-021-00220-7

Zavala, J. A., Casteel, C. L., DeLucia, E. H., & Berenbaum, M. R. (2008). Anthropogenic increase in carbon dioxide compromises plant defense against the invasive insect. *PNAS, 105*(13), 5129–5133.

Zhang, Q., Liu, H., Wu, X., et al. (2020). Identification of drought-tolerant mechanisms in a drought-tolerant maize mutant based on physiological, biochemical and transcriptomic analyses. *BMC Plant Biology, 20*, 315.

Zhang, X., Yang, F., Ma, H., & Li, J. (2022). Evaluation of the saline–alkaline tolerance of Rice (Oryza sativa L.) mutants induced by heavy-ion beam mutagenesis. *Biology, 11*, 126.

Ziska, L. H., & Dukes, J. S. (2010). *Weed biology and climate change* (p. 248). Blackwell Publishing Ltd.

Zu, X., Yanke, L., Qianqian, W., Yumei, L., Xinyue, H., Feng, T., Jiayu, N., Huihui, X., Yufeng, W., Shaoxia, Z., Kun, L., Huhui, C., Sheng, Q., Qi, R., Huaqi, W., & Honggui, L. (2021). Increased Drought Resistance 1 Mutation Increases Drought Tolerance of Upland Rice by Altering Physiological and Morphological Traits and Limiting ROS Levels. *Plant and Cell Physiology, 62*(7), 1168–1184.

# Induced Mutagenesis-A Reliable Technology to Overcome the Limitations of Low Genetic Variability in Lentils


**Mohammad Rafiq Wani, Aamir Raina, Nasya Tomlekova, Rafiul Amin Laskar, Mohammad Feroz, and Samiullah Khan**

**Abstract** Practices of agriculture and plant breeding approaches are indispensable for feeding the populaces of the world. In agriculture, the grain legumes occupy a unique position for their value as food and fodder, their role in biological nitrogen fixation, and as industrial raw materials. There are several reasons for the low productivity of pulses, which include a lack of high yielding genotypes, the vagaries of the monsoon, sowing on marginal lands under rain-fed conditions, negligence of plant protection, and imbalances of plant nutrients. Lack of genetic variability limits the scope of selection for better genotypes. For improvement in seed yield, genetic reconstitution of such crops is required to evolve better plant types. Mutation breeding has proven beneficial to upsurge the existing germplasm variability for improving certain specific traits of the varieties. By integrating molecular high throughput

M. R. Wani (✉)
Department of Botany, Abdul Ahad Azad Memorial Degree College Bemina, Cluster University, Srinagar, Jammu and Kashmir, India

A. Raina
Mutation Breeding Laboratory, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

Botany Section, Women's College, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

N. Tomlekova
Molecular Biology Laboratory, Maritsa Vegetable Crops Research Institute, Agricultural Academy, Plovdiv, Bulgaria

R. A. Laskar
Department of Botany, Pandit Deendayal Upadhyaya Adarsha Mahavidyalaya (PDUAM), Eraligool, Karimganj, Assam, India

M. Feroz
Department of Zoology, Abdul Ahad Azad Memorial Degree College Bemina, Cluster University, Srinagar, Jammu and Kashmir, India

S. Khan
Mutation Breeding Laboratory, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India



A. Raina et al. (eds.), *Advanced Crop Improvement, Volume 2*,
https://doi.org/10.1007/978-3-031-26669-0_9

mutation screening techniques, induced mutations could increase the required genetic diversity for the improvement of pulses, particularly lentils.

**Keywords** Food security · Mutagenesis · Mutagens · In vitro technologies · Soma clonal variation · Tissue culture

## 1 Introduction

Pulses, or grain legumes, are important crops that provide high-quality proteins in developing countries, including India. Pulses, designated as the chief components of agricultural food crops, are consumed by the predominantly substantial vegetarian population of India. In dietetic terms, pulses match cereals in terms of protein and minerals, besides serving as a rotation crop with cereals, thereby lessening the soil pathogens and improving the physical properties of the soil. Pulses build up a mechanism for fixing atmospheric nitrogen to meet their nitrogen requirements (Wani et al., 2021; Raina et al., 2022a). In India, pulses are generally grown with minimum resource inputs, so they are less pricey than animal proteins.

Cultivated in rain-fed environments, pulses usually do not require rigorous irrigation facilities which qualifies them to grow even in such soils that are not favorable for the cultivation of cereals and cash crops. Moreover, pulses possess several other useful qualities, such as improving soil fertility, fitting in mixed and intercropping systems, and providing green pods as vegetables for humans and feedstuff for livestock. Despite limitations like an unfavorable environment, a dearth of superior seeds, a lack of proper post-harvest management, and deficient marketability, India has been successful to raise the annual pulse production from 8.41 to 23.02 million tonnes owing to an area expansion from 19.09 million hectares in 1950–51 to 27.87 million hectares in 2019–20 and filling a yield gap from 441 to 826 kg/ha (Table 1). In world agriculture, legumes are specially cultivated for food proteins (Khadke & Kothekar, 2011; Raina et al., 2022b; Rasik et al., 2022).

A large number of legume species, hitherto unexplored, have great potential for not only contributing as a major source of dietary protein for humans but also providing excellent fodder for livestock. The Food and Agriculture Organization (FAO) of the United Nations recognizes 10 primary and 5 minor pulse crops cultivated globally in over 105 countries. From the production perspective, dry beans (26.8 mt), dry pea (14.3 mt), chickpea (12.0 mt), cowpea (7.69 mt), lentil (6.3 mt), and pigeon pea (4.4 mt) are of utmost importance (FAO, 2017). Among the ten primary pulse crops recognized by the FAO, lentil is indispensable. In 1950–51, the percent share of pulses in the total food-grain basket in India, vis-à-vis area, production, and productivity, was 19.62, 16.55, and 84.48, respectively. This trend continued till 1960–61 and started dwindling from 1970–71 due to non-advancement in the production technologies of pulses as compared to other food grains. In 2019–20, the

**Table 1** Share of pulses to total food grains in India

| | Pulses | | | Food grains | | | Pulses % to food grains | | |
|---|---|---|---|---|---|---|---|---|---|
| Year | A | P | Y | A | P | Y | A | P | Y |
| 1950–51 | 19.09 | 8.41 | 441 | 97.32 | 50.82 | 522 | 19.62 | 16.55 | 84.48 |
| 1960–61 | 23.56 | 12.70 | 539 | 115.58 | 82.02 | 710 | 20.38 | 15.48 | 75.92 |
| 1970–71 | 22.54 | 11.82 | 524 | 124.32 | 108.42 | 872 | 18.13 | 10.90 | 60.09 |
| 1980–81 | 22.46 | 10.63 | 473 | 126.67 | 129.59 | 1023 | 17.73 | 8.20 | 46.24 |
| 1990–91 | 24.66 | 14.26 | 578 | 127.84 | 176.39 | 1380 | 19.29 | 8.08 | 41.88 |
| 1995–96 | 22.28 | 12.31 | 552 | 121.01 | 180.42 | 1491 | 18.41 | 6.82 | 37.02 |
| 2000–01 | 20.35 | 11.08 | 544 | 121.05 | 196.81 | 1626 | 16.81 | 5.63 | 33.46 |
| 2001–02 | 22.01 | 13.37 | 607 | 122.78 | 212.85 | 1734 | 17.93 | 6.28 | 35.01 |
| 2002–03 | 20.50 | 11.13 | 543 | 113.86 | 174.77 | 1535 | 18.00 | 6.37 | 35.37 |
| 2003–04 | 23.46 | 14.91 | 635 | 123.45 | 213.19 | 1727 | 19.00 | 6.99 | 36.77 |
| 2004–05 | 22.76 | 13.13 | 577 | 120.00 | 198.36 | 1652 | 18.97 | 6.62 | 34.93 |
| 2005–06 | 23.39 | 13.39 | 598 | 121.60 | 208.60 | 1715 | 18.41 | 6.42 | 34.87 |
| 2006–07 | 23.76 | 14.11 | 594 | 124.07 | 211.78 | 1707 | 19.15 | 6.66 | 34.80 |
| 2007–08 | 23.63 | 14.76 | 625 | 124.07 | 230.78 | 1860 | 19.05 | 6.40 | 33.58 |
| 2008–09 | 22.09 | 14.57 | 660 | 122.83 | 234.47 | 1909 | 17.98 | 6.21 | 34.55 |
| 2009–10 | 23.28 | 14.66 | 630 | 121.33 | 218.11 | 1798 | 19.19 | 6.72 | 35.03 |
| 2010–11 | 26.40 | 18.24 | 691 | 126.67 | 244.49 | 1930 | 20.84 | 7.46 | 35.80 |
| 2011–12 | 24.46 | 17.09 | 699 | 124.76 | 259.32 | 2079 | 19.61 | 6.59 | 33.61 |
| 2012–13 | 23.25 | 18.34 | 789 | 120.77 | 257.12 | 2129 | 19.25 | 7.13 | 37.06 |
| 2013–14 | 25.21 | 19.25 | 764 | 125.04 | 265.04 | 2120 | 20.16 | 7.26 | 36.03 |
| 2014–15 | 23.10 | 17.16 | 743 | 122.07 | 252.67 | 2069 | 18.92 | 6.79 | 35.91 |
| 2015–16 | 24.91 | 16.35 | 656 | 123.22 | 251.57 | 2042 | 20 | 7 | 32 |
| 2016–17 | 29.45 | 23.13 | 786 | 129.23 | 275.11 | 2129 | 23 | 8 | – |
| 2017–18 | 29.81 | 25.42 | 853 | 127.52 | 285.01 | 2234 | 23 | 9 | – |
| 2018–19 | 29.16 | 22.08 | 757 | 124.78 | 285.21 | 2286 | 23 | 8 | – |
| 2019–20* | 27.87 | 23.02 | 826 | 124.77 | 291.95 | 2340 | 22 | 8 | – |

Source: http://dpd.dacnet.nic.in; DES, Ministry of Agri. & FW (DAC&FW), Govt. of India
*III Advance Estimate
*A* million hectares, *P* million tonnes, *Y* kg/ha

production has gone down to 8% as compared to other food grains (Table 1). Even though this crop group is imperative from a nutritional perspective, there has been no significant rise in area and production recorded from 1950–51 to 2009–10. Nevertheless, substantial progress in area and production was recorded from 2010–11 to 2018–19. Due to the progression in infrastructural and irrigation amenities, the pulse crops get sidelined treatment, which pushes them to nutrient deficient and marginal land pieces (http://dpd.dacnet.nic.in), thereby leading to the emergence of poor crops with deficient productivity and poor seed quality.

## 2 Origin, Area, Production, and Productivity of Lentil

Lentil (*Lens culinaris* Medik) is an annual herb, erect in growth, light green in color, freely branched, with a slender stem and soft, hairy foliage (Fig. 1). Being one of the oldest cultivated legume crops, lentil originated in the Fertile Crescent of the Near East and then spread to Europe, the Middle East, Northern Africa, and the Indo-Gangetic plains (Ford et al., 2007); and it was domesticated in the Near East arc in early Neolithic times (Ladizinsky, 1979a). Lentil is an important crop of dry-land agriculture and a valuable human food, mostly consumed as dry seeds. The straw and pod walls have a high food value, and the husk is used as livestock feed.

India stood first in the area and second in production of lentils with 43% and 23% of the global area and production, respectively. New Zealand recorded the highest yield of 2667 kg/ha followed by China with a yield of 2239 kg/ha. Canada ranked first in production (38%) producing a yield of 1971 kg/ha followed by India (23%) producing a yield of 600 kg/ha (Table 2). However, as per the average data of 2014–18 (Table 3), India ranked second in area and production, with 30% and 20% of world area and production, respectively.

Lentil (2n = 14), having a large genome of 41,063 Mbp (Arumuganathan & Earle, 1991), is an important pulse crop of the winter season and grows in nearly all parts of India as an intercrop or pure crop. The large chromosome size and small chromosome number make lentils suitable material for cytogenetic studies. In India, the area, production, and productivity of lentils during 2019–20* were 1.32 million hectares, 1.18 million tonnes, and 894 kg/ha, respectively (Table 4). Being a cool-season crop, lentil production is mostly narrowed to northern and central India. Uttar Pradesh, Madhya Pradesh, West Bengal, Bihar, and Jharkhand are the major lentil-producing states of India on the basis of their percentage share of production during 2019–20* (Table 5; Fig. 2).

**Fig. 1** Lentil sprout buds. (Source: pixabay.com)

**Table 2** Global ranking in area, production, and yield of lentil: major countries

| | Area (Lakh ha) | | | Production (Lakh tonnes) | | | Yield (kg/ha) | |
|---|---|---|---|---|---|---|---|---|
| Rank | Country | Area | % to world | Country | Production | % to world | Country | Yield |
| 1 | India | 18.90 | 43.50 | Canada | 18.805 | 37.98 | New Zealand | 2667 |
| 2 | Canada | 9.542 | 21.96 | India | 11.340 | 22.90 | China | 2239 |
| 3 | Turkey | 2.812 | 6.47 | Turkey | 4.170 | 8.42 | Australia | 2237 |
| 4 | Nepal | 2.065 | 4.75 | Australia | 3.241 | 6.55 | Egypt | 2167 |
| 5 | Australia | 1.449 | 3.34 | USA | 2.277 | 4.60 | Canada | 1971 |
| 6 | USA | 1.404 | 3.23 | Nepal | 2.269 | 4.58 | USA | 1621 |
| 7 | Syrian Arab | 1.280 | 2.95 | China | 1.500 | 3.03 | France | 1613 |
| 8 | Iran | 1.200 | 2.76 | Ethiopia | 1.298 | 2.62 | Turkey | 1483 |
| 9 | Ethiopia | 1.081 | 2.49 | Syrian Arab | 1.250 | 2.52 | Armenia | 1308 |
| 10 | Bangladesh | 0.898 | 2.07 | Bangladesh | 0.930 | 1.88 | Argentina | 1250 |
| 11 | Australia | 1.449 | 3.34 | USA | 2.277 | 4.60 | Canada | 1971 |
| | | | | | | | India | 600 |
| | **World** | **43.447** | | **World** | **49.517** | | **World** | **1140** |

Source: FAO Statistics 2013

**Table 3** Area (lakh hectares), production (lakh tonnes), and yield (kg/ha) of lentils in major countries (Average: 2014–18)

| Country | Area | % Contribution | Production | % Contribution | Yield |
|---|---|---|---|---|---|
| Canada | 17.98 | 34 | 27.03 | 46 | 1503 |
| India | 15.92 | 30 | 11.50 | 20 | 722 |
| Turkey | 2.53 | 5 | 3.65 | 6 | 1441 |
| USA | 2.74 | 5 | 3.33 | 6 | 1218 |
| Nepal | 2.04 | 4 | 2.31 | 4 | 1131 |
| **All above** | **41.21** | **(79%)** | **47.82** | **(81%)** | **1160** |
| **World** | **52.23** | | **58.84** | | **1127** |

Source: Lentil.cdr (dacnet.nic.in); Ministry of Agriculture and Farmers Welfare, Department of Agriculture, Cooperation and Farmers Welfare, Directorate of Pulses Development, Vindhyachal Bhavan, Bhopal, Madhya Pradesh

The country's area under lentil cultivation was 13.90 lakh hectares, with a production of 10.93 lakh tonnes (twelfth plan 2012–15; Fig. 3, Table 6). Madhya Pradesh was ranked first with respect to acreage at 39.59% (5.50 lakh hectares), followed by Uttar Pradesh at 33.95% and Bihar at 11.29%. Regarding production, Uttar Pradesh stood first with 34.36% (3.76 lakh tonnes) followed by Madhya Pradesh with 30.73% (3.36 lakh tonnes) and Bihar with 17.35% (1.90 lakh tonnes). The state of Bihar recorded the highest yield of 1209 kg/ha followed by Rajasthan and West Bengal with 962 kg/ha and 960 kg/ha, respectively. The national yield average was 786 kg/ha. The lowest yield of 327 kg/ha was recorded in the state of Chhattisgarh, followed by Maharashtra (400 kg/ha) and Madhya Pradesh (610 kg/ha).

**Table 4** All-India area, production, and yield of lentil

| Year | Area | Production | Yield |
|---|---|---|---|
| 2000–01 | 1.48 | 0.92 | 619 |
| 2001–02 | 1.47 | 0.97 | 664 |
| 2002–03 | 1.38 | 0.87 | 634 |
| 2003–04 | 1.40 | 1.04 | 743 |
| 2004–05 | 1.47 | 0.99 | 675 |
| 2005–06 | 1.51 | 0.95 | 629 |
| 2006–07 | 1.47 | 0.91 | 621 |
| 2007–08 | 1.31 | 0.81 | 622 |
| 2008–09 | 1.38 | 0.95 | 693 |
| 2009–10 | 1.48 | 1.03 | 697 |
| 2010–11 | 1.60 | 0.94 | 591 |
| 2011–12 | 1.56 | 1.06 | 678 |
| 2012–13 | 1.42 | 1.13 | 797 |
| 2013–14 | 1.34 | 1.02 | 761 |
| 2014–15 | 1.47 | 1.04 | 705 |
| 2015–16 | 1.28 | 0.98 | 765 |
| 2016–17 | 1.46 | 1.22 | 838 |
| 2017–18 | 1.55 | 1.62 | 1047 |
| 2018–19 | 1.36 | 1.23 | 901 |
| 2019–20* | 1.32 | 1.18 | 894 |

Source: Directorate of Economics & Statistics, DAC&FW; Agricultural Statistics at a Glance – 2020 (English version).pdf (dacnet.nic.in)

*4th Advance Estimates

Area – million hectares, Production – million tonnes, Yield – kg/ha

## 3 Nutrient Composition and Growth Habit of Lentil

Lentil is a valuable protein-rich human food. The protein content ranges from 24 to 100 g. It is an excellent dietary supplement due to its high protein content and nutrient density, which stabilize the nutritive insufficiencies of a cereal-based diet. The pulse plants, during their cultivation, augment nutrient status by accumulating nitrogen, carbon, and organic matter in the soil besides increasing the farmer's revenue with high market returns. It also has high levels of dietary fiber, vitamins, and carbohydrates (Erskine et al., 1990). The young pods are eaten as vegetables and ground into flour to make a variety of preparations. In the Indian subcontinent, lentil is commonly consumed as "dal." Moreover, bold and attractive-looking lentil grains have a high demand for exportation at premium prices. Lentils are supposed to prevent constipation and represent a major source of lectins, which are used for treating the prophylaxis of retroviral infections, including HIV. Also, lentils have anti-carcinogenic, blood pressure-lowering, hypocholesterolemic and hypoglycemic

**Table 5** Area, production, and yield in the major lentil producing states of India during 2018–19 and 2019–20*

| 2019–20* | | | | | | 2018–19 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| State | Area | % to All-India | Production | % to All-India | Yield | Area | % to All-India | Production | % to All-India | Yield |
| Uttar Pradesh | 0.46 | 35.17 | 0.45 | 38.47 | 978 | 0.48 | 34.93 | 0.49 | 39.78 | 1026 |
| Madhya Pradesh | 0.38 | 28.79 | 0.32 | 26.95 | 837 | 0.42 | 30.89 | 0.33 | 26.85 | 783 |
| West Bengal | 0.17 | 13.07 | 0.16 | 13.88 | 950 | 0.17 | 12.49 | 0.14 | 11.52 | 831 |
| Bihar | 0.15 | 11.57 | 0.12 | 10.26 | 793 | 0.15 | 10.85 | 0.15 | 12.06 | 1001 |
| Jharkhand | 0.06 | 4.73 | 0.05 | 4.59 | 867 | 0.06 | 4.20 | 0.05 | 4.08 | 875 |
| Others | 0.09 | 6.67 | 0.07 | 5.84 | 782 | 0.09 | 6.63 | 0.07 | 5.72 | 777 |
| **All-India** | **1.32** | 100.00 | **1.18** | 100.00 | **894** | **1.36** | 100.00 | **1.23** | 100.00 | **901** |

Source: Directorate of Economics & Statistics, DAC&FW; Agricultural Statistics at a Glance – 2020 (English version).pdf (dacnet.nic.in); *4th Advance Estimate

Area – million hectares, Production – million tonnes, Yield – kg/ha

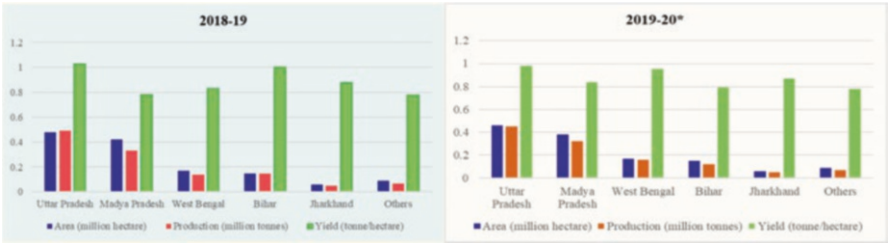



**Fig. 2** Comparative area (million hectares), production (million tonnes), and yield (tonnes/ha) in major lentil producing states of India during 2018–19 and 2019–20*. (Source: Directorate of Economics & Statistics, DAC&FW; *4th Advance Estimate)

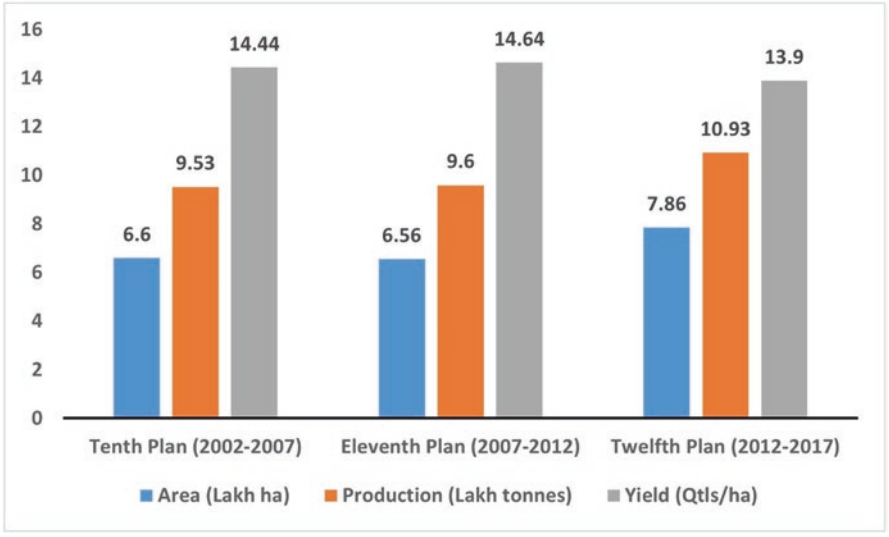



**Fig. 3** Plan wise national scenario of lentil. (Source: http://dpd.dacnet.nic.in)

effects (Faris et al., 2012). The comprehensive nutritional composition of lentils is given in Tables 7 and 8.

Lentil plants are slender, semi-erect, with compound leaves (4–7 pairs of leaflets) that terminate at apices with a tendril. Plants normally range from 20–30 cm tall. Flowering begins from lower to upper branches and continues till harvest. Pods are oblong and smooth, about 1.3 cm long, and contain one or two lens-shaped seeds. Flowers are self-pollinated and are white or pale blue in color. Plants tend to lodge at maturity due to their weak stems. Germination is hypogeal.

**Table 6** Plan-wise lentil scenario – states

| State | Xth Plan | % to AI | XIth Plan | % to AI | XIIth Plan | % to AI | State |
|---|---|---|---|---|---|---|---|
| Assam | A | 0.20 | 1.39 | 0.22 | 1.50 | 0.29 | 2.12 |
| | P | 0.11 | 1.15 | 0.11 | 1.15 | 0.20 | 1.80 |
| | Y | 548 | | 511 | | 668 | |
| Bihar | A | 1.72 | 11.91 | 1.81 | 12.36 | 1.57 | 11.29 |
| | P | 1.35 | 14.17 | 1.59 | 16.56 | 1.90 | 17.35 |
| | Y | 787 | | 878 | | 1209 | |
| Chhattisgarh | A | 0.17 | 1.18 | 0.16 | 1.09 | 0.14 | 1.00 |
| | P | 0.05 | 0.52 | 0.05 | 0.52 | 0.05 | 0.42 |
| | Y | 212 | | 322 | | 327 | |
| Haryana | A | 0.06 | 0.42 | 0.04 | 0.27 | 0.05 | 0.39 |
| | P | 0.05 | 0.52 | 0.03 | 0.31 | 0.05 | 0.46 |
| | Y | 900 | | 783 | | 935 | |
| Madhya Pradesh | A | 5.06 | 35.04 | 5.5 | 37.57 | 5.50 | 39.59 |
| | P | 2.43 | 25.50 | 2.33 | 24.27 | 3.36 | 30.73 |
| | Y | 481 | | 424 | | 610 | |
| Maharashtra | A | 0.07 | 0.48 | 0.07 | 0.48 | 0.04 | 0.26 |
| | P | 0.03 | 0.31 | 0.03 | 0.31 | 0.01 | 0.13 |
| | Y | 368 | | 431 | | 400 | |
| Punjab | A | 0.03 | 0.21 | 0.01 | 0.07 | 0.01 | 0.061 |
| | P | 0.02 | 0.21 | 0.01 | 0.10 | 0.01 | 0.050 |
| | Y | 560 | | 673 | | 647 | |
| Rajasthan | A | 0.19 | 1.32 | 0.28 | 1.91 | 0.31 | 2.23 |
| | P | 0.19 | 1.99 | 0.25 | 2.60 | 0.30 | 2.72 |
| | Y | 995 | | 917 | | 962 | |
| Uttar Pradesh | A | 5.96 | 41.27 | 5.56 | 37.98 | 4.72 | 33.95 |
| | P | 4.65 | 48.79 | 4.44 | 46.25 | 3.76 | 34.36 |
| | Y | 781 | | 799 | | 796 | |
| Uttarakhand | A | 0.16 | 1.11 | 0.15 | 1.02 | 0.11 | 0.82 |
| | P | 0.08 | 0.84 | 0.09 | 0.94 | 0.10 | 0.89 |
| | Y | 494 | | 605 | | 847 | |
| West Bengal | A | 0.65 | 4.50 | 0.55 | 3.76 | 0.65 | 4.66 |
| | P | 0.45 | 4.72 | 0.44 | 4.58 | 0.62 | 5.68 |
| | Y | 686 | | 791 | | 960 | |
| All-India | A | 14.44 | | 14.64 | | 13.90 | |
| | P | 9.53 | | 9.60 | | 10.93 | |
| | Y | 660 | | 656 | | 786 | |

Source: http://dpd.dacnet.nic.in
*AI* All-India, *A* lakh hectares, *P* lakh tonnes, *Y* kg/ha

**Table 7** Nutrient composition of lentil (raw seeds)

| Nutrient | Unit | Value per 100 g |
|---|---|---|
| **Proximates** | | |
| Water | g | 8.26 |
| Energy | kcal | 352 |
| Total lipid (fat) | g | 1.06 |
| Carbohydrate | g | 63.35 |
| Fiber, total dietary | g | 10.7 |
| Sugars, total | g | 2.03 |
| **Minerals** | | |
| Calcium, Ca | mg | 35 |
| Iron, Fe | mg | 6.51 |
| Magnesium, Mg | mg | 47 |
| Phosphorus, P | mg | 281 |
| Potassium, K | mg | 677 |
| Sodium, Na | mg | 6 |
| Zinc, Zn | mg | 3.27 |
| **Vitamins** | | |
| Vitamin C, total ascorbic acid | mg | 4.5 |
| Thiamin | mg | 0.873 |
| Riboflavin | mg | 0.211 |
| Niacin | mg | 2.605 |
| Vitamin B-6 | mg | 0.540 |
| Folate, DFE | µg | 479 |
| Vitamin B-12 | µg | 0.00 |
| Vitamin A, RAE | µg | 2 |
| Vitamin A, IU | IU | 39 |
| Vitamin E (alpha-tocopherol) | mg | 0.49 |
| Vitamin D (D2 + D3) | µg | 0.0 |
| Vitamin D | IU | 0 |
| Vitamin K (phylloquinone) | µg | 5.0 |
| **Lipids** | | |
| Fatty acids, total saturated | g | 0.154 |
| Fatty acids, total monounsaturated | g | 0.193 |
| Fatty acids, total polyunsaturated | g | 0.526 |
| Fatty acids, total trans | g | 0.000 |
| Cholesterol | mg | 0 |
| **Others** | | |
| Caffeine | mg | 0 |

Nutrient values and weights are for edible portions
Source: https://www.nutritionvalue.org/Lentils%2C_raw_nutritional_value.html

**Table 8** Amino acid composition of lentil (g/100-g protein)

| Amino acid | Value per 100 g |
|---|---|
| Protein | 24.63 g |
| Alanine | 1.029 g |
| Arginine | 1.903 g |
| Aspartic acid | 2.725 g |
| Cysteine | 0.322 g |
| Glutamic acid | 3.819 g |
| Glycine | 1.002 g |
| Histidine | 0.693 g |
| Isoleucine | 1.065 g |
| Leucine | 1.786 g |
| Lysine | 1.720 g |
| Methionine | 0.210 g |
| Phenylalanine | 1.215 g |
| Proline | 1.029 g |
| Serine | 1.136 g |
| Threonine | 0.882 g |
| Tryptophan | 0.221 g |
| Tyrosine | 0.658 g |
| Valine | 1.223 g |

Source: https://www.nutritionvalue.org/Lentils%2C_raw_nutritional_value.html

## 4 Varieties, Climatic Conditions, Insect Pests, and Diseases of Lentil

The varieties of lentils are broadly classified as *microsperma* and *macrosperma* types. *Microsperma* is small, with round seeds about 2–6 mm in diameter, cotyledons are yellow or orange, and the testa ranges from pale yellow to black in color. *Macrosperma,* on the other hand, has large, flattened seeds, 6–9 mm in diameter, having yellow cotyledons and pale green testa. In India, large seeded (*macrosperma*) types are mostly cultivated in the central zone, such as the Bundelkhand regions of Uttar Pradesh, Madhya Pradesh, and Maharashtra, whereas small seeded (*microsperma*) types are grown in Indo-Gangetic plains such as Bihar, Eastern Uttar Pradesh, West Bengal, and Assam. Bold seeded types are generally poor yielders.

Lentil is adapted to cool growing conditions. It is a resilient crop, tolerating frost and severe winters to a greater extent. Well-drained loam soils are best suited for lentil cultivation. Extreme drought and high temperatures during the flowering and pod-filling stages reduce the yield. The world is expecting 30% population growth by 2050, which puts an unprecedented demand on already climate threatened agricultural production. Pulses, especially lentils, have great potential to solve global food insecurity in changing climates. Lentil is infected by many insect pests and aphids. *Aphis craccivora* is dominant among aphid species that attack and damage

the crop. Spiny pod borer (*Etiella zinckenella*) causes minor to moderate damage to lentil pods. The major diseases of lentils are wilt (*Fusarium oxysporum*) and rust (*Uromyces fabae*). However, the occasional incidence of stem root rot, powdery mildew, and *Alternaria* blight are also reported, particularly under humid climatic conditions.

## 5 Limitations and Scope of Traditional and Modern Plant Breeding

In the late ’70 s and early ’80 s, inheritance studies of several morphological and agronomical traits, viz., seed coat, epicotyl and flower color, dehiscence of the pod, etc., showed that most of the traits are monogenic and useful as morphological markers (Haddad et al., 1978; Ladizinsky, 1979b; Muehlbauer & Slinkard, 1981). In lentil, Zamir and Ladizinsky (1984) reported the first genetic linkage analysis. The first use of recombinant inbred lines (RILs) for mapping lentil markers was reported by Tahir et al. (1994), who determined six linkage groups in which they mapped four morphological and 17 isozyme markers. The use of intraspecific crosses was rare in the past for the development of linkage maps due to the limited availability of variation in cultivated species. However, segregating populations of lentils from intraspecific crosses has been utilized in several classical mapping experiments to establish linkage groups among the morphological markers. Linkage relationships among seed coat pattern, pod pubescence, and flowering time (Sarker et al., 1999), leaf color, and plant pubescence (Hoque et al., 2002) were demonstrated in lentil. Kumar et al. (2004, a, b) found two linkage groups among different morphological markers in lentils, namely, leaf pigmentation, stem pigmentation, pod pigmentation, erect growth habit and color of the leaf, pubescence of the plant, number of leaflets per leaf, and plant height.

Breeding objectives of lentils usually differ depending on the difficulties and primacies of farmers and consumers of the specific regions. Higher stable seed yield, disease resistance, and better seed quality are the main breeding goals of the key exporting countries; however, for import-dependent countries like India, increased yield per hectare remains the key resolve (Muehlbauer et al., 1995). Local factors and several biotic and abiotic stresses are the main impediments toward the global yield improvement of lentils, particularly in resource-deprived countries (Tivoli et al., 2006; Muehlbauer et al., 2006; Sinclair & Vadez, 2012). Though in lentils, traditional breeding has been efficacious over the past in addressing main production constraints and developing varieties resistant to major biotic or abiotic stresses (Muehlbauer et al., 2006; Materne & McNeil, 2007), its scope is limited attributable to low genetic variability, scarceness of genetic information, and accurate selection methods.

Molecular breeding has provided plant breeders with a reliable means to overcome these limitations for rapidly improving the crop. PCR-based markers have been proven to be a useful tool for indirect selection of desirable traits with

high accuracy that would otherwise be difficult or time-consuming using conventional methods. Currently, the regular use of markers in lentil breeding programs is very limited. In the near future, the sustainable implementation of advanced molecular techniques to develop novel markers for highly sought-after traits would accelerate the lentil improvement programs.

Being a self-pollinated crop, lentil predominantly has a low rate of outcrossing, which results in low genetic variability and restricts the trait improvement programs. Due to the dearth of sufficient natural variability, conventional methods of plant breeding had a limited scope for the improvement of lentils. In these circumstances, mutation breeding, a well-functioning branch of plant breeding, supplements the conventional methods in a favorable manner (Gottschalk, 1986). Mutation breeding is widely exploited to modify one or a few traits in an otherwise outstanding variety without altering its original genetic makeup and other phenotypic traits (Raina & Khan, 2020; Raina & Danish, 2018). In that sense, it provides a rapid method to improve indigenous crop varieties without going through exhaustive procedures of hybridization and backcrossing (Raina et al., 2019; Sellapillaibanumathi et al., 2022). Induced mutagenesis is a powerful tool to generate new genetic variability in the traits of interest for boosting the breeding programs has already been established in different crop plants (Sharma, 1990; Reddy & Annadurai, 1992; Wani & Khan, 2003; Solanki, 2005; Wani et al., 2017; 2021; Wani, 2018, 2020, 2021; Amin et al., 2016, 2019; Goyal et al., 2019a, b, 2020a, b, 2021a, b; Raina et al., 2020a, b, 2021, 2022d) and with the advent of marker-based selection techniques; the possibilities of improving the crop plants in general, and lentils in particular, have tremendously increased.

## 6 Mutagenesis in Lentil

### *6.1 Mutagenesis and Biological Damage*

In biological material, mutagens induce biological damage, gene mutations, and chromosomal aberrations in the $M_1$ generation. Out of these, gene and chromosomal mutations may pass on to the subsequent generations, while biological damage may remain confined to only the $M_1$ generation. The study of biological damage in the $M_1$ generation is normally used to appraise the mutagenic potency and sensitivity of the biological material. Biological effects represent injuries that can be determined cytologically and measured by growth reduction and death of the plant. The induction of mutations and their use in the development of mutant varieties in lentils are well documented by Sharma (1997) with different doses of gamma rays and NMU in *microsperma* and *macrosperma* lentils. A progressive reduction in seed germination, pollen fertility, seedling growth, and plant survival was reported with increasing doses of mutagens in the $M_1$ generation. Root length was more affected than shoot length concerning mutagenic treatments.

Based on $M_1$ biological parameters, Sinha and Godward (1972) found *macrosperma* lentils to be more sensitive to mutagens than *microsperma* lentils.

Sarker and Sharma (1989) reported that mutagenic treatment induced significant biological damage in $M_1$ parameters. However, the trend of mutagenic damage varied with different doses and durations of mutagenic treatments. Gamma rays drastically affected fertility and seedling height, while EMS and NEU severely impacted fertility, germination, and plant survival.

Different mutagenic treatments revealed differential trends vis-à-vis plant survival, plant height, and seeds per pod in variety K-85 of lentils (Tripathi & Dubey, 1992). Tufail et al. (1998) reported that the proportion of plant emergence and plant survival at maturity in varieties Pant L406, Masoor 85, Precoz, and L605 decreased with a corresponding increase in radiation doses. Variety L605 appeared to be the most sensitive, followed by Pant L406, Masoor 85, and Precoz. Sharma and Sharma (1986), Sinha and Chaudhary (1987), and Kalia and Gupta (1988) reported greater radiosensitivity of *macrosperma* lentil, whereas Singh et al. (1989) reported that the *microsperma* variety Pant L639 was more sensitive than the *macrosperma* variety RAU101 to 5 to 25 kR doses of gamma rays. The difference in the radiosensitivity of varieties from both groups was attributed to the difference in their genetic backgrounds (Rajput & Siddiqui, 1981; Malik et al., 1988). Tyagi and Sharma (1981) reported that differences in radiosensitivity exist within and between *microsperma* and *macrosperma* groups and concluded that the varietal differences were more conspicuous than the inter-group ones.

## *6.2 Cytological Effects*

The diploid chromosome number of lentils is $2n = 2\times = 14$. Two pairs of chromosomes are metacentric, two pairs are submetacentric, and three pairs are acrocentric. Cytological effects of physical and chemical mutagens were studied by Dixit (1985) in a variety of T-36 of lentils and reported direct linking of mitotic abnormalities with mutagenic dose. In comparison to gamma rays, NMU induced a lesser proportion of anomalous cells. Combination treatments of gamma rays and NMU showed the direct additive effect. Similarly, NMU induced the highest percentage of abnormal cells, as reported by Dixit and Dubey (1984). There was a direct association between the anomalies induced and the concentrations of mutagens applied. This statement agrees with Sinha and Godward's (1968) observation of lentils. Mitotic anomalies were directly proportional with increasing mutagen doses in variety K-333 (Tripathi, 1995). The anomalies detected included clumping, fragmentation, bridges, laggards, and an unequal distribution of chromosomes at anaphase. Chromosomal aberrations play an important role in inducing sterility, thereby influencing the recovery of mutations. Meiotic anomalies increased with increasing irradiation doses of gamma rays in variety T-36 of lentils. However, gamma rays and NMU in combination did not exhibit a synergistic effect on inducing meiotic abnormalities (Dixit & Dubey, 1983a).

## *6.3 Chlorophyll and Morphological Mutations*

The proficiency of different mutagens in bringing genetic variability for crop improvement is evaluated with the help of chlorophyll mutations, which are utilized as genetic markers in elementary and applied research. In NEU treatments, the incidence of chlorophyll mutants such as viridis, xantha, and chlorina was reported to be higher as compared to EI and gamma rays (Solanki & Sharma, 2001; Sarker & Sharma, 1989). Gamma rays at a dose of 15 kR, induced xantha, albo-xantha, and tigrina types of chlorophyll mutants (Paul & Singh, 2002). Chemical mutagens induced a higher frequency of chlorophyll mutations than radiation (Sharma & Sharma, 1981b; Tripathi & Dubey, 1992; Reddy et al., 1993; Vandana et al., 1994). Varied effectiveness and efficiency of mutagens in inducing chlorophyll mutations were reported by Dixit and Dubey (1986) in lentils. Sharma and Sharma (1979) compared the effectiveness and efficiency of NMU using *microsperma* genotypes as test symbols. In all the varieties, the mutation rate per unit dose of NMU was approximately three times higher than that of gamma rays.

Singh and Singh (1989) confirmed three categories of chlorophyll mutations, viz., albina, xantha, and viridis, in *microsperma* and *macrosperma* varieties of lentils following mutagenic treatments with gamma rays, EMS, and hydroxylamine (HA). As compared to gamma rays and HA, EMS was found to be the most efficient mutagen. Moreover, *microsperma* variety appeared more sensitive toward mutagenic agents than *macrosperma* variety indicating a possible role of seed size in the mutagenesis. Laskar and Khan (2017) studied the mutagenic effects of gamma rays and HZ in DPL-62 and Pant L-406 varieties of lentils, which resulted in the isolation of several kinds of mutants with altered phenotypes. Gamma rays and HZ at moderate doses showed higher effectiveness and efficiency, whereas, for combination treatments with some inter-varietal exceptions, lower doses were found to be most effective and efficient. The frequency of induced mutations in the $M_2$ generation appeared to have a direct association with mutagen-sensitive parameters in the $M_1$ generation (Dixit, 1985; Tripathi, 1995). Hence, the extent of induced mutagen damage through the reduction in germination, seedling growth, plant survival, chromosomal anomalies, and pollen and ovule sterility could be interconnected with mutational efficiency.

Induction of morphological mutations by physical or chemical mutagens in lentils was reported by various workers (Ramesh & Dhananjay, 1996; Solanki & Sharma, 1999; Laskar et al., 2018a, b; Wani et al., 2021). Sharma and Sharma (1979) studied the leaf mutation by treating the dry seeds of lentil with NMU and gamma rays. Leaf mutants isolated in $M_2$ included the boat leaf mutant (3–4 boat-shaped leaflets per leaf) and the crinkled leaf mutant (short leaf having 6–8 small, overlapping, and irregularly shaped leaflets). The segregation pattern showed that the crinkled leaf mutation was controlled by a single recessive gene designated as *"crl*." Sarker and Sharma (1986) studied the chlorophyll and morphological mutations in lentils after treatments with gamma rays, EMS, NEU, and SA. Of all the four mutagens, NEU was found to be more effective in inducing chlorophyll as well

as morphological mutations. Among the morphological mutations, narrow leaf and tendrilled mutations were induced more frequently by NEU and EMS, whereas broadleaf and bushy dwarf mutants were higher in gamma rays and SA treatments.

As compared to gamma rays in the $M_2$ and $M_3$ generations, the frequency of macromutations was higher in EMS treatments (Tyagi & Gupta, 1991). Mutants for growth habit and foliage types were induced by EMS treatments, whereas mutants for flowering behavior, maturity, duration, and plant height were induced by SA treatments (Solanki et al., 2004; Khan et al., 2006; Solanki, 2005; Solanki & Phogat, 2005). NMU induced sterile mutants and the mutants with tendrils instead of terminal two to three leaflets (Sharma & Sharma, 1978a). Sterile plants with elongated peduncles and multi-floret inflorescences were also reported by Sharma and Sharma (1981a).

In lentil, mutations for plant height, growth habit, branching, stem structure, leaf morphology, inflorescence, calyx, flower, pod, fertility, and seed colour were reported by different workers (Sharma & Sharma, 1983; Sinha et al., 1987; Tyagi & Gupta, 1991; Ashutosh & Dubey, 1992; Vandana et al., 1994; Ramesh & Dhananjay, 1996; Tyagi & Ramesh, 1998; Solanki & Sharma, 1999; Jeena & Singh, 2000). Based on the extent of height reduction, plant mutants were classified into dwarf, semidwarf, and bushy-dwarf types (Sharma & Sharma, 1982; Dixit & Dubey, 1983b; Vandana et al., 1994). At places of emergence, the branches were fused with the main stem, and the plant looked like a bunch of closely merged branches, giving it a "bunchy top" appearance. The mutation was controlled by a single recessive gene, *"fa,"* besides inducing disease resistance in mutant lines (Bravo, 1983).

## *6.4 Induced Variability for Quantitative Traits*

Due to the absence of adequate natural variability, conventional methods of plant breeding, i.e., introduction, selection, and hybridization, had a limited scope for crop improvement, particularly in pulses. New genetic variability demands could be achieved by crossing landraces with exotic material and/or through mutation breeding. Mutation breeding is a potent tool for creating genetic variability, particularly in species where hybridization is difficult or naturally existing variability has been exhausted (Raina et al., 2016, 2022c; Khursheed et al., 2016; Laskar et al., 2015; Tantray et al., 2017; Sellapillai et al., 2022). It is a sustainable technique available with plant breeders to broaden the genetic bases of crop plants and to create a gene pool of numerous desirable agro-economic traits (Raina et al., 2017) and is relatively cheaper to perform at a large scale (Siddiqui & Khan, 1999). The conventional mutagenesis technique for crop improvement is undergoing a renaissance due to progressions in contemporary cutting-edge technologies. Under a changing climate, mutation induction is a recognized technique to create diversity in existing crop varieties to expand the degree of adaptability for crop biomass enhancement (Laskar et al., 2019).

The mutation technique is considered better than other methods of crop improvement because it requires the least investment of land and labor (Gustaffson,

1947). In recent times, a lot of work has been done on induced mutagenesis in various crop plants. In these experimental crops, the mutational effect varied with varying mutagens and mutagenic doses. Thus, selecting an optimum dose of a mutagen for a genotype is an important step in mutation breeding programs (Khursheed et al., 2015, 2018a, b, c). Improvement of high yielding varieties is the basic necessity of the time. Plant breeders over and across the country have adopted various crop improvement strategies for generating variability and designing genotypes with high yield potential. Among the various breeding methodologies adopted, mutation has been considered a potent tool in the generation of requisite variability. The use of mutations resulting from irradiation or chemical mutagens has not received much attention as a breeding method in lentils. However, genetic variability has been created for many qualitative and quantitative characters such as pod and seed size, plant height, number of branches per plant, number of pods per plant, number of seeds per pod, dwarfing, early maturity, seed yield, days to flowering, and plant type (Sharma & Sharma, 1978b, 1981c; Dixit & Dubey, 1986).

Complex traits such as yield or seed size may be influenced by several genes, each with a very small effect, as suggested by classical quantitative genetic theory. These genes are commonly known as polygenes (Mather & Jinks, 1971). The mutation in polygenes is known as micromutation, and its usefulness in crop breeding has been emphasized by several workers (Lawrence, 1965; Scossiroli, 1966; Sindhu & Slinkard, 1983; Sinha & Chowdhary, 1984; Sarker & Sharma, 1988; Kalia & Gupta, 1989; Swarup et al., 1991; Ashutosh & Dubey, 1992; Khan et al., 2004; Khan & Wani, 2005; Khan & Wani, 2006; Khursheed et al., 2019). Experiments demonstrated that random mutations in quantitative traits could be induced in both positive and negative directions with the increase in variances. Such changes are due to increased genetic variation in the population (Yamaguchi, 2005). There are, however, conflicting reports as to whether mutations are induced equally in plus and minus directions or are unidirectional. Jalil and Yamaguchi (1964) observed in gamma-irradiated progenies of rice that, without selection, the mean values for seed size decreased due to successive irradiation. Moreover, subsequent irradiation with selection shifted the mean values toward the desired direction.

Physical and chemical mutagens in lentils have been used in the past, and as a result, varietal development has come up. Globally, up to the thirtieth of January 2022 (https://mvd.iaea.org accessed on 30 January 2022), mutation breeding has been successful in developing 3348 mutant varieties of crop plants (Fig. 4), including 466 varieties of pulses and 18 varieties of lentil. The principal contribution is from cereals (1596), followed by ornamental flowers (666), pulses (466), and edible oil crops (103). Among the 18 released mutant varieties of lentils, two varieties, namely, Ranjan and Rajendra Masoor 1 have been developed in India for various improved traits, particularly high yield, resistance to diseases, early maturity, and tolerance to cold. The description of lentil varieties released globally through mutagenesis is depicted in Table 9.

This study concludes that although there are numerous ways of improving the varieties of lentil and other pulse crops through conventional and contemporary breeding methods, the methods need to be improved in such directions to accomplish better success in breeding programs for these nutritionally important crops.

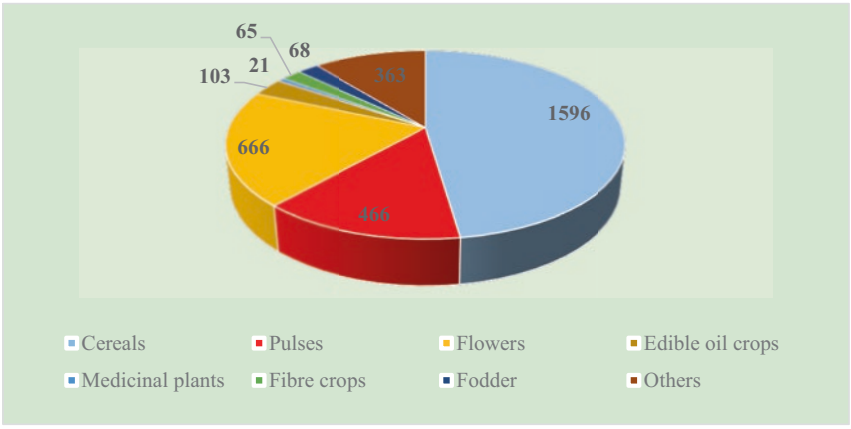



**Fig. 4** Number of mutant varieties of crop plants released in the world (Source: Joint FAO/IAEA, Vienna Mutant Variety Database (MVD); http://mvgs.iaea.org accessed on 30 January 2022)

**Table 9** Details of lentil varieties developed through mutation breeding

| Mutant variety name | Country | Year of registration | Developed by | Main improved attributes |
|---|---|---|---|---|
| S-256 (Ranjan) | India | 1981 | Irradiation | High yield, spreading type |
| Rajendra Masoor 1 | India | 1996 | 100 Gy gamma rays | Low-temperature tolerance, early maturity, good for late sowing |
| Mutant 17 MM | Bulgaria | 1999 | 40 Gy gamma rays | Vigorous growth habit, large leaflet, pods, and seeds, resistance to anthracnose, *Stemohylium,* and viruses, high yield, drought tolerance, improved cooking quality |
| Zornitsa | Bulgaria | 2000 | 0.1% EMS | High yield, high protein content (28.7%), good culinary and organoleptic quality, and resistance to anthracnose, viruses, and *Ascochyta* blight |
| Djudje | Bulgaria | 2000 | 30 Gy gamma rays | High yield, dwarf bushy habit, non-shattering, resistance to *Fusarium* and *Botrytis*, high protein content (27.9%), good culinary and organoleptic quality, suitable for mechanized harvesting |
| Binamasur-1 | Bangladesh | 2001 | Chemical mutagen | High yield, tolerant to rust and blight, black seed coat |
| Elitsa | Bulgaria | 2001 | 40 Gy gamma rays | High yield (34.4%), resistance to major disease |

(continued)

**Table 9** (continued)

| Mutant variety name | Country | Year of registration | Developed by | Main improved attributes |
|---|---|---|---|---|
| NIAB Masoor-2002 | Pakistan | 2002 | Irradiation | Erect growth habit, early maturity (120 days), black seed coat color, high grain yield, disease resistance, synchronous pod maturity |
| Verzuie | Moldova, Republic | 2004 | 250 Gy gamma rays | The main improved attributes are drought resistance, vegetative period, proteins, oils, fructose, glucose, starch, and cellulose. |
| Aurie | Moldova, Republic | 2005 | 250 Gy gamma rays | Drought resistance, high yield, early maturity, high protein content |
| Binamasur-2 | Bangladesh | 2005 | 200 Gy gamma rays | High yield, early maturity, and tolerance to rust and blight |
| Binamasur-3 | Bangladesh | 2005 | 0.5% EMS | High yield, early maturity, rust, and blight tolerance |
| NIAB Masoor-2006 | Pakistan | 2006 | 200 Gy gamma rays | A higher number of pods, resistance to lodging, blight, and rust, and 20–60% higher seed yield |
| Binamasur-5 | Bangladesh | 2011 | 200 Gy gamma rays | Early maturity, high yield |
| Binamasur-6 | Bangladesh | 2011 | 250 Gy gamma rays | Early maturity, high yield |
| Binamasur-8 | Bangladesh | 2014 | 200 Gy gamma rays | Early maturity, high yield |
| Binamasur-9 | Bangladesh | 2014 | 200 Gy gamma rays | Early maturity, high yield |
| Binamasur-11 | Bangladesh | 2017 | 200 Gy gamma rays | Early maturity, high yield, and plant architecture |

Source: Joint FAO/IAEA, Vienna Mutant Variety Database (MVD); https://mvd.iaea.org accessed on 30 January 2022

## References

Amin, R., Laskar, R. A., Khursheed, S., Raina, A., & Khan, S. (2016). Genetic sensitivity towards MMS mutagenesis assessed through *in vitro* growth and cytological test in *Nigella Sativa* L. *Life Sciences International Research Journal, 3*, 2347–8691.

Amin, R., Wani, M. R., Raina, A., Khursheed, S., & Khan, S. (2019). Induced morphological and chromosomal diversity in the mutagenized population of black cumin *(Nigella sativa* L.) using single and combination treatments of gamma rays and ethylmethane sulfonate. *Jordan Journal of Biological Sciences, 12*(1), 23–30.

Arumuganathan, K., & Earle, E. D. (1991). Nuclear DNA content of some important plant species. *Plant Molecular Biology, 9*(3), 208–218.

Ashutosh, T., & Dubey, D. K. (1992). Effects of separate and simultaneous application of gamma rays and N-nitroso-N-methyl urea on germination, growth, fertility and yield of two lentil varieties. *LENS Newsletters, 19*(1), 9–13.

Bravo, A. (1983). *Development of disease-resistant lines of grain legumes through mutation breeding. Induced mutations for disease resistance in crop plants II* (pp. 153–156). IAEA.

Dixit, P. (1985). *Studies on mutagenesis and polygenic variability induced by separate and synergistic action of a chemical and physical mutagen in lentil (Lens culinaris Medik) var. T-36*. Ph. D. Thesis, Kanpur University, Kanpur.

Dixit, P., & Dubey, D. K. (1983a). The meiotic consequences of chromosomal aberrations induced by separate and simultaneous applications of gamma rays and NMU in lentil (*Lens culinaris* Med.). *Advances in Life Sciences, 2*(1), 47–51.

Dixit, P., & Dubey, D. K. (1983b). Seedling mutations induced by separate and simultaneous application of gamma rays and NMU in lentil (*Lens culinaris* Med.) var. T-36. *Pulse Crops Newsletters, 3*, 14–17.

Dixit, P., & Dubey, D. K. (1984). Effect of chemical mutagens on the somatic chromosome and mitosis in lentil (*Lens culinaris* Med.). *Advances in Life Sciences, 11*, 13–15.

Dixit, P., & Dubey, D. K. (1986). Chlorophyll and seedling morphology mutations induced by separate and simultaneous applications of gamma rays and NMU in lentil. *LENS Newsletters, 13*(1), 5–8.

Erskine, W., Rihawe, S., & Capper, B. S. (1990). Variation in lentil straw quality. *Animal Feed Science and Technology, 28*, 61–69.

FAO. (2017). *Food and Agriculture Organization of the United Nations. 2017*. Food and Agriculture Organization of the United Nations.

Faris, M. A. E., Takruri, H. R., & Issa, A. Y. (2012). *Role of lentils (Lens culinaris L.) in human health and nutrition: A review*. Springer.

Ford, R., Redden, R. J., Materne, M., & Taylor, P. W. J. (2007). Lentil. In C. Kole (Ed.), *Pulses, sugar and tuber crops* (pp. 91–108). Springer.

Gottschalk, W. (1986). Experimental mutagenesis in plant breeding. In A. B. Prasad (Ed.), *Mutagenesis: Basic and applied* (pp. 81–96). Print House.

Goyal, S., Wani, M. R., Laskar, R. A., Raina, A., & Khan, S. (2019a). Assessment on cytotoxic and mutagenic potency of gamma rays and EMS in *Vigna mungo* L. Hepper. *Biotecnología Vegetal, 19*, 193–204.

Goyal, S., Wani, M. R., Laskar, R. A., Raina, A., Amin, R., & Khan, S. (2019b). Induction of morphological mutations and mutant phenotyping in black gram [*Vigna mungo* (L.) Hepper] using gamma rays and EMS. *Vegetos, 32*(4), 464–472.

Goyal, S., Wani, M. R., Laskar, R. A., Raina, A., & Khan, S. (2020a). Mutagenic effectiveness and efficiency of individual and combination treatments of gamma rays and ethyl methanesulfonate in black gram [*Vigna mungo* (L.) Hepper]. *Advances in Zoology and Botany, 8*(3), 163–168.

Goyal, S., Wani, M. R., Laskar, R. A., Raina, A., & Khan, S. (2020b). Performance evaluation of induced mutant lines of black gram (*Vigna mungo* (L.) Hepper). *Acta Fytotechnica et Zootechnica, 23*(2), 70–77.

Goyal, S., Wani, M. R., Raina, A., Laskar, R. A., & Khan, S. (2021a). Phenotypic diversity in mutagenized population of urdbean (*Vigna mungo* (L.) Hepper). *Heliyon, 7*, e06356.

Goyal, S., Wani, M. R., Raina, A., Laskar, R. A., & Khan, S. (2021b). Quantitative assessments on induced high yielding mutant lines in urdbean (*Vigna mungo* (L.) hepper). *Legume Science*, e125.

Gustaffson, A. (1947). Mutations in agricultural plants. *Hereditas, 33*, 1–100.

Haddad, N., Muehlbauer, F. J., & Hampton, R. O. (1978). Inheritance of resistance to pea seed-borne mosaic virus in lentils. *Crop Science, 18*, 613–615.

Hoque, M. E., Mishra, S. K., Kumar, Y., Kumar, R., Tomar, S. M. S., & Sharma, B. (2002). Inheritance and linkage of leaf colour and plant pubescence in lentil (*Lens culinaris* Medik.). *Indian Journal of Genetics, 62*, 140–142.

Jalil, M. A., & Yamaguchi, H. (1964). Experiments on the induction of polygenic mutations with successive irradiation in rice. *Phyton, 21*(2), 149–155.

Jeena, A. S., & Singh, L. S. (2000). Field evaluation of wild relatives of lentil. *Indian Journal of Pulses Research, 1*, 50–51.

Kalia, N. R., & Gupta, V. P. (1988). Differential radiosensitivity in microsperma and macrosperma lentils. *LENS Newsletters, 16*(1), 16–18.

Kalia, N. R., & Gupta, V. P. (1989). Induced polygenic variations in lentil. *LENS Newsletters, 16*(1), 16–18.

Khadke, S. G., & Kothekar, V. S. (2011). Genetic improvement of moth bean (*Vigna aconitifolia* (Jacq.) Marechal) through mutation breeding. In S. Khan & M. I. Kozgar (Eds.), *Breeding of pulse crops* (pp. 34–54). Kalyani Publishers.

Khan, S., & Wani, M. R. (2005). Genetic variability and correlations studies in chickpea mutants. *Journal of Cytology and Genetics, 6*, 155–160.

Khan, S., & Wani, M. R. (2006). MMS and SA induced genetic variability for quantitative traits in mungbean. *Indian Journal of Pulses Research, 19*(1), 50–52.

Khan, S., Wani, M. R., & Parveen, K. (2004). Induced genetic variability for quantitative traits in *Vigna radiata* (L.) Wilczek. *Pakistan Journal of Botany, 36*(4), 845–850.

Khan, S., Wani, M. R., & Parveen, K. (2006). Sodium azide induced high yielding early mutant in lentil. *Agricultural Science Digest, 26*(1), 65–66.

Khursheed, S., Laskar, R. A., Raina, A., Amin, R., & Khan, S. (2015). Comparative analysis of cytological abnormalities induced in *Vicia faba* L. genotypes using physical and chemical mutagenesis. *Chromosome Science, 18*(3–4), 47–51.

Khursheed, S., Raina, A., & Khan, S. (2016). Improvement of yield and mineral content in two cultivars of *Vicia faba* L. through physical and chemical mutagenesis and their character association analysis. *Archives of Current Research International, 4*(1), 1–7.

Khursheed, S., Raina, A., Amin, R., Wani, M. R., & Khan, S. (2018a). Quantitative analysis of genetic parameters in the mutagenized population of faba bean (*Vicia faba* L.). *Research on Crops, 19*(2), 276–284.

Khursheed, S., Raina, A., Laskar, R. A., & Khan, S. (2018b). Effect of gamma radiation and EMS on mutation rate: their effectiveness and efficiency in faba bean (*Vicia faba* L.). *Caryologia, 71*(4), 397–404.

Khursheed, S., Raina, A., & Khan, S. (2018c). Physiological response of two cultivars of faba bean using physical and chemical mutagenesis. *International Journal of Advanced Research in Engineering, 7*(4), 897–905.

Khursheed, S., Raina, A., Parveen, K., & Khan, S. (2019). Induced phenotypic diversity in the mutagenized populations of faba bean using physical and chemical mutagenesis. *Journal of the Saudi Society of Agricultural Sciences, 18*(2), 113–119. https://doi.org/10.1016/j.jssas.2017.03.001

Kumar, Y., Mishra, S. K., Tyagi, M. C., & Sharma, B. (2004). Detection of two linkage groups in lentil (*Lens culinaris* Medik.). *Indian Journal of Genetics, 64*, 306–309.

Kumar, Y., Mishra, S. K., Tyagi, M. C., Singh, S. P., & Sharma, B. (2005a). Inheritance of genes for three pigmentation traits in lentil (*Lens culinaris* Medik.). *Journal of Genetics and Breeding, 59*, 107–111.

Kumar, Y., Mishra, S. K., Tyagi, M. C., Singh, S. P., & Sharma, B. (2005b). Linkage between genes for leaf colour, plant pubescence, number of leaflets and plant height in lentil (*Lens culinaris* Medik.). *Euphytica, 145*, 41–48.

Ladizinsky, G. (1979a). The genetics of several morphological markers in lentil. *The Journal of Heredity, 70*, 135–137.

Ladizinsky, G. (1979b). The origin of lentil and its wild gene pool. *Euphytica, 28*(1), 179–187.

Laskar, R. A., & Khan, S. (2017). Mutagenic effectiveness and efficiency of gamma rays and HZ with phenotyping of induced mutations in lentil cultivars. *International Letters of Natural Sciences, 64*, 17–31.

Laskar, R. A., Khan, S., Khursheed, S., Raina, A., & Amin, R. (2015). Quantitative analysis of induced phenotypic diversity in chickpea using physical and chemical mutagenesis. *Journal of Agronomy, 14*(3), 102–111.

Laskar, R. A., Laskar, A. A., Raina, A., & Amin, R. (2018a). Induced mutation analysis with biochemical and molecular characterization of high yielding lentil mutant lines. *International Journal of Biological Macromolecules, 109*, 167–179.

Laskar, R. A., Wani, M. R., Raina, A., Amin, R., & Khan, S. (2018b). Morphological characterization of gamma rays induced multipodding mutant (mp) in lentil cultivar Pant L- 406. *International Journal of Radiation Biology, 94*(11), 1049–1053.

Laskar, R. A., Khan, S., Deb, C. R., Tomlekova, N., Wani, M. R., Raina, A., & Amin, R. (2019). Lentil (*Lens culinaris* Medik.) diversity, cytogenetics and breeding. In J. M. Al-Khayri et al. (Eds.), *Advances in plant breeding: Legumes*. Springer. https://doi.org/10.1007/978-3-030-23400-3_9

Lawrence, C. W. (1965). Radiation-induced polygenic mutations. The use of induced mutations in plant breeding. *Radiation Botany, 5*(Suppl), 491–496.

Malik, I. A., Ali, G. S. Y., & Saleem, M. (1988). Serrated leaf mutants in *Vigna radiata* (L.) Wilczek. *Mutation Breeding Newsletter, 32*, 11–12.

Materne, M., & McNeil, D. (2007). Breeding methods and achievements. In S. S. Yadav, D. McNeil, & P. C. Stevenson (Eds.), *Lentil – An ancient crop for modern times* (pp. 241–253). Spinger.

Mather, K., & Jinks, J. (1971). *Biometrical genetics* (2nd ed.). Chapman and Hall.

Muehlbauer, F. J., & Slinkard, A. E. (1981). Genetic and breeding methodology. In C. Webb & G. C. Hawtin (Eds.), *Lentils* (pp. 69–90). CAB.

Muehlbauer, F. J., Kaiser, W. J., Clement, S. L., & Summerfield, R. J. (1995). Production and breeding of lentil. *Advances in Agronomy, 54*, 283–332.

Muehlbauer, F. J., Cho, S., Sarker, A., McPhee, K. E., Coyne, C. J., Rajesh, P. N., & Ford, R. (2006). Application of biotechnology in breeding lentil for resistance to biotic and abiotic stress. *Euphytica, 147*, 149–165.

Paul, A., & Singh, D. P. (2002). Induced chlorophyll mutations in lentil (*Lens culinaris* Medik). *Indian Journal of Genetics, 62*(3), 263–264.

Raina, A., & Danish, M. (2018). Mutagenesis in plant breeding for disease and pathogen resistance. *Agricultural Research & Technology, 13*(1), 12–13.

Raina, A., & Khan, S. (2020). Increasing rice grain yield under biotic stresses: Mutagenesis, transgenics and genomics approaches. In C. Aryadeep (Ed.), *Rice research for quality improvement: Genomics and genetic engineering* (pp. 149–178). Springer. https://doi.org/10.1007/978-981-15-5337-0_8

Raina, A., Laskar, R. A., Khursheed, S., Amin, R., Tantray, Y. R., Parveen, K., & Khan, S. (2016). Role of mutation breeding in crop improvement-past, present and future. *Asian Research Journal of Agriculture, 2*(2), 1–13.

Raina, A., Laskar, R. A., Khursheed, S., Khan, S., Parveen, K., Amin, R., & Khan, S. (2017). Induced physical and chemical mutagenesis for improvement of yield attributing traits and their correlation analysis in chickpea. *International Letters of Natural Sciences, 61*, 14–22.

Raina, A., Khan, S., Laskar, R. A., Wani, M. R., & Mushtaq, W. (2019). Chickpea (*Cicer arietinum* L.) cytogenetics, genetic diversity and breeding. In J. M. Al-Khayri et al. (Eds.), *Advances in plant breeding: Legumes*. Springer. https://doi.org/10.1007/978-3-030-23400-3_3

Raina, A., Laskar, R. A., Tantray, Y. R., Khursheed, S., Wani, M. R., & Khan, S. (2020a). Characterization of induced high yielding cowpea mutant lines using physiological, biochemical and molecular markers. *Scientific Reports, 10*(1), 1–22.

Raina, A., Khan, S., Sahu, P., & Roa, R. (2020b). Increasing rice grain yield under abiotic stresses: Mutagenesis, transgenics and genomics approaches. In C. Aryadeep (Ed.), *Rice research for quality improvement: Genomics and genetic engineering* (pp. 753–777). Springer. https://doi.org/10.1007/978-981-15-4120-9_31

Raina, A., Sahu, D., Parmeshwar, K., Laskar, R. A., Rajora, N., Soa, R., Khan, S., & Ganai, R. A. (2021). Mechanisms of genome maintenance in plants: playing it safe with breaks and bumps. *Frontiers in Genetics, 12*, 675686. https://doi.org/10.3389/fgene.2021.675686

Raina, A., Laskar, R. A., Wani, M. R., Jan, B. L., Ali, S., & Khan, S. (2022a). Gamma rays and sodium azide induced genetic variability in high yielding and biofortified mutant lines in

cowpea (*Vigna unguiculata* (L.) Walp.). *Frontiers in Plant Science, 13*, 911049. https://doi.org/10.3389/fpls.2022.911049

Raina, A., Laskar, R. A., Wani, M. R., Jan, B. L., Ali, S., & Khan, S. (2022b). Comparative mutagenic effectiveness and efficiency of gamma rays and sodium azide in inducing chlorophyll and morphological mutants of cowpea. *Plants, 11*, 1322. https://doi.org/10.3390/plants11101322

Raina, A., Laskar, R. A., Wani, M. R., & Khan, S. (2022c). Chemical mutagenesis: Role in breeding and biofortification of lentil (*Lens culinaris* Medik) mutant lines. *Molecular Biology Reports, 49*(12), 11313–11325. https://doi.org/10.1007/s11033-022-07678-6

Raina, A., Laskar, R. A., Wani, M. R., & Khan, S. (2022d). Plant breeding strategies for abiotic stress tolerance in cereals. In A. Roychoudhury, T. Aftab, & K. Acharya (Eds.), *Omics approach to manage abiotic stress in cereals* (pp. 151–177). Springer. https://doi.org/10.1007/978-981-19-0140-9_8

Rajput, M. A., & Siddiqui, K. A. (1981). Mutation breeding of soybean for high yield and oil content. In: *Proceedings of induced mutations for improvement of grain legume production. 2nd research coordination meeting, Thailand*, pp. 117–124.

Ramesh, B., & Dhananjay, S. (1996). Developmental morphology of induced semidwarf and stunted mutants in lentil. *Indian Journal of Genetics, 56*(3), 335–340.

Rasik, S., Raina, A., Laskar, R. A., Wani, M. R., Reshi, Z., & Khan, S. (2022). Lower doses of Sodium azide and methyl methanesulphonate improved yield and pigment contents in vegetable cowpea (*Vigna unguiculata* (L.) Walp.). *South African Journal of Botany, 148*, 727–736. https://doi.org/10.1016/j.sajb.2022.04.034

Reddy, V. R. K., & Annadurai, M. (1992). Cytological effects of different mutagens in lentil (*Lens culinaris* Medik). *Cytologia, 57*, 213–216.

Reddy, V. R. K., Thresiamma, P. J., & Edwin, R. (1993). A comparative study of microsperma and macrosperma lentils I. Chlorophyll mutations. *The Journal of Indian Botanical Society, 72*, 25–28.

Sarker, A., & Sharma, B. (1986). *Induced macro-mutations in lentil (Lens culinaris Medik)*. Bangladesh association for the advancement of science, Dhaka Conference. Section 1 Dhaka, BAAS, 1986–87.

Sarker, A., & Sharma, B. (1988). Efficiency of early generation selection for induced polygenic mutations in lentil (*Lens culinaris* Medik.). *Indian Journal of Genetics, 48*(2), 155–159.

Sarker, A., & Sharma, B. (1989). Frequency and spectrum of chlorophyll mutations in lentil (*Lens culinaris* Medik). *Thai Journal of Agricultural Science, 22*(2), 107–111.

Sarker, A., Erskine, W., Sharma, B., & Tyagi, M. C. (1999). Inheritance and linkage relationships of days to flowering and morphological loci in lentil (*Lens culinaris* Medikus subsp. *culinaris*). *The Journal of Heredity, 90*, 270–275.

Scossiroli, R. E. (1966). The use of induced genetic variability for quantitative traits after seed irradiation in *Triticum durum* Savremen. *Poljopr, 14*, 221–234.

Sellapillai, L., Dhanarajan, A., Raina, A., & Ganesan, A. (2022). Gamma ray induced positive alterations in morphogenetic and yield attributing traits of finger millet (*Eleusine coracana* (L.) Gaertn.) in $M_2$ generation. *Plant Science Today, 9*(4), 939–949.

Sellapillaibanumathi, L., Dhanarajan, A., Raina, A., & Ganesan, A. (2022). Effects of gamma radiations on morphological and physiological traits of finger millet (*Eleusine coracana* (L.) Gaertn.). *Plant Science Today, 9*(1), 89–95.

Sharma, S. K. (1990). Mutation breeding in lentil. In I. A. Khan & S. A. Farook (Eds.), *Genetic improvement of pulse crops* (pp. 251–270). Premier Pub. House.

Sharma, B. (1997). Effective use of induced polygenic variability for quantitative traits in plant breeding. In R. L. Kapoor & M. L. Saini (Eds.), *Plant breeding and crop improvement* (Vol. II, p. 11). CBS Publishers and Distributors.

Sharma, S. K., & Sharma, B. (1978a). Induction of tendril mutations in lentil (*Lens culinaris* Medik). *Current Science, 47*(22), 864–866.

Sharma, S. K., & Sharma, B. (1978b). Homologous leaf mutations induced in large and small seeded lentils and their effect on quantitative characters. *Indian Journal of Agricultural Sciences, 48*, 751–756.

Sharma, S. K., & Sharma, B. (1979). Leaf mutations induced with NMU and gamma rays in lentil (*Lens culinaris* Medik). *Current Science, 48*(20), 916–917.

Sharma, S. K., & Sharma, B. (1981a). Induced mutations of physiological nature in lentil. *Indian Journal of Genetics, 40*(1), 290–294.

Sharma, S. K., & Sharma, B. (1981b). Induced chlorophyll mutations in *Lens culinaris* var. microsperma and macrosperma. *Indian Journal of Genetics, 41*, 328–333.

Sharma, S. K., & Sharma, B. (1981c). Note on the induced leaf variants in lentil. *Indian Journal of Agricultural Sciences, 51*, 805–807.

Sharma, S. K., & Sharma, B. (1982). NMU induced dwarf mutations in lentil. *Science and Culture, 47*, 230–231.

Sharma, S. K., & Sharma, B. (1983). Induced fasciation in lentil. *Genetica Agraria, 37*, 319–326.

Sharma, S. K., & Sharma, B. (1986). Mutagen sensitivity and mutability in lentil. *Theoretical and Applied Genetics, 71*, 820–825.

Siddiqui, B. A., & Khan, S. (1999). *Breeding in crop plants: mutations and in vitro mutation breeding* (1st ed.). Kalyani Publishers.

Sinclair, T. R., & Vadez, V. (2012). The future of grain legumes in cropping systems. *Crop Pasture, 63*, 501–512.

Sindhu, J. S., & Slinkard, A. E. (1983). Lentils and their wild relatives. *LENS Newsletters, 10*, l.

Singh, D., & Singh, J. (1989). Effect of gamma rays, EMS and hydroxylamine on type and frequency of chlorophyll mutations in lentil (*Lens culinaris* Medik). *Lentil Experimental News Service, 16*(2), 3–5.

Singh, D., Singh, R. M., & Singh, J. (1989). Effect of gamma rays, ethylmethane sulphonate and hydroxylamine on type and frequency of chlorophyll mutations in lentil. *LENS Newsletters, 6*, 3–4.

Sinha, R. P., & Chaudhary, S. K. (1987). Sensitivity of lentil genotypes to radiation. *LENS Newsletters, 14*, 7–9.

Sinha, R. P., & Chowdhary, S. K. (1984). Variability in lentil germplasm. *LENS Newsletters*, 13–14.

Sinha, S. S. N., & Godward, M. B. E. (1968). Radiation studies in *Lens culinaris*. *Journal of Cytology and Genetics, 3*, 80–91.

Sinha, S. S. N., & Godward, M. B. E. (1972). Radiation studies in *Lens culinaris*. Meiotic abnormalities induced due to gamma radiation and its consequences. *Cytologia, 37*, 685–695.

Sinha, R. P., Chaudhary, S. K., & Sharma, R. N. (1987). Inheritance of cotyledon colour in lentil. *LENS Newsletters, 4*, 3–6.

Solanki, I. S. (2005). Isolation of macromutations and mutagenic effectiveness and efficiency in lentil (*Lens culinaris* Medik). *National Journal of Plant Improvement, 7*(2), 81–84.

Solanki, I. S., & Phogat, D. S. (2005). Chlorophyll mutation induction and mutagenic effectiveness and efficiency in lentil (*Lens culinaris* Medik). *Indian Journal of Genetics, 65*(4), 264–268.

Solanki, I. S., & Sharma, B. (1999). Induction and isolation of morphological mutations in different damage groups in lentil (*Lens culinaris* Medik). *Indian Journal of Genetics, 59*(4), 479–485.

Solanki, I. S., & Sharma, B. (2001). Frequency and spectrum of chlorophyll mutations in macrosperma lentil (*Lens culinaris* Medik). *Indian Journal of Genetics, 61*(3), 283–286.

Solanki, I. S., Phogat, D. S., & Waldia, R. S. (2004). Frequency and spectrum of morphological mutations and effectiveness and efficiency of chemical mutagens in macrosperma lentil. *National Journal of Plant Improvement, 6*(1), 22–25.

Swarup, I., Goswami, H. K., & Lai, M. S. (1991). Genetic analysis of yield and its contributing characters in lentil. *LENS*, 7–10.

Tahir, M., Muehlbauer, F. J., & Spaeth, S. C. (1994). Association of isozyme markers with quantitative trait loci in random single seed derived lines of lentil (*Lens culinaris* Medik.). *Euphytica, 75*, 111–119.

Tantray, A. Y., Raina, A., Khursheed, S., Amin, R., & Khan, S. (2017). Chemical mutagen affects pollination and locule formation in capsules of black cumin (*Nigella sativa* L.). *International Journal of Agricultural Science, 8*(1), 108–117.

Tivoli, B., Baranger, A., Avila, C. M., Banniza, S., Barbetti, M., Chen, W. D., Davidson, J., Lindeck, K., Kharrat, M., Rubiales, D., Sadiki, M., Sillero, J. C., Sweetingham, M., & Muehlbauer, F. J. (2006). Screening techniques and sources of resistance to foliar diseases caused by major necrotrophic fungi in grain legumes. *Euphytica, 147*, 223–253.

Tripathi, A. (1995). *Studies on macro and micro mutations induced by individual and combined action of ethylmethane sulphonate and gamma rays in lentil (Lens culinaris Med.)*. Ph. D. Thesis, Kanpur University, Kanpur.

Tripathi, A., & Dubey, D. K. (1992). Frequency and spectrum of mutations induced by separate and simultaneous applications of gamma rays, ethylmethane sulphonate (EMS) in two microsperma varieties of lentil. *LENS, 19*(1), 3–8.

Tufail, M., Malik, I. A., Choudhary, M., Ashraf, M., & Saleem, M. (1998). *Genetic resources and breeding of lentil in Pakistan*. Symposium-Indian Institute of Pulses Research, Kanpur.

Tyagi, B. S., & Gupta, P. K. (1991). Induced mutations for fasciation in lentil (*Lens culinaris* Medik). *Indian Journal of Genetics, 51*(3), 326–331.

Tyagi, N. K., & Ramesh, B. (1998). Characteristic and development morphology of reduced plant height mutants in lentil. *LENS Newsletters, 21*, 6–10.

Tyagi, M. C., & Sharma, B. (1981). Effect of photoperiod and vernalization on flowering and maturity in macrosperma lentils. *Pulse Points Newsletters, 1*(2), 40–41.

Vandana, Tripathi, A., & Dubey, D. K. (1994). Frequency and spectrum of mutations induced by ethylmethane sulphonate (EMS) and diethyl sulphate (DES) in lentil var. K-85. *LENS, 21*(1), 16–19.

Wani, M. R. (2018). Early maturing mutants of chickpea (*Cicer arietinum* L.) induced by chemical mutagens. *Indian Journal of Agricultural Sciences, 88*(4), 635–640.

Wani, M. R. (2020). Characterization of chlorophyll deficient mutants in mungbean (*Vigna radiata* (L.) Wilczek). *Bangladesh Journal of Botany, 49*(4), 1013–1019.

Wani, M. R. (2021). Comparative biological sensitivity and mutability of chemo-mutagens in lentil (*Lens culinaris* Medik). *Legume Research, 44*(1), 26–30. https://doi.org/10.18805/LR-4058

Wani, M. R., & Khan, S. (2003). Chlorophyll mutations in lentil. *Tropical Agriculturist, 154*, 21–26.

Wani, M. R., Dar, A. R., Tak, A., Amin, I., Shah, N. H., Rehman, R., Baba, M. Y., Raina, A., Laskar, R., Kozgar, M. I., & Khan, S. (2017). Chemo-induced pod and seed mutants in mungbean (*Vigna radiata* L. Wilczek). *SAARC Journal of Agriculture, 15*(2), 57–67.

Wani, M. R., Laskar, R. A., Raina, A., Khan, S., & Khan, T. U. (2021). Application of chemical mutagenesis for improvement of productivity traits in lentil (*Lens culinaris* Medik). *Annals of Biology, 37*(1), 69–75.

Yamaguchi, H. (2005). Use of induced mutations for crop improvement: Revisited. In S. K. Datta (Ed.), *Role of classical mutation breeding in crop improvement* (pp. 1–19). Daya Publishing House.

Zamir, D., & Ladizinsky, G. (1984). Genetics of allozyme variants and linkage groups in lentil. *Euphytica, 33*, 329–336.

# Abiotic Stress Tolerance and Nutritional Improvement in Chickpeas Through Recombination, Mutation, and Molecular Breeding


**G. Misra and Archana Joshi-Saha**

**Abstract** The chickpea (*Cicer areitinum* L.), an old domesticated crop, is presently one of the top three legumes cultivated and consumed globally. It is a rich protein source for vegetarians and/or economically poor populations that cannot afford to buy meat or meat products on a daily basis. Chickpeas are cultivated in more than 55 countries with varied climatic conditions. Depending on the area of cultivation, the crop faces varying abiotic stresses. The cultivation areas, specific abiotic stresses, adaptive mechanisms of chickpea plants, selection of relevant traits and their screening, as well as the conventional and molecular breeding strategies to develop climate-smart chickpeas and some success stories, are discussed in the present chapter.

Zinc (Zn) and iron (Fe) deficiency majorly contribute to "hidden hunger" in over two billion people worldwide. Due to the wider cultivation and affordability of chickpeas, it is a staple crop in many countries and hence a good candidate for nutritional enhancement through "biofortification" programs. It is a newer area in chickpea breeding. Biofortification through agronomic as well as genetic methods is being tried. The state of the art, research strategies, and future prospects in chickpea biofortification are highlighted. Molecular insights into the mechanisms for abiotic stress tolerance and nutritional quality improvement have also identified links between the two. We discuss these aspects and the future strategic research needed to develop more robust plant types along with better grain quality.

**Keywords** Abiotic stress tolerance · Chickpeas · Recombination · Mutation breeding · Molecular breeding

G. Misra
Nuclear Agriculture and Biotechnology Division, Bhabha Atomic Research Centre, Mumbai, India

A. Joshi-Saha (✉)
Nuclear Agriculture and Biotechnology Division, Bhabha Atomic Research Centre, Mumbai, India

Homi Bhabha National Institute, Mumbai, India
e-mail: archanaj@barc.gov.in



A. Raina et al. (eds.), *Advanced Crop Improvement, Volume 2*,
https://doi.org/10.1007/978-3-031-26669-0_10

## 1 Introducing Chickpeas

One of the oldest domesticated legumes, chickpeas, needs no introduction. Many ancient manuscripts mention this grain, supporting its early origin and importance in many civilizations (Van der Maesen, 1987). *Cicer arietinum* L. is the only cultivated species from the genus *Cicer* and tribe, *Cicereae* (Lucas & Fuller, 2014). Three wild annual *Cicer* species, *C. reticulatum* Lad, *C. echinospermum* P.H. Davis, and *C. bijugum* K.H. Rech, were found in present-day Syria and southeastern Turkey and are considered to be the places where chickpeas originated (Van der Maesen, 1987). Very recently, another new species, *C. turcicum* Toker, Berger, & Goktur, endemic to East Anatolia, Turkey, was identified, which, based on the internal transcribed spacer (ITS) region sequencing, was found to be closely related to *C. reticulatum* and *C. echinospermum*. Thus, there are now 10 annual and 36 perennial species in the genus *Cicer* that can be divided into three gene pools (Toker et al., 2021). *C. reticulatum,* present in the primary gene pool along with *C. arietinum,* is thought to be its progenitor (Van der Maesen, 1987; Toker, 2009). *C. echinospermum* belongs to the secondary gene pool. This species can also be crossed with *C. arietinum* to obtain some fertile progeny, while most of the other species are in the tertiary gene pool, and it is not easy to cross successfully with domesticated *C. arietinum* (Toker et al., 2021). The cultivated chickpeas have two subtypes: macrosperma (also called *kabuli,* a term of Indian origin, derived from Kabul, possibly as an indication of its arrival in India apparently through Afghanistan) and microsperma (also called *desi*, another term of Indian origin, implying 'local') types. While, *Kabuli* chickpeas are beige to whitish in color, large in size, have a typical owl's head shape, *desi* are often small seeded, angular, and have varying seed coat colors. In addition, pea-shaped chickpeas are also available in the germplasm; they also often result from *desi* X *kabuli* or vice-versa crosses (Fig. 1).

These subtypes not only differ in seed morphology but also differ with respect to the plant architecture, flower color and conditions of cultivation (Wood et al., 2011; Purushothaman et al., 2014). The chickpea has not lost its importance since ancient times, as indicated by present-day chickpea cultivation. Together with dry beans and peas, it is among the three major pulses produced worldwide and ranks second and third in terms of area under cultivation and production, respectively. Globally, it is cultivated in an area of around 13.7 Mha with a production of 13.6 Mt (averaged values from 2015 to 2019, FAOSTAT, 2021). Although it is cultivated in more than 50 countries, India contributes the most both in the point of area (9.27 Mha, 67.6% of total area: averaged values from 2015 to 2019) and production (9.01 Mt, 66.2% of global production; averaged values from 2015 to 2019). Australia, Myanmar, and Ethiopia contribute around 14%, 4%, and 4% of global chickpea production, respectively, while Turkey and Russia account for around 3% share in global production (Merga & Haji, 2019).

Chickpeas, generally termed as the "poor man's meat," are highly protein-rich and a primary protein source for a large vegetarian population as well as an economically poor population that cannot afford to buy meat or meat products on a daily basis. Moreover, it contains significant amounts of carbohydrates, minerals,

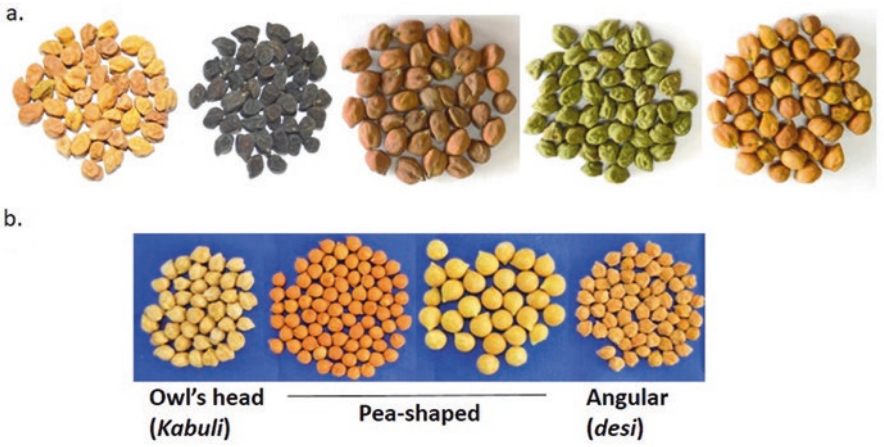



**Fig. 1** Variation for: (**a**) seed coat color and (**b**) seed shape in chickpea germplasm and breeding lines. The pea-shaped lines are derived from a cross between ICCV2 (*kabuli*) and a white flower mutant of cv. Vijay (*desi*)

and dietary fiber contributing toward low fat meals with health benefits (Jukanti et al., 2012). Because chickpea is affordable and cultivated in many places across the globe, it is a staple crop in many countries and hence a good candidate for developing nutritionally improved genotypes either through classical or molecular breeding approaches. These aspects are covered in detail in this chapter.

The chickpea belongs to the "Hologalegina/galegoid clade" that contains the "cool season" temperate legumes, which are often vernalized, requiring long-day plants (Summerfield & Roberts, 1985; Lee et al., 2017). Being cultivated worldwide, the crop also faces various abiotic stresses depending on the area under cultivation. The cultivation areas and the specific abiotic stresses, as well as the strategies to develop climate-smart chickpeas, are also discussed in depth.

As we understand more and more about the molecular mechanisms that govern abiotic stress tolerance and nutritional quality improvement, we have also started to decipher the links between the two. We explore these links in a separate section, as well as the future strategic research needed to develop more robust plant types, along with better grain quality.

## 2 Abiotic Stresses and Impact on Chickpea Cultivation

The chickpea is cultivated worldwide in varied climatic conditions (Fig. 2). Therefore, depending on the area of cultivation, the crop faces varying abiotic stresses (Table 1).

Being a winter crop, low temperature, chilling, or frost is a major stress. In many parts of North Asia and southern Australia, it is encountered during the reproductive phase, while, in other regions of the Mediterranean, seedling and the early

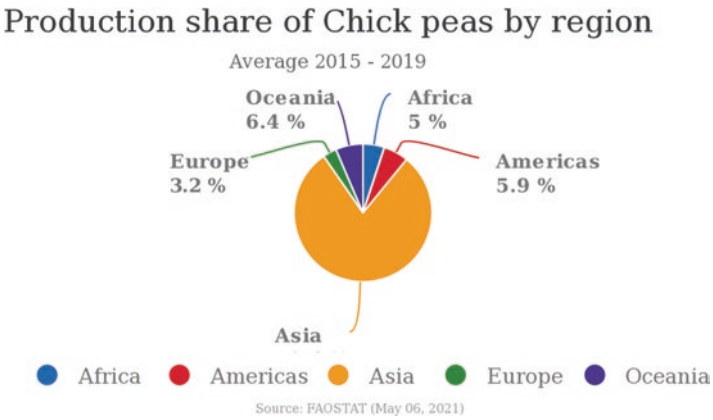



**Fig. 2** Worldwide regions cultivating chickpeas and their production share. Values are the average of 5 years (2015–2019). (Data Source: FAOSTAT (May 6, 2021))

**Table 1** Major environmental stresses encountered by chickpeas

| Sr. no. | Stress | Area/climate | Season sown | Stage of growth when stress is encountered | References |
|---|---|---|---|---|---|
| 1. | Chilling temperature | South Australia, North India | Winter | Reproductive phase | Berger et al. (2011) |
| 2. | Freezing temperature | Higher elevations of Mediterranean countries and temperate regions such as northern Iraq, Iran, Russia, North America, Turkey, and north India | Winter | Early seedling stage, vegetative phase<br>Isolated frost events can damage other stages as well | Singh et al. (1984) |
| 3. | Terminal drought | South Asia<br>Mediterranean and temperate regions,<br>West Asia and North Africa (WANA) regions | Winter (post monsoon rain)<br>Spring (in season rain, that declines toward autumn) | Reproductive phase | Johansen et al. (1996) |
| 4. | Terminal Heat | Mediterranean regions receiving rainfall during crop season, south Asia, and regions where sowing is done in springs of low latitude areas of WANA regions | | Reproductive phase | Berger et al. (2011) and Johansen et al. (1996) |
| 5. | Erratic rainfall | USA | Spring | Extension of vegetative phase, failure to timely maturation | McVay et al. (2013) |

vegetative period encounter this stress (Berger et al., 2005, 2012). Since chickpea has an indeterminate growth habit, rain toward the end of the crop cycle may exceed the crop duration, which may lead to the failure of the crop to mature (McVay et al., 2013). Chickpea cultivation is primarily rainfed, and therefore, it encounters water deficits toward the reproductive or pod filling stages – often termed as "terminal drought." This terminal drought is often accompanied by an increase in temperature (terminal heat stress), which can cause major yield losses of up to 50% (Devasirvatham & Tan, 2018). India, which contributes the most in terms of area under cultivation and production, encounters all the above abiotic stresses depending on the area of cultivation. In the northern parts of the country, chilling or frost is the major abiotic stress, while in the northeast, excessive rainfall, especially at maturity can be devastating. Majority of chickpea growing areas in India are in central and southern states, which inherently have arid/semiarid climates with very short winters. Hence, two major stresses chickpeas face in these areas are heat stress and terminal drought.

In addition, the arid and semiarid regions are also prone to salinity, which can limit chickpea growth and development, causing yield losses of around 8–10% (Flowers et al., 2010), which might increase further considering the increasing demands for food and expanding the cultivation areas to saline lands (Ladeiro, 2012). Collectively, the abiotic stresses are estimated to cause higher yield losses as compared to the biotic stresses (Ryan, 1997).

In order to breed plants that are resilient to various abiotic stresses, it is imperative to understand the basic adaptive mechanisms of the plants against those stresses. Understanding these mechanisms allows us to choose the traits that can be selected for or against for crop improvement. Broadly, the adaptive mechanisms are classified into three categories: escape, avoidance, and tolerance strategies. In the "escape" strategies, plants regulate their phenology such that the stress scenario is not encountered by the plants; "avoidance mechanisms" imply that the strategies are such that though the stress is encountered, its effects are countered, while in "tolerance mechanisms" the plant tries to minimize the damage caused by the stress scenario (Joshi-Saha & Reddy, 2018). In the following section, these adaptive mechanisms and thus the target traits for breeding are discussed for each of the major stresses encountered by chickpeas. In addition, screening for these target traits, breeding approaches, and some of the recent success stories are also described in the following sections.

## 3 Abiotic Stresses, Adaptive Mechanisms, and Target Traits in Chickpea

### *3.1 Low-Temperature Stress*

Although chickpeas are a cool season legume of temperate regions, sub-optimal low temperatures can be detrimental. For chickpeas, sub-optimal temperatures are categorized in two ranges: freezing temperature (<−1.5 °C, based on the average

freezing point of plant tissue) and, chilling temperature (between −1.5 °C and −15 °C) (Croser et al., 2003). Freezing temperature stress is encountered in the early seedling/vegetative stages of the crop as well as any stage of growth due to occasional falls in temperatures, while chilling stress is more severe in the reproductive/flowering and pod filling stages (Srinivasan et al., 1998; Clarke, 2001; Berger et al., 2006; Kumar et al., 2011) (Table 1). Under different sowing conditions, grain yield losses due to freezing/chilling temperatures have been reported ranging from 15–20% (Chaturvedi et al., 2009) to 30–40% (Rani et al., 2020).

Frost damages the crop by forming ice crystals extracellularly in the plant tissue, which dehydrates the cells and causes injury (Snyder & de Melo-Abreu, 2005). Freezing temperatures can affect chickpea germination and seedling vigor, and if encountered at a later stage, can also cause flower drop and pod abortion. Moreover, it also affects the seed size and harvest index, possibly due to its effect on metabolism and the translocation of metabolites from source to sink (Saxena, 1990; Croser et al., 2003). In general, chickpeas can survive at a minimum temperature of −8 °C; however, it is seen that post germination, some lines can tolerate up to −12 °C (Wery, 1990; Croser et al., 2003). Cold temperatures can advance the vegetative period and delay the commencement of the reproductive phase. Under controlled conditions, treatment of chickpea cultivars with freezing temperatures (−3 and −5 °C) decreased shoot length, leaf number, fresh and dry mass, and relative water content (RWC). The plants could not survive when treated at −7 °C. Freezing treatment also reduced the chlorophyll and carotenoid content, which led to a reduction in photosynthetic efficiency (Arslan et al., 2018).

In chickpea, most of the work pertaining to cold tolerance has been focused on chilling stress. An extreme temperature mainly affects the reproductive phase. Microsporogenesis and the development and growth of male gametophytes are more sensitive than female gametophytes in cold-sensitive chickpea genotypes at a temperature <10 °C (Clarke & Siddique, 2004; Sharma & Nayyar, 2014; Kiran et al., 2019). Based on field screening, the wild *Cicer* species, *C. arietinum,* is less tolerant than *C. echinospermum, C. bijugum,* and *C. judaicum.* Moreover, *C. arietinum* showed a delay in pod setting at temperatures of around 14 °C. In addition, no difference in cold sensitivity between *desi* and *kabuli* types was reported (Berger et al., 2012). Seedling germination, establishment, and vigor are also affected by chilling temperature, and the variation for these traits has been observed (Bakht et al., 2006). The effect of cold stress also varies within the reproductive growth stage. For example, cold exposure at the late pod filling stage affects different properties of seeds like their number, growth rate, fill duration, and average weight and size. Additionally, a decrease in content of proteins, fat, starch, soluble sugars, crude fiber, and storage protein fractions was also observed, which was thought to be associated with a decrease in sucrose content, sucrose synthase activity, as well as activities of invertase and starch synthase. Whereas, exposure to cold at the early pod filling stage showed inhibition in germination and growth potential (Kaur et al., 2008).

At the cellular level, damages caused by cold stress include a decrease in membrane stability, a change in rate of respiration and photosynthesis, changes in the conformations of proteins and lipids, reduced enzyme activity, and oxidative stress, which ultimately causes plant wilting (Croser et al., 2003). This was majorly

attributed to the accumulation of ROS (Nayyar et al., 2005a; Awasthi et al., 2015). Moreover, increases in abscisic acid (ABA) and reductions in bioactive gibberellic acid (GA) are also implicated in distorting the carbohydrate pools in the anther, affecting pollen development and consequent flower abortion (Nayyar et al., 2005b; Thakur et al., 2010; Sharma & Nayyar, 2016).

The adaptive mechanism for cold tolerance in chickpea plants includes enhanced activities and regulation of anti-oxidative enzymes (Kumar et al., 2011; Turan & Ekmekci, 2011; Farooq et al., 2017; Karami-Moalem et al., 2018) and in the pod wall and developing seeds (Kaur et al., 2009). Moreover, accumulation of nonenzymatic metabolites like proline, soluble phenolics, polyamines, and gamma-aminobutyric acid (GABA) was also observed under cold stress in chickpea. Higher levels of GABA were correlated with increased activity of the polyamine catabolic enzymes diamine oxidase and polyamine oxidase, which provide the raw material for the synthesis of GABA (Saghfi & Eivazi, 2014; Amini et al., 2021). The role of carbohydrate metabolism in the cold tolerance of chickpea is also elucidated through gene expression as well as biochemical analysis (Saghfi & Eivazi 2014; Sharma & Nayyar, 2014). Most of the screening for cold stress has been done under field conditions with visual rating scales based on leaf necrosis (Table 2).

**Table 2** Examples of rating scales developed for screening of various abiotic stress in chickpea under field conditions

| Abiotic stress | Rating scale | References |
|---|---|---|
| Cold | 1–9 Visual (evaluated after 100% mortality of susceptible check) Where, 1 = no visible symptoms; 2 = highly tolerant (≤10% leaflets with withering & drying but no killing); 3 = tolerant (11–20% leaflets with withering; ≤20% branches show withering and drying but no killing); 4 = moderately tolerant (21–40% leaflets and ≤20% branches with withering and drying but no killing); 5 = intermediate (41–60% leaflets and 21–40% branches with withering and drying and ≤5% plant killing); 6 = moderately susceptible (61–80% leaflets & 41–60% branches with withering and drying and 6–25% plant killing); 7 = susceptible (81–99% leaflets and 61–80% branches with withering and drying and 26–50% plant killing); 8 = highly susceptible (100% leaflets and 81–99% branches with withering and drying and 51–99% plant killing); 9 = 100% plant killing | Singh et al. (1989) |
| Drought and heat | 1–9 Visual (evaluated after 100% mortality of susceptible check) Where, 1 = very highly resistance (no effect of drought and high temperature with early flowering, very good plant vigor, 100% pod setting), 2 = highly resistance (with early flowering, good plant vigor, 96–99% pod setting), 3 = resistance (with early flowering, good plant vigor, 86–95% pod setting), 4 = moderately resistance (with early flowering, moderate plant vigor, 76–85% pod setting), 5 = tolerant (with medium flowering, poor plant vigor, 51–75% pod setting), 6 = moderately susceptible (with medium flowering, no plant vigor, 26–50% pod setting), 7 = susceptible (with late flowering, no plant vigor, 11–25% pod setting), 8 = highly susceptible (with late flowering, no plant vigor, 1–10% pod setting), and 9 = very highly susceptible (with no flowering, no pod setting, 100% plant killed) | Singh et al. (1997) |

(continued)

**Table 2** (continued)

| Abiotic stress | Rating scale | References |
|---|---|---|
| Salinity | 1–10 Visual (1 = green & healthy plant with no symptoms; 2 = Beginning of yellowing or necrotic bottom leaves, 3 = necrosis on 25% of bottom leaves and yellowing on the rest of the bottom half of the plant, 4 = necrosis on 50% (bottom half) of plant, 5 = necrosis on bottom half and yellowing to the top half of the plant, 6 = necrosis on > 50% < 75% of the plant, 7 = necrosis on 75% of plant, 8 = necrosis on whole plant with still green or yellowing apical leaves, 9 = only stem and the shoot tips green, 10 = plant is dead | Maliro et al. (2008) |

In addition to field screening, an artificial "freeze test" on excised plantlets has been designed for high precision and high throughput screening of large populations (Nezami et al., 2012).

## *3.2 High-Temperature (Heat) Stress*

In warmer climates or in late sown conditions, chickpea growth and development, as well as grain yield, can be severely affected by high temperature stress. Particularly, the reproductive phase is very sensitive to high temperatures of >30/35 °C (Summerfield et al., 1984; Gaur et al., 2013; Devasirvatham et al., 2015). During this phase, heat stress (≥32/20 °C as day/night temperatures) causes severe yield losses ranging from 39% (Devasirvatham et al., 2015) to almost 100% (Canci & Toker, 2009a) depending on the genotype and intensity of stress. Yield losses of about 10–15% per degree rise in temperature above optimum are estimated to occur in southern India, where the crop faces terminal heat stress (Upadhyaya et al., 2011). Different physiological stages in the reproductive phase of chickpea are very sensitive to high temperature stress (Table 3).

High temperature also affects growth and development in chickpeas by targeting various physiological and metabolic processes like nutrient and water uptake, membrane mobility, sucrose metabolism, photosynthesis, photosynthetic assimilate partitioning, pigment degradation, transpiration, respiration, induction of oxidative damage, and disturbing source-to-sink relationships (Kaushal et al., 2013; Fahad et al., 2017).

Humidity in the air is greatly reduced by extreme heat stress, which in turn raises the vapour pressure deficit (VPD) at the leaf-air interface, an important parameter that determines photosynthetic efficiency as well as its effect on vegetative growth (Yuan et al., 2019). Due to the VPD, water is transpired by plants through stomata. In response to increased VPD, as an adaptive mechanism, plants close their stomata to reduce the rate of transpiration. Consequently, the entry of carbon dioxide, which also happens through stomata, is affected, which in turn reduces photosynthesis and causes a consequent reduction in plant productivity and yield (Grossiord et al., 2020).

**Table 3** Physiological stages in reproductive phase of chickpea affected by heat stress

| Physiological stage | Effect | References |
|---|---|---|
| Flowering | Reduction in the vegetative phase and acceleration of flowering<br>Flower drop | Devasirvatham et al. (2015), Clarke and Siddique (1998), and Devasirvatham and Tan (2018) |
| Anther and pollen development | Anther epidermis thickening, change in locule number<br>Reduced viability, germination, and load in pollen | Devasirvatham et al. (2013) and Kaushal et al. (2013) |
| Stigma function | Reduced receptivity of the stigma | Kaushal et al. (2013) |
| Ovary and ovule development | structural abnormalities | Devasirvatham et al. (2013) |
| Fertilization and seed setting | Reduced fertilization, abnormal pod development, and reduced seed set | Wang et al. (2006) and Devasirvatham et al. (2013) |

The activity of various photosynthetic complexes is highly sensitive to temperature, and, therefore, is directly affected by heat stress (Moore et al., 2021). An elevated temperature negatively impacts ribulose bisphosphate (RuBP) regeneration by damaging the photosynthetic pigments that are important for the functioning of photosystem II (PSII), thereby reducing plant growth and productivity (Moore et al., 2021). A comparative study based on membrane stability and PSII function in leaves, showed that chickpea exhibits high sensitivity to high atmospheric temperatures (Srinivasan et al., 1996). In addition, increased degradation of chlorophyll and its reduced biosynthesis are also observed in heat-sensitive chickpea genotypes (Parankusum et al., 2017; Makonya et al., 2019).

An increase in temperature leads to an increased rate of respiration. This causes carbon starvation because even a high photosynthetic rate cannot compensate for this carbon loss (Levitt, 1980). Not much is known about the rate of respiration in chickpeas at high temperatures. Cellular respiration in chickpea seedlings increases with increasing temperatures, from 30/25 °C (day/night, control) to 35/30 °C and 40/35 °C (day/night, heat stress conditions). Higher temperature stress 45/40 °C reduced the cellular respiration in these plants (Kumar et al., 2012a). Moreover, at the reproductive stage, high-temperature stress also reduced the cellular oxidizing ability of chickpea plants (Kumar et al., 2013).

Heat stress leads to oxidative damage to proteins, DNA, RNA, and membrane lipids. Generation of reactive oxygen species (ROS) like hydroxyl ($OH^{\cdot}$), superoxide ($O_2^{-}$) and alkoxyl radicals as well as hydrogen peroxide ($H_2O_2$) and singlet oxygen ($^1O_2$) (Suzuki & Mittler, 2006; Wahid et al., 2007). The redox state in chickpea is affected by heat stress during seed germination and seedling formation (Kumar et al., 2012a), as well as at the time of flowering (Kaushal et al., 2011; Kumar et al., 2013; Awasthi et al., 2015). In heat-sensitive chickpea genotypes, oxidative injury through an increase in lipid peroxidation and $H_2O_2$ levels in the leaves was more of than in tolerant genotypes (Kaushal et al., 2011; Kumar et al., 2012a, 2013; Awasthi et al., 2015).

In chickpea, sucrose metabolism is also affected by heat stress (Kaushal et al., 2013). Starch and sucrose biosynthetic enzymes like invertase, sucrose phosphate synthase, and adenosine diphosphate-glucose pyrophosphorylase are also greatly affected by high temperatures (Kaushal et al., 2013; Fahad et al., 2017). According to Awasthi and co-workers (2017), this reduction caused by the inactivation of enzymes is correlated with a decrease in seed weight. In addition to sucrose metabolism, phloem-mediated photosynthate translocation from the source (photosynthetic tissues) to the sink (non-photosynthetic tissues) is also affected by heat stress (Prasad et al., 2017). Compared to the tolerant genotypes, the sucrose content in the heat-sensitive genotypes of chickpea was significantly lower in anthers and pollen grains under late sown conditions. This was also correlated with lower pollen viability and, finally, reduced yields due to heat stress (Kaushal et al., 2013).

Heat-stress-induced adaptive mechanisms, can be broadly classified into three types: escape, avoidance, and tolerance. Among the "escape" mechanisms, early flowering and maturity is an important phenological mechanism in chickpea, particularly in spring sown Mediterranean or winter sown southern Indian conditions where the crop faces terminal heat (Toker et al., 2007a; Gaur et al., 2010). Other than escape mechanisms, emphasis should be given to relatively less studied processes like the mechanisms controlling heat avoidance in chickpea. These mechanisms include leaf reflectance, canopy, and transpiration-mediated interception and reduction of non-photosynthetic energy (Devasirvatham et al., 2012). Oxidative damage is caused by ROS generated during heat stress. Therefore, several "tolerance" mechanisms that preserve the integrity of the membranes, chlorophyll, and other biomolecules also play an important role in chickpea. Proline is known to impart partial tolerance to heat by elevating the activities of anti-oxidative enzymes and molecules such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and the antioxidants ascorbate (ASC) and glutathione (GSH) (Kaushal et al., 2011). The amino acid proline has multifaceted roles. It can act as an osmolyte as well as a molecular chaperon to stabilize protein structures and prevent their unfolding. Proline acts as a storehouse of carbon and nitrogen, maintains cytosolic pH. and communicates stress signals (Verbruggen & Hermans, 2008). Additionally, phytohormones like abscisic acid (ABA) and salicylic acid (SA) also contribute toward the heat tolerance in chickpea. Exogenously applied ABA reduced the effect of heat stress on the growth of chickpea seedlings and showed an increase in endogenous ABA and osmolytes (Kumar et al., 2012b). Chakraborty and Tongden (2005) showed that application of SA reduced membrane damage induced by heat stress in chickpeas and induced peroxidase, ascorbate peroxidase, and catalase. Several heat-shock proteins, metabolites, and other heat-shock responsive transcription factors have also been identified for which detailed molecular analysis is in progress (Parankusum et al., 2017; Chidambaranathan et al., 2018; Pareek et al., 2019).

### *3.3 Drought Stress*

Being a rainfed crop, in most chickpea-growing areas, particularly in the semiarid tropics, high evapotranspiration demands lead to the depletion of stored soil moisture toward the reproductive/maturation phase of plant growth. This causes a water deficit or terminal drought stress to the crop. Apart from terminal drought, transient drought stress can also occur at any stage of crop growth, including germination, seedling establishment, or the vegetative phase (Maqbool et al., 2017). However, the reproductive phase (flowering and pod development) is more sensitive than the vegetative phase (Pushpavalli et al., 2015a). Moreover, in many cases, the genotypes that showed drought tolerance at early seedling stages did not later reflect tolerance to terminal drought and were not good yielders (Shah et al., 2020). Drought stress affects the morphology and physiology as well as biochemical and molecular parameters in chickpea (Leport et al., 1999; Maqbool et al., 2017; Pang et al., 2017).

The morphological effects include early seedling establishment and reduction in seed germination (Vessal et al., 2012), reduction in biomass accumulation and stunted growth (Siddique et al., 1999), reduction in plant height, decrease in the number of primary and secondary branches (Arif et al., 2021), reduction in leaf length, leaflet length, and width (Maqbool et al., 2017), and effects on root length and biomass depending on the age of plants (Randhawa et al., 2014; Kashiwagi et al., 2015). Terminal drought may have drastic effects on reproductive biology and can affect the anthesis, pollination, pollen germination viability, fertility, growth of the pollen tube, pistil function, reduced number of flowers and pod formation, and increased flower and pod abortion (Siddique et al., 1999; Fang et al., 2009; Pang et al., 2017). Depending on the severity, various physiological and biochemical processes affected by drought stress include stomatal conductance, rate of transpiration, $CO_2$ assimilation, electrolyte leakage, lipid peroxidation and membrane injury, chlorophyll and carotenoids synthesis, nutrient and water uptake, ABA concentration, water use efficiency (WUE), loss of turgor due to reduced water potential, relative water content (RWC), and finally overall plant growth. Rate of $CO_2$ assimilation is reduced in drought-susceptible chickpea genotypes due to closure of stomata, decline in $CO_2$ level inside leaf, inhibition of RUBISCO, and ATP synthase activity (Mafakheri et al., 2010, 2011; Rahbarian et al., 2011; Devasirvatham & Tan, 2018; Kaloki et al., 2019). Additionally, water-deficiency can also lead to adverse effects on nodule development and symbiotic $N_2$ fixation (Nasr Esfahani et al., 2014).

Escape, avoidance, and tolerance are the three broad classes of adaptive mechanisms for drought stress (Upadhyaya et al., 2012). Similar to heat stress, early phenology (early flowering and maturity) is an important and very successful breeding strategy for escaping terminal drought (Gaur et al., 2008a; Devasirvatham & Tan, 2018). Early phenology should be accompanied by a shorter vegetative phase and a longer grain filling period to produce a higher grain yield (Soltani & Sinclair, 2012). Moreover, high initial growth vigor associated with early flowering and a faster rate of partitioning are suggested to be useful contributing traits toward drought escape (Krishnamurthy et al., 1999; Sabaghpour et al., 2003). Among the drought

"avoidance" mechanisms, where plants try to maintain their hydration state, are broadly two types: water saving and water spending mechanisms. Stomatal conductance, root traits, and water use efficiency are some of the important traits to consider in this regard. To maintain a high water potential, plants either reduce transpiration or increase water uptake. Stomatal conductance regulates transpiration to minimize water loss. An infrared thermal camera or thermometer can easily monitor canopy temperature depression (CTD), which is a surrogate trait for stomatal conductance (Purushothaman et al., 2016). Many root traits are part of the drought avoidance mechanism, including higher root biomass in the early growth stages and rooting depth (Kashiwagi et al., 2015). Mechanisms for drought tolerance include the sustenance of growth with the least injury by activating physiological and molecular responses such as the synthesis of osmoprotectants, soluble sugars, osmotic adjustment, activating antioxidative system and defense mechanisms, cell membrane stability, the synthesis of stress proteins, and hormonal signaling. However, such mechanisms, though useful for plant survival under stress, are not often correlated with crop performance in terms of grain yield (Upadhyaya et al., 2012).

### *3.4 Salinity Stress*

Chickpea is highly sensitive to salinity. All the stages of chickpea growth and development, from seed germination to seedling and plant establishment, vegetative growth, the reproductive phase, as well as nitrogen fixation, are affected due to salinity. Different genotypes differ with respect to both the rate and extent of germination in response to salinity; however, the reasons for these differences are not known (Flowers et al., 2010). Also, emergence from soil (salinized versus control) is slower and with a lower final germination percentage as compared to experiments done in petri plates with the same salt concentrations, indicating that early seedling growth regulating shoot emergence from soil is more sensitive than radicle emergence, that is, usually easily observed in petri plates (Esechie et al., 2002; Flowers et al., 2010). Vegetative growth is severely reduced in chickpea under saline conditions, with sensitive genotypes not surviving even 25 mM NaCl under hydroponic conditions with 55% relative humidity (Lauter & Munns, 1987). However, it is also noteworthy that the soil type and conditions affect the relative growth of different genotypes under salt stress, while the relative ranking of sensitivity of those genotypes might not be affected (Krishnamurthy et al., 2011a).

Salt stress imposes a typical biphasic response in plants, which includes early occurring osmotic stress followed by accumulation of $Na^+$ and $Cl^-$, causing ion toxicity (Munns & Tester, 2008; Teakle & Tyerman, 2010). The osmotic stress is due to the salt present outside the root zone that disturbs the water-balance, which in turn affects physiological processes such as stomatal conductance and photosynthesis (Munns & Tester, 2008; Yang & Guo, 2017). Stress induced by $Na^+$ and $Cl^-$ ion hyper accumulation of mainly in older leaves, causes premature senescence,

which affects the development of young leaves due to a limited supply of carbohydrates that ultimately reduces plant yield and can also lead to plant death (Munns & Tester, 2008). In glycophytes, hyper-accumulated $Na^+$ competes with the uptake of $K^+$ and disturbs the stomatal regulation that eventually causes water loss, while the $Cl^-$ ions basically inhibit chlorophyll production that leads to chlorosis and leaf senescence (Wakeel, 2013; Geilfus, 2018). However, experiments where sensitive and tolerant chickpea genotypes were exposed to an osmotic potential iso-osmotic to 60 mM NaCl showed that such a solution had no detrimental effects on chickpea growth, while plant dry mass was reduced by NaCl treatments. Growth was not affected by $Cl^-$ salts, which suggests that in chickpea genotypes salt sensitivity is mainly caused by $Na^+$ ion toxicity (Khan et al., 2016a). Measurement of seed yield under high salt concentration in a chickpea minicore collection and its comparison with seed yield under nonsaline conditions showed a relation between the two, indicating that seed yield under salt stress can be explained partly by the yield potential and by salinity tolerance. Moreover, shoot dry weight ratio did not show any significant correlation with yield ratio under saline versus nonsaline conditions, and therefore, these parameters at the vegetative phase indicated that there was no difference in the salinity tolerance among genotypes. The ability to maintain a large number of filled pods was found to be the major trait related to salinity tolerance. Moreover, salinity tolerance and shoot $Na^+$ or $K^+$ accumulation were not related (Vadez et al., 2007). In contrast, in other studies, both the vegetative and reproductive phases were found to be sensitive to salinity. The pod formation was particularly sensitive (Samineni et al., 2011). Salt sensitive genotypes also showed higher pod abortion. Yield under salt stress as an indication of salt tolerance exhibited a positive association with higher shoot biomass as well as higher pod and seed numbers. Moreover, the sensitive genotypes accumulated more $Na^+$ and $K^+$ in the seeds as compared to the tolerant ones (Turner et al., 2013). A low assimilate supply at flowering due to sub-optimal photosynthesis and reduced shoot growth reduces leaf area and branching, and, therefore, these parameters contribute to reproductive failure in chickpeas under salt stress (Khan et al., 2017).

Plants have broadly three mechanisms to deal with salinity stress: (a) osmotic adjustment of cells, (b) avoidance by $Na^+$ and $Cl^-$ exclusion; and (c) "tissue tolerance," which means the tissues maintain their physiological function even in relatively high internal concentrations of $Na^+$ and $Cl^-$ (Munns & Tester, 2008). Chickpea plants show substantial osmotic adjustments, so that the osmotic component of salt stress has limited effect on vegetative growth (Khan et al., 2016a). Recently, it has been shown that "exclusion" of Na, particularly from photosynthetically active mesophyll cells, and its compartmentalization in epidermal cells reduces structural damage to chloroplast, maintain photosynthesis and hence contribute to salinity tolerance in chickpea (Kotula et al., 2019). Selection for salinity tolerance is largely based on the performance of chickpea plants for yield under salt stress conditions. However, depending on the soil type and salinity levels, early seedling establishment and vegetative growth should also be given importance during selection.

### 3.5 Selection and Screening of Traits: Conventional Methods to Modern Tools

The ease of screening a large number of germplasm and/or populations for the target traits or traits that can serve as "proxies" for the target traits is an important consideration in breeding for various abiotic stress tolerances. Several traits have been targeted depending on the type of stress. They are listed in Table 4.

Many contributing characters regulate abiotic stress tolerance. Traits like days-to-flowering and maturity are easy to screen and select in large populations. Several chickpea cultivars with early phenology have been developed. For example, the breeding line ICCV 92944, is a heat tolerant early maturing line and has been released in many countries, including India, as JG14. This variety is suitable for late sown conditions, particularly in cereal-based cropping systems (Gaur et al., 2019).

There should be a good correlation of the trait with grain yield under stress conditions. For example, grain yield under drought was closely associated with the rate of partitioning, phenology, crop growth rate, shoot biomass at reproductive stage, canopy temperature depression, leaf area index at mid-pod fill stage, and pod number $m^{-2}$ at maturity (Purushothaman et al., 2016). Among the root traits, a combination of profuse root length density at surface soil depth and total root dry weight was considered a good selection strategy for efficient water use and enhanced terminal drought tolerance (Purushothaman et al., 2017). Also, these traits should have high heritability and a lower yield penalty under non-stress conditions (Blum, 2011).

In many environments, drought is often accompanied by high temperature; therefore, the two should also be evaluated simultaneously. A rating scale for such evaluations has also been formulated (Table 2). Also, many of the contributing traits can be used for selection; however, it is important to set up the right stress scenario (Tardieu, 2012).

### 3.6 Breeding Approaches for Abiotic Stresses Tolerance and Some Success Stories

Methodologies of breeding for "climate resilient crops" include conventional, mutational, and molecular breeding. In addition, genetic engineering approaches have also been found useful in some cases.

Conventional breeding that involves introduction, selection, and hybridization has been extensively used in chickpea breeding programs around the globe. Examples of some of the successes are taken from Indian chickpea breeding programs. Some of the early success stories in chickpea breeding were through selection from germplasm or genetic stocks. Some of the notable abiotic stress tolerant varieties developed through this method include: JG315 (resistant to Fusarium wilt: Jha et al., 2020; tolerant to drought stress: Maheswari et al., 2019); karnal Chana 1 (tolerant to salinity: Flowers et al., 2010), Annigeri 1 (tolerant to drought: Purushothaman et al., 2016).

**Table 4** Various traits, phenotyping methods, and criteria for selection in chickpea under different abiotic stresses

| Stress scenario | Traits | Mechanism | Phenotyping | Basis of selection | Correlation with yield under stress | References |
|---|---|---|---|---|---|---|
| Terminal Drought, heat, salinity | Shoot biomass | Tolerance | Dry weight measurement | More shoot biomass at the reproductive stage | Yes, shoot biomass at reproductive stage | Maliro et al. (2008), Purushothaman et al. (2016), and Atieno et al. (2017) |
| Terminal drought | Root biomass | Avoidance | Dry weight | Higher total root biomass | Yes, higher total root biomass at early growth stage | Kashiwagi et al. (2015) |
| Terminal drought | Root length density (RLD) | Avoidance | In cylinders and the field (monolith sampling) | Higher RLD | Yes, higher RLD at 15–30 cm depth | Kashiwagi et al. (2006a) |
| Terminal drought Heat at reproductive phase | Flowering and maturity | Escape | Visual | Early flowering, early maturity | Selection in combination with rate of partitioning and crop growth rate | Berger et al. (2003), Gaur et al. (2008a), Canci and Toker (2009a), and Purushothaman et al. (2016) |
| Drought at vegetative and reproductive phase | Chlorophyll content | Tolerance | Lab- and field-based (SPAD chlorophyll meter readings (SCMR)) | Higher chlorophyll content, stay green phenotype | NC | Kaushal et al. (2013), Ulemale et al. (2013), and Parankusam et al. (2017) |
| Drought and heat | Chlorophyll fluorescence PSII photochemical efficiency | Tolerance | Chlorophyll fluorometer | Higher Fv/Fm ratio in heat | NC | Rahbarian et al. (2011) and Makonya et al. (2019) |

(continued)

**Table 4** (continued)

| Stress scenario | Traits | Mechanism | Phenotyping | Basis of selection | Correlation with yield under stress | References |
|---|---|---|---|---|---|---|
| Drought and heat | Stomatal conductance, Canopy temperature depression (CTD) | Avoidance | Leaf porometer, thermal imaging (high throughput) | High stomatal Conductance Higher CTD at the reproductive stage, Lower stomatal conductance at the vegetative phase | CTD positive association with yield under heat and drought stress | Zaman-Allah et al. 2011, Devasirvatham et al. (2015), Purushothaman et al. (2016), Kumar et al. (2017), and Priya et al. (2018) |
| Drought and heat | ABA concentration | Tolerance | Direct measurement | seedling stage: facilitates production of osmoprotectants reproductive phase: closes stomata, reduces assimilate production, pod development, abortive pods | No | Kumar et al. (2012b) and Pang et al. (2017) |
| Drought, heat, salt cold | Osmoprotectants (Proline, phenols, and sugars) and Antioxidant enzyme activity | Tolerance | Enzymatic and nonenzymatic measurements | Higher proline, total phenolics, and trehalose content | No | Nayyar et al. (2005a), Eyidogan and Oz (2007), Mafekheri et al. (2011), Kaushal et al. (2011), Kaur et al. (2017), and Farooq et al. (2018) |
| Terminal drought | Rate of partition | Tolerance | Yield measurement | More biomass partitioning to the grains | Yes | Kashiwagi et al. (2013) |
| Terminal drought | Water use efficiency (WUE) | Tolerance | Carbon isotope discrimination ($\Delta^{13}C$) | Lower WUE till flowering | $\Delta^{13}C$ positively correlated with seed yield under drought stress | Kashiwagi et al. (2006b) and Krishnamurthy et al. (2013) |

| | | | | | | |
|---|---|---|---|---|---|---|
| Drought, heat, salt | Relative water content (RWC) | Tolerance | Gravimetric measurement | Higher RWC | NC | Macar and Ekmekci (2009) and Kaushal et al. (2013) |
| Drought | Specific leaf area (SLA) and leaf area index (LAI) | Avoidance | Scanning and image analysis | Higher SLA and LAI, while no clear association with drought | LAI at mid pod fill stage and SLA at physiological maturity | Purushothaman et al. (2016) |
| Heat, cold | Pollen traits | Tolerance | Microscopy | Pollen viability, fertility, germination of pollen tube | No | Clarke et al. (2004), Devasirvatham et al. (2013), and Devasirvatham (2015) |
| Heat, salt | Membrane stability | Tolerance | Electrolyte leakage | Higher membrane stability | No | Hasanuzzaman et al. (2013) and Singh et al. (2018) |
| Heat, drought, salinity and cold | Pod traits | Tolerance | Counting | Pod number, pod production, pod abortion and seed filling | Yes | Kaur et al. (2008), Awasthi et al. (2014), and Turner et al. (2013) |
| Heat and drought | Early vigor | Avoidance | Growth measurement | High vigor: TR restriction under high VPD, helpful to conserve soil water under high evaporative demand | NA | Sivasakthi et al. (2017) |

*NC* not clear, *NA* not available, *TR* Transpiration rate, *VPD* vapour pressure deficit

Although rich germplasm collections are available for a crop, a lack of comprehensive information on the variability of economically important traits often restricts their utilization. This is often limited due to the introduction of the concept of "core" and "mini-core" collections, which have helped circumvent this problem. The core collection consists of about 10% of the entire collection and represents at least 70% of the genetic variability of the entire collection, while the mini-core collection consists of 10% of the core and 1% of the entire germplasm (Upadhyaya et al., 2012). In chickpea, such collections have extensively been screened for a number of characters such as root traits (Kashiwagi et al., 2005; Lalitha et al., 2015), SPAD chlorophyll content (Kashiwagi et al., 2006c), early maturity (Upadhyaya et al., 2007), salinity stress (Serraj et al., 2004; Vadez et al., 2007), and heat stress (Krishnamurthy et al., 2011b; Upadhyaya et al., 2011) that are important in breeding for abiotic stress tolerance. After assessing the genetic diversity for the required trait, parental lines are chosen for hybridization. Various types of populations can be developed, of which the majority of cultivars released for commercial cultivation in self-pollinated crops like chickpea are developed through the pedigree method. This method consists of developing segregating populations of desirable parents, followed by advancing the generation through selfing and selection till the advanced lines with characters from both parents are obtained (Breseghello & Coelho, 2013). Additionally, three-way crosses and multiparent crosses have resulted in the development of abiotic stress tolerant cultivars in chickpea (Table 5). Also, multiparent advanced generation intercross (MAGIC) populations and lines have also been developed in chickpea breeding to make use of diverse founder parents and combine useful genes, including those for abiotic stress tolerance. A MAGIC population using 8 founder parents is developed by ICRISAT, and another using 12 founder parents has been developed by ICARDA (Gaur et al., 2019; Roorkiwal et al., 2020).

In addition to the pedigree method, ideotype breeding is another strategy to breed for abiotic stress tolerance. In this method, desirable traits that are correlated with or associated with complex traits are incorporated, which often involves the usage of wild/exotic germplasm/landraces. This requires backcrossing toward elite

**Table 5** List of Indian varieties of chickpea with tolerance to multiple stresses developed through hybridization

| Sr. No. | Cultivar[a] | Stress[a] | Pedigree[b] |
|---|---|---|---|
| 1 | JG 14 | Heat and drought | [(GW 5/7 X P327) X ICCL 83149] |
| 2 | Indira Chana | Heat and drought | JG 74 X ICCL 83105 |
| 3 | JG 11 | Heat and drought | [(Phule G5 X Narsinghpur bold) X ICCC 37] |
| 4 | JAKI 9218 | Heat and drought | [(ICCV37X GW 5/7) X ICCV 107] |
| 5 | JG 6 | Heat and drought | [(ICCV10 X K850) x (H 208 X RS11)] |
| 6 | RSG 888 | Heat and drought | RSG 44 X E 100Y |
| 7 | GNG 663 | Heat and drought | GNG 16 X GNG 146 |
| 8 | PDG 4 | Drought and Cold | [(GL 769 X GF 88421) X GF 8976] |

[a]Source: Maheswari et al. (2019)

[b]Source: Project Coordinator's Report 2018–2019, All India Coordinated Research project on Chickpea, ICAR-Indian Institute of Pulses research, Kanpur-208024

parents or pre-breeding so that the linkages between undesirable traits and traits of interest can be broken (Breseghello & Coelho, 2013). Several positive alleles contributing toward yield attributing traits from *C. reticulatum* were transferred to cultivated *C. arietinum* cv. GPF2 by backcrossing. As compared to the backcrossed progenies, $F_2$ and $F_3$ were inferior due to linkage drag on undesirable traits like prostrate growth habit, seed shape, and dull seed color. Two-to-three backcrosses reduced the linkage drag (Bhavyasree et al., 2018). Screening among wild species of chickpea has led to the identification of several potential sources of tolerance to different abiotic stresses that are useful for the introgression of desirable traits in the elite background (Table 6). Breeding strategies like advanced backcross QTL can be used wherein, the QTL analysis is done in advanced backcross generation (BC2 onwards) to reduce linkage drag (Tanksley & Nelson, 1996).

Domesticated chickpea has a narrow genetic base due to the repeated use of a few parental lineages over the years (Srivastava et al., 2017). Induced mutagenesis to create genetic variability in the existing gene pool or to develop characters unavailable or lost in the existing gene pool is an important supplementary breeding methodology. Mutation breeding, where the mutants can be directly evaluated or can be used in hybridization programs, is particularly advantageous where improvement is needed in only one or two traits in cultivars that are otherwise very well adapted (Joshua, 2000; Raina et al., 2016, 2017). Induced mutagenesis is the most

**Table 6** Wild chickpea species showing abiotic stress tolerance

| Stress | Accessions screened/location | Species (no. of accessions with resistance/ tolerance) | References |
|---|---|---|---|
| Heat and drought at reproductive phase | 68 lines of eight wild annual *Cicer* species at the Antalya location (approximately 30°44′E, 36°52′N, 51 m above sea level), Turkey, were sown 2 months later than normal sowing time so that the plants get exposed to heat and drought during the reproductive phase | *C. reticulatum* (4)<br>*C. pinnatifidum* (1) | Canci and Toker (2009b) |
| Heat stress at reproductive phase | 10 *Cicer* species, Akdeniz University campus, Antalya, Turkey (30°44′E, 36°52′N, 51 m asl) | *C. turtium* (1) | Toker et al. (2021) |
| Cold: Chilling tolerance at vegetative phase | 137 lines of eight wild annual *Cicer* species 1987–1988 and 1988–1989 seasons at the International Center for Agricultural Research in the Dry Areas (ICARDA), Syria | *C. bijugum*<br>*C. echinospermum*<br>*C. reticulatum* | Singh et al. (1990) |
| | 857 breeding lines, 4284 *kabuli* and 2137 *desi* germplasm lines, and 59 lines of seven annual wild *Cicer* species were evaluated during the winter seasons of 1987–1992, at ICARDA sites at Tel Hadya and Breda in Syria | *C. reticulatum* (26)<br>*C. bijugum* (10)<br>*C. echinospermum* (4)<br>*C. pinnatifidum* (2)<br>*C. judaicum* (1) | Singh et al. (1995) |

(continued)

**Table 6** (continued)

| Stress | Accessions screened/location | Species (no. of accessions with resistance/ tolerance) | References |
|---|---|---|---|
| Cold: Chilling tolerance at reproductive phase | Wide range of domesticated ($n$ = 1762) and wild *Cicer* ($n$ = 200) germplasm, field trials over a wide range of sites ($n$ = 26) in southwest Australia, northern India, and northern Syria over years and/or sowing dates to maximize the likelihood of encountering a wide temperature range during the reproductive phase | *C. echinospermum* (1)<br>*C. bijugum*<br>*C. judaicum* | Berger et al. (2012) |
| Cold: Freezing tolerance at young plant stage Seedlings were covered by snow for 25 days in the season. The number of days with freezing temperatures was 69 | 43 accessions of eight annual wild *Cicer* species at the Urkutlu location (30E, 37N and 860 m from sea level) in Bucak city of Burdur province in the west Mediterranean region of Turkey | *C. bijugum* (3)<br>*C. reticulatum* (2)<br>*C. echinospermum* (2) | Toker (2005) |
| Drought | 7 perennial wild species, 3 annual wild species, at Antalya (approximately 30°44′E, 36°52N, 51 m from sea level), Turkey, pots, Drought stress was applied three times consecutively, and seedlings were wilted as dried out. | Perennials:<br>*C. anatolicum*<br>*C. microphyllum*<br>*C. songaricum*<br>Annuals:<br>*C. pinnatifidum*<br>*C. reticulatum* | Toker et al. (2007b) |
| Salinity | Total 600 accession of which, 43 annual wild species, pot level, green house, salt treatment (NaCl: 6 $dSm^{-1}$) commenced at 21 days after sowing and were scored on the basis of necrosis at 42 days after sowing | *C. reticulatum* (6) | Maliro et al. (2008) |

efficient technique to greatly increase genetic variation in a short period of time and has been employed in various crops such as cowpea (Raina et al., 2020a, Rasik et al., 2022); lentil (Laskar et al., 2018a, b; Wani et al., 2021) faba bean (Khursheed et al., 2018), mungbean (Wani et al., 2017), urdbean (Goyal et al., 2019a, b), chickpea (Laskar et al., 2015; Raina et al., 2019), black cumin (Tantray et al., 2017, Amin et al., 2019), and finger millet (Sellapillaibanumathi et al., 2022). Because natural mutations occur sporadically, artificial mutations are generated, and genetic gain is best achieved by using mutagens (Raina & Khan, 2020, Raina et al., 2020b, 2022a, b, c, d; Sellapillai et al., 2022). Various physical and chemical mutagens have been used both individually and in combination to introduce mutation in various crop plants. The success of mutation breeding is evident from "mutant variety database" created by FAO & International Atomic Energy Agency (IAEA) that enlists over

>3000 mutant varieties in more than 175 crop plants (https://mvd.iaea.org/#!Search). The application of mutation breeding in chickpea is still underexploited, as so far only a handful of chickpea varieties (27 listed in mutant variety database) have been developed through mutation breeding. A number of chickpea mutants have been reported (Kharkwal, 1998, 1999; Khan et al., 2005; Wani, 2009; Kamble & Patil, 2014), of which many have been isolated with unique agro-morphological traits such as variations in leaf shape, size, and type (Kozgar, 2014; Joshi-Saha et al., 2015), stem fasciation (Gaur & Gour, 1999), disease resistance (Shah et al., 2009), broad few leaflets, compact growth habit (Gaur et al., 2008b), outwardly curved wings (Gaur & Gour, 2003),variegated leaf and apical chlorosis (Gaur et al., 2004), iron deficiency chlorosis resistant mutant (Toker et al., 2012), and determinate growth habit (Yildirim et al., 2013). "Escape traits" of early phenology (early flowering, early maturity) can be an easy target to achieve using mutation breeding in an otherwise elite, high yielding background (Fig. 3).

The advent of molecular techniques, especially the development of molecular markers, has facilitated faster and better selection, particularly for the transfer of quantitative trait loci (QTLs). The first genetic map of chickpea was developed in 1997 by combining the mapping results from three interspecific (*C. arietinum* X *C. reticulatum*) mapping populations. This map consisted of 9 morphological, 27 isozyme, 10 RFLP, and 45 RAPD markers covering 550 cM (Simon & Muehlbauer, 1997). Since then, there has been a tremendous development in the identification of newer marker types and their numbers (Fig. 4).

Early generation markers (like RAPD, AFLP) were based on scanning the whole genome randomly, while, major milestones were achieved when large-scale development of both nongenic as well as genic SSRs and SNPs were developed. The marker development was boosted by advancements in next generation sequencing (NGS) and whole genome sequencing and resequencing of chickpea genomes. Various quantitative trait loci and linked or associated markers have been identified in chickpea that are useful for marker assisted breeding (Table 7).

Advancements in NGS technologies and the complete genome sequence availability of cultivated as well as wild chickpea (Varshney et al., 2013; Jain et al., 2013; Gupta et al., 2017) have great promises for "genomics assisted breeding" (GAB).

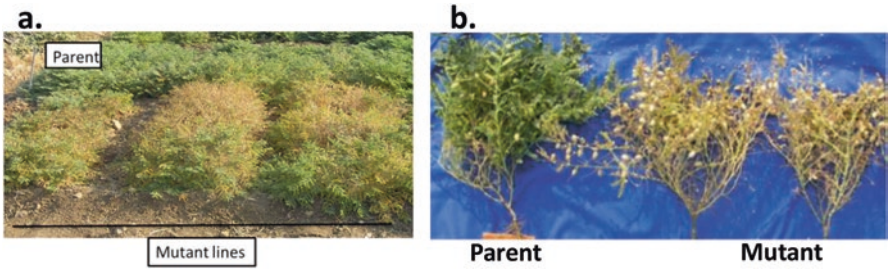



**Fig. 3** (**a**) Development of early maturing chickpea mutant lines through mutation breeding using electron beam irradiation. (**b**) Single plants of parent and mutant. Parent matures in 100–110 days, while mutant matures in 85–90 days at Trombay, Mumbai, India

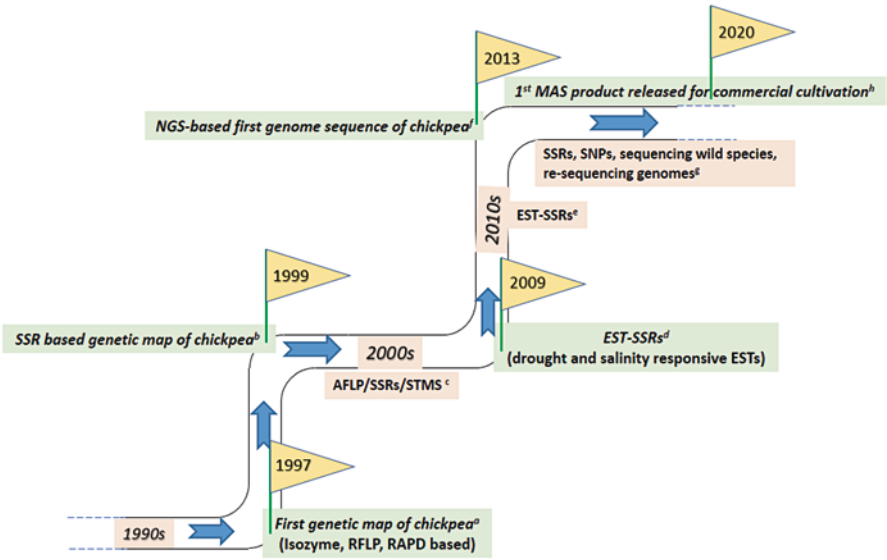



**Fig. 4** Development, usage, and major milestones for molecular breeding in chickpea. (**a**) Simon and Muehlbauer (1997); (**b**) Winter et al. (1999); (**c**) Nguyen et al. (2004), Lichtenzveig et al. (2005), Sethy et al. (2003, 2006); (**d**) Varshney et al. (2009); (**e**) Hiremath et al. (2011), Garg et al. (2011), Deokar et al. (2011); (**f**) Varshney et al. (2013), Jain et al. (2013); (**g**) Deokar et al. (2014), Parida et al. (2015), Gupta et al. (2017), Varshney et al. (2019); (**h**) https://icar.org.in/content/development-two-superior-chickpea-varieties-genomics-assisted-breeding. RFLP: Restriction Fragment Length, RAPD: Random amplified fragment length polymorphism, AFLP: Amplified fragment length polymorphism, SSRs: simple sequence repeats, STMS: sequence tagged microsatellites, EST-SSR: Expressed sequence tag- simple sequence repeat, SNPs: single nucleotide polymorphisms, NGS: next generation sequencing, MAS: marker assisted selection

Two main approaches can be taken for genomic-assisted breeding: (a) genomic selection (GS) and (b) marker assisted selection (MAS). For MAS, markers that are strongly associated with the trait of interest are needed to be found. Identified markers can then be used for selection in breeding programs. MAS can be employed in marker assisted back crossing (MABC) and marker assisted recurrent selection (MARS) (Thudi et al., 2014b; Khan et al., 2016b). In genomic selection (GS), selection models are developed based on dense genetic markers that are spread across the whole genome as well as the phenotyping of a training population that helps select individuals with high genome-estimated breeding values (GEBVs) in the breeding population (Khan et al., 2016b). It is an advanced breeding approach; unlike MABC and MARS, it predicts the genomic assisted breeding values of a line by analyzing historical genotyping as well as phenotyping data and thus selecting lines using genotyping data before carrying out phenotyping analysis of the line in the field. The rate of genetic gains can potentially be augmented using this method (Roorkiwal et al., 2018).

Very recently, two chickpea varieties developed through marker assisted breeding have been released for commercial cultivation in India (https://icar.org.in/

**Table 7** List of QTLs and markers associated with or linked to abiotic stress tolerance in chickpea

| Abiotic stress | Population | Traits[a] | QTLs and location | Markers | Method | References |
|---|---|---|---|---|---|---|
| Drought | 115 RILs (ILC 588$^{DT}$ × ILC3279$^{DS}$) | HI, flowering, maturity, higher SC, lower canopy temperature | 18, of which 2, LG3 and LG1, affected many traits related to drought | 97 SSRs | Linkage mapping, CIM | Rehman et al. (2011) |
| | 181 RILs (ILC588 $^{DT}$ × ILC3279 $^{DS}$) | drought resistance score, PH, DF, DM, GY, BY, HI, numbers of pod and seed/3 plants, empty pods %, 100 SW | 96, of which 4 common, possible pleiotropic on LG3 and LG4 | SSR markers H6C07 (LG3) and H5G01 (LG4) associated with QTLs for multiple traits | Linkage mapping, ICIM-EPI | Hamwieh et al. (2013) |
| | 264 RILs (ICC 4958$^{DT}$ × ICC 1882) and 288 RILs (ICC 283 × ICC 8261 $^{DT}$) | 20 DT traits related to root, yield, and yield-related, morphological, phenological, and physiological traits | 45 main effect, 973 epistatic, and 9 clusters of QTL for drought traits of which 1 cluster (QTL hotspot) on LG4 | SSRs (ICCM0249, NCPGR127, TAA170, NCPGR21, TR11, GA24 and STMS11) | Linkage mapping, CIM | Varshney et al. (2014) |
| | 264 RILs (ICC 4958$^{DT}$ × ICC 1882) | Same as Varshney et al. (2014) | 164 main-effect QTLs including 24 novel QTLs | GBS of RILs derived SNPs, 49 SNP markers in the QTL-hotspot region | Fine mapping of QTL hotspot identified by Varshney et al. (2014), CIM | Jaganathan et al. (2015) |
| | 232 RILs (ICC 4958$^{DT}$ × ICC 1882) | 17 drought related traits from Varshney et al. (2014) | QTL-hotspot region (Varshney et al., 2014) fine mapped into 2 subregions, namely, QTL-hotspot_a(15 genes) and QTL-hotspot_b (11 genes) | SNPs | High density bin mapping, ICIM-ADD mapping Gene enrichment analysis using significant marker trait associations | Kale et al. (2015) |

(continued)

**Table 7** (continued)

| Abiotic stress | Population | Traits[a] | QTLs and location | Markers | Method | References |
|---|---|---|---|---|---|---|
| | RILs (ICC 4958$^{DT}$ × ICC 1882), 15 RILs pooled for each contrasting trait | 100SW, RTR under rainfed conditions. | 2 genomic regions: on CaLG01 (1.08 Mb) and CaLG04 (2.7 Mb) linkage groups for 100SDW. Another genomic region on CaLG04 (1.10 Mb) for RTR | SNPs, putative candidate genes associated with 100SDW (4 genes) and RTR (5 genes), respectively. | Simple interval mapping | Singh et al. (2016) |
| | 190 $F_8$ RILs (*C. arietinum* desi accession ICC 4958 × *C. reticulatum* wild accession ICC 17163) | YP and HI | 6 QTLs, 5 root-specific genes with non-synonymous and regulatory SNPs governing drought-responsive yield traits | Genic SSRs and SNPs from transciptome of *C. microphyllum* and comparison with *C. arietinum*, ICC 4958 | 490 genic SSR- and SNP markers-anchored genetic linkage map, CIM | Srivastava et al. (2016) |
| | 232 RILs (ICC 4958$^{DT}$ × ICC 1882) | 16 phenotypic traits categorized into 3 groups: (i) Canopy traits (ii) Transpiration traits (iii) Biomass traits<br>Traits measured under well-watered conditions | 21 major QTLs for plant vigour and canopy conductance traits 13 QTLs for plant vigour traits on CaLG04, 1 QTL for canopy conductance on CaLG03 earlier reported (Varshney et al., 2014) drought tolerance hotspot identified as a vigour locus | – | ultra-high density bin map, GMM and CIM | Sivasakthi et al. (2018) |

| | | | | | | |
|---|---|---|---|---|---|---|
| Drought and heat | Of 300 accessions of reference set | Root traits:RL; RLD; RDW; RD; RSA; RV; RDW/TDW; AD; PA, Morphological traits: PH, PW, apical primary & secondary branches, basal primary and secondary branches, Tertiary branches, SDW Phenological traits: DF50, DM, and FD, GY, and yield related traits: seeds/pod, pods/plant, 100SW, GY, yield/plant, biomass, HI, seeds/area, TDW transpiration efficiency related traits: Δ carbon ratio, and SPAD chlorophyll meter reading | 312 significant MTAs of which 70 for 100 SW | Diversity Array Technology (DArT), SNP, SSR 18 SNPs from 5 genes (ERECTA, ASR, DREB, CAP2 promoter, and AMDH) significantly associated with different traits | Association mapping, MLM | Thudi et al. (2014a) |
| | 272 accessions | 20 drought and heat tolerance related traits | 262 markers and several candidate genes for 13 traits | SNPs, | Genome wide association using (GLM)/(CMLM) | Varshney et al. (2019) |

(continued)

**Table 7** (continued)

| Abiotic stress | Population | Traits[a] | QTLs and location | Markers | Method | References |
|---|---|---|---|---|---|---|
| Heat stress | 292 RILs (ICC $4567^{HS}$ × ICC $15614^{HT}$ | Under heat stress: Number of filled pods, total number of seeds, and GY (per plot basis), HI, biomass and pod setting %. visual score on podding behavior | 2 genomic regions with 4 QTLs each, on CaLG05 and CaLG06, 4 major QTLs for number of filled pods, total number of seeds, and GY (per plot basis) and pod setting %, located in the CaLG05, 19 pairs of epistatic QTLs with significant epistatic effect, and nonsignificant QTL × environment effect, 25 putative candidate genes identified | GBS derived SNPs | CIM | Paul et al. (2018) |
| | 71 genotypes | | MTA for membrane stability index and leaf chlorophyll content measured | SSRs | Association analysis, GLM, MLM | Jha et al. (2018) |
| | 206 $F_2$ (DCP 92-3 × ICCV 92944) | 17 traits under heat stress conditions | 1 QTL on LG3 for primary branch number and another QTL on LG6 related to chlorophyll content | SSRs | | Jha et al. (2019) |
| Cold | 129 RILs (*C. arietinum* desi accession ICC $4958^{CS}$ × *C. reticulatum* wild accession PI 489777) | Visual damage to leaf by cold<br>Freezing test | 3 significant QTL were found on (LGs) 1B, 3, and 8 | GBS derived SNPs | CIM | Mugabe et al. (2019) |

| | | | | | | |
|---|---|---|---|---|---|---|
| Salinity | 126 RILs ($JG62^{ST} \times ICCV2^{SS}$) | Seed number, seed yield, in early and late flowering group | A QTL for GY under saline conditions, and a cluster of QTLs for GY components in LG 6, including a QTL for seed number | SSRs | CIM | Vadez et al. (2012) |
| | 188 RILs (ICCV $2^{SS} \times$ JG $11^{ST}$) | DF50, DM, 7 yield-related traits: aboveground dry matter/plant stem & leaf weight/plant, total pod number/plant, seed number/plant, yield/plant, HI, 100 SW, filled pods number, empty pods number | 6 QTLs on CaLG05and 5 on CaLG07 for different salinity tolerance associated traits and either higher plant vigor (CaLG05) or higher reproductive success (CaLG07). 48 putative salinity stress responsive candidate genes found on CaLG05 (31 genes) and CaLG07 (17 genes) in a distance of 11.1 Mb and 8.2 Mb | SSRs, SNPs | Linkage map, CIM | Pushpavalli et al. (2015b) |
| | 201 RILs (DCP92-$3^{SS} \times ICCV10^{ST}$) | GY and other yield contributing traits | 2 genomic regions for for salinity tolerance QTLs in a ~3.3 Mb region on CaLG03 and ~0.1 Mb region on CaLG06 with major QTLs for yield and salinity tolerance | Axiom® CicerSNParray | | Soren et al. (2020) |

(continued)

**Table 7** (continued)

| Abiotic stress | Population | Traits[a] | QTLs and location | Markers | Method | References |
|---|---|---|---|---|---|---|
| | 200 RILs (Rupali$^{SS}$ ×Genesis 836$^{ST}$) | Imaging-based: PSA, RGR, days to first flower, sodium and potassium content in the youngest fully expanded leaves, PH, GY and yield components including shoot biomass at maturity, seed number, total pod number, empty pod number, filled pod number, and 100 SW | 42 QTL for RGR, GY, and yield component traits under both control and saline conditions, and leaf tissue ion accumulation under salt stress, 6 QTL on CaLG04, CaLG05, and CaLG06 associated with tolerance per se, 21 QTL mapped to two distinct regions on CaLG04; The first distinct region controlled: number of filled pods, leaf necrosis, seed number, and GY specifically under salinity, and co-located with 4 QTL linked to salt tolerance per se. The second distinct region controlled 100SW and growth-related traits, independent of salinity treatment | DArTseq, KASP-based genotyping assay | Linkage mapping, whole genome average interval mapping | Atieno et al. (2021) |

*RILs* recombinant inbred lines, *DT* drought tolerant, *DS* drought susceptible, *MTA* marker-trait association, *SNPs* single nucleotide polymorphisms, *GBS* genotype by sequencing, *CIM* composite interval mapping, *ICIM-EPI* inclusive composite interval mapping-epistatic mapping, *MLM* mixed linear model, *ICIM-ADD* Inclusive Composite Interval Mapping of Additive (and dominant) QTL, *GMM* genotype matrix mapping, *GLM* generalized linear model, *CMLM* compressed mixed linear model, *HS* heat-sensitive, *HT* heat tolerant, *CS* cold-sensitive, *CT* cold tolerant, *SS* salt-sensitive, *ST* salt tolerant, [a]trait abbreviations used: *HI* harvest Index, *SC* stomatal conductance, *PH* plant height, *DF* days to flowering, *DM* days to maturity, *GY* grain yield, *BY* biological yield, *100SW* 100 seed weight, *DT* drought tolerant, *RTR* total dry root weight to total, plant dry weight ratio, *YP* yield (g) per plant, *RL* rot length, *RLD* root length density, *RDW* root dry weight, *RD* rooting depth, *RSA* root surface area, *RV* root volume, *TDW* total dry weight, *AD* average diameter, *PA* projected area, *PW* plant width, *SDW* shoot dry weight, *DF50* days to 50% flowering, *FD* flowering days, *PSA* projected shoot area, *RGR* relative growth rates

content/development-two-superior-chickpea-varieties-genomics-assisted-breeding). The first variety, "Pusa Chickpea 10216," is a drought-tolerant variety developed after introgression of "QTL-hotspot" using molecular markers in the genetic background of Pusa-372. On the other hand, another variety, "Super Annigeri 1," is developed by the introgression of a resistance segment for Fusarium wilt from the WR 315 genotype of chickpea in the genetic background of Annigeri-1.

Moreover, recent developments in CRISPR-Cas based genome editing tools can be very useful and expedite precision breeding (Veillet et al., 2020). In chickpea, very recently, using this technology, the first report of gene-knockouts for two genes associated with drought tolerance, namely, 4-caumarate ligase (4-CL, a gene of the phenylpropanoid pathway controlling lignin biosynthesis) and Reveille 7 (RVE7, a Myb transcription factor regulating circadian rhythms in plants) has been reported (Badhan et al., 2021). Presently, the work has been done in chickpea protoplast; further work is needed at the plant level, and the effect of such knockouts in terms of drought stress response is yet to be explored.

## 4 Developing Nutritionally Improved Chickpeas

"Hidden hunger" denoting micronutrient malnutrition, particularly iron (Fe), zinc (Zn), and vitamin A, affects more than 2 billion people globally (Saltzman et al., 2017; Gödecke et al., 2018). Several measures have been proposed to tackle the problem of hidden hunger, including food fortification, supplementation of the diet with micronutrients, and diet diversification (Bamji et al., 2020). In addition, biofortification, i.e., enhancing the nutrients in the grain or other edible part of the plant, either with agronomic practices (agronomic biofortification) or through conventional plant breeding or transgenic means (genetic biofortification), seems a more viable approach to reaching a larger population that cannot afford fortified foods. Genetic biofortification through conventional breeding is considered the most acceptable, cost-effective, and sustainable strategy (Bouis et al., 2011; Saltzman et al., 2013; Garg et al., 2018). Being staple crops, rice, wheat, maize, potato, and cassava are preferred targets for genetic biofortification (Saltzman et al., 2017). Pulses (grain legumes), including chickpea are also staple food crops in many Asian and African countries. Often called "poor man's meat," they serve as a cheap and rich source of proteins for a large population. Moreover, pulses and cereals complement each other with respect to the essential amino acids viz., lysine and methionine, respectively (Singh, 2017). The biofortification programs for nutritionally improved pulses, including chickpea, are relatively recent. Most of the agronomic biofortification strategies for chickpea are in the research stage. The type of application (soil versus foliar), nature of inorganic fertilizer, and time of application are being standardized. Soil or foliar application of Zn, Fe, and/or urea showed increased Fe and Zn content in the grains (Hidoto et al., 2017; Pal et al., 2019a, b). Zinc–ethylenediaminetetraacetic acid (Zn-EDTA) was found to be better than zinc sulfate heptahydrate. Also, foliar application of Zn was proved to be better than soil

application. When foliar sprays of Zn-EDTA at three stages (active vegetative, flowering, and grain-filling) were given, the following positive changes were observed in chickpea: highest grain and straw yields, greatest Zn biofortification of grains, and highest Zn uptake. Agronomic efficiency was greatest (kg grain increased $kg^{-1}$ Zn applied) following two sprays of Zn-EDTA at vegetative and flowering stages) (Shivay et al., 2015). Recently, a tank mix application of $ZnSO_4$ (0.5%), $FeSO_4$ (0.5%), and urea (2%) at flowering and pod formation stages of chickpea showed improvement in the Zn and Fe contents of chickpea seeds (Pal et al., 2021). Agronomic biofortification is labor-intensive and less cost-effective due to the requirement that the chemicals need to be applied at particular stages of plant growth, although some cost can be reduced by combining foliar sprays with pesticide applications (Wang et al., 2016). However, the extensive use of chemicals is a cause for concern due to the environmental pollution attributed to their indiscriminate use and consequent leaching into groundwater. Moreover, soil quality parameters and crop physiology also influence the success of agronomic biofortification strategies. In addition, the interaction of various chemical species with each other can also influence their uptake and nutritional status in crops (Prasad et al., 2016).

Mineral micronutrient deficiency in humans is a global challenge. Several strategies have been developed to combat this menace. Although transgenic- and agronomic-based strategies of biofortification are being used, biofortification through conventional breeding is considered to be more acceptable than the other strategies (Garg et al., 2018). As a first step toward nutritionally improved chickpeas, there is a need to establish a baseline status for Fe and Zn in the crop. This requires studies ascertaining the diversity of this trait in the available germplasm and cultivars. Such studies have been conducted in chickpeas, and a wide variation for both Fe and Zn content has been observed (Thavarajah & Thavarajah, 2012; Diapari et al., 2014; Upadhyaya et al., 2016; Tan et al., 2018; Joshi-Saha et al., 2018; Misra et al., 2020; Joshi-Saha et al., 2021). The studies have also shown a strong genotype X environment (G X E). The overall contribution toward variation was highest for the location (environment) effect, followed by genotype, and least for genotype X location in 19 chickpea genotypes grown in four locations belonging to three different agroclimatic zones of chickpea cultivation in India (Misra et al., 2020). Moreover, a positive correlation between Fe and Zn content in chickpea was observed, suggesting that the two traits can be co-selected (Tan et al., 2018; Vandemark et al., 2018; Misra et al., 2020). Another important aspect to consider in breeding for high mineral micronutrient traits is their correlation with yield. In chickpea, many studies have reported a negative correlation of Zn with yield (Diapari et al., 2014; Vandemark et al., 2018; Misra et al., 2020). Therefore, in breeding for high Zn combined with high yield, such deleterious linkages should be broken by either the appropriate selection of parents for hybridization programs or the use of induced mutagenesis and mutation breeding. Use of tightly linked molecular markers with high zinc content is also important in selecting for high mineral content in a high yielding genetic background.

There is immense scope for using molecular tools, particularly marker assisted selection (MAS), genome-wide association studies (GWAS), and genomic selection

**Table 8** Markers linked/associated with high iron/zinc content in chickpea

| Population | Method | Identified markers and locations | References |
|---|---|---|---|
| 94 accessions | Association mapping | 8 SNPs loci, Chr 1 (1, Fe and Zn), Chr 4 (3 Zn, 2 Fe), Chr 6 (1 Fe), Chr 7 (1, Zn) | Diapari et al. (2014) |
| ICC 4958 X ICC 8261 (RIL, 277 individuals), 92 accessions | Linkage and Association mapping | 8 QTLs<br>16 SNPs, of which, 11 trait-associated SNPs linked tightly with 8 QTLs validated by QTL mapping and expression analysis | Upadhyaya et al. (2016) |
| MNK-1 X Annigeri 1 (185, $F_{2:3}$ individuals) | Linkage mapping | 11 QTLs in CaLG03, CaLG04, and CaLG05 | Sab et al. (2020) |

*SNPs* Single nucleotide polymorphisms, *RIL* recombinant inbred line

(GS), in chickpea breeding for improvement in mineral micronutrient content. However, there are only a few studies so far that have identified markers linked to or associated with high grain Fe and/or Zn content in chickpea (Table 8).

## 5 Co-improving Stress Tolerance and Quality Parameters-Paradox or Possibility?

Many quality improvement programs emphasize breeding for reduced antinutritional factors such as phytic acid (PA) and the raffinose family of oligosaccharides in pulse crops. However, these compounds play an important role in general plant physiology and performance under various stress conditions (Joshi-Saha & Reddy, 2016; Salvi et al., 2016, 2018). In cereals and pulses, including chickpea, increased PA content reduces the bioavailability of mineral micronutrients by chelation owing to its negatively charged phosphate groups and lack of phytase enzyme in non-ruminant animals, including humans (Schlemmer et al., 2009). Therefore, breeding for low phytic acid is an important consideration in improving of nutritional quality. However, PA seems to play a role in biotic and abiotic stress tolerance (Murphy et al., 2008; Joshi-Saha and Reddy, 2015), and perturbations in its biosynthetic pathways seem to affect the stress signaling pathways and responses (Lemtiri-Chlieh et al., 2000, 2003; Doria et al., 2009; Raboy, 2009). Many breeding lines with reduced phytic acid content show reduced seed germination and vigor, especially in tropical environments that are prone to abiotic stresses (Meis et al., 2003; Oltmans et al., 2005; Naidoo et al., 2012; Raboy et al., 2015). Therefore, depending on the crop, a threshold value of PA content should be decided so that plant performance under stress is not affected. A recent multi-environment study in chickpea indicates that some of the popular cultivars of India have naturally been selected for low PA during their development (Misra et al., 2020).

## 6 Conclusion and Future Prospects

Modern breeding approaches require accelerated breeding programs. This can be achieved through multifaceted approaches that not only save time but also can handle large-sized breeding populations. A "speed breeding" approach in a long-day plant like chickpea, can markedly reduce the breeding cycle time by increasing the number of generations from only one generation per year to up to seven generations per year (Samineni et al., 2020). In addition, "phenomics," involving large-scale high-throughput phenotyping (HTP) methods, can help in the screening of a large number of populations. Many of the imaging-based systems can provide a nondestructive means coupled with a quicker screening. Such HTP methods have been used in chickpea for root traits in a mini rhizotron (Pratap et al., 2019), 3D leaf area, projected leaf area, plant height (Kar et al., 2020), canopy temperature, plant vigor under water stress (Sivasakthi et al., 2017), and image-based analysis under salinity stress (Atieno et al., 2017). However, such large-scale phenotyping methods for nutritional traits, particularly for estimation of micronutrients like Fe and Zn, are lacking and are a major bottleneck in breeding for nutritionally enhanced chickpeas. Presently, atomic absorption spectrometry (AAS) and inductively coupled plasma optical emission spectrometry (ICP-OES) are used for estimation. Although these techniques are very sensitive, they are labor-intensive, expensive, and time consuming. There is a need to explore nondestructive energy dispersive X-ray fluorescence (EDXRF) in a high throughput mode for rapid screening of Fe and Zn. In addition, to quantifying the nutrient levels in the crop, there is also a need to develop robust methods to assess their bioavailability. Such methods will not only be useful for setting up a reliable baseline for nutritional enhancement but also will be useful for assessing biofortified crops once they are developed. Combining these with genomics-assisted breeding will also help in the rapid and efficient incorporation of novel/useful alleles in chickpea breeding programs.

## References

Amin, R., Wani, M. R., Raina, A., Khursheed, S., & Khan, S. (2019). Induced morphological and chromosomal diversity in the mutagenized population of black cumin (*Nigella sativa* L.) using single and combination treatments of gamma rays and ethylmethane sulfonate. *Jordan Journal of Biological Sciences, 12*(1), 23–30.

Amini, S., Maali-Amiri, R., Kazemi-Shahandashti, S.-S., et al. (2021). Effect of cold stress on polyamine metabolism and antioxidant responses in chickpea. *Journal of Plant Physiology, 258–259*, 153387. https://doi.org/10.1016/j.jplph.2021.153387

Arif, A., Parveen, N., Waheed, M. Q., et al. (2021). A comparative study for assessing the drought-tolerance of chickpea under varying natural growth environments. *Frontiers in Plant Science, 11*, 607869. https://doi.org/10.3389/fpls.2020.607869

Arslan, Ö., Eyidoğan, F., Ekmekçi, Y., et al. (2018). Freezing tolerance of chickpea: Biochemical and molecular changes at vegetative stage. *Biologia Plantarum, 62*, 140–148. https://doi.org/10.1007/s10535-017-0760-5

Atieno, J., Li, Y., Langridge, P., et al. (2017). Exploring genetic variation for salinity tolerance in chickpea using image-based phenotyping. *Scientific Reports, 7*, 1300. https://doi.org/10.1038/s41598-017-01211-7

Atieno, J., Colmer, T. D., Taylor, J., et al. (2021). Novel salinity tolerance loci in chickpea identified in glasshouse and field environments. *Frontiers in Plant Science, 12*, 667910. https://doi.org/10.3389/fpls.2021.667910

Awasthi, R., Kaushal, N., Vadez, V., et al. (2014). Individual and combined effects of transient drought and heat stress on carbon assimilation and seed filling in chickpea. *Functional Plant Biology, 41*, 1148–1167. https://doi.org/10.1071/FP13340

Awasthi, R., Bhandari, K., & Nayyar, H. (2015). Temperature stress and redox homeostasis in agricultural crops. *Frontiers in Environmental Science, 3*. https://doi.org/10.3389/fenvs.2015.00011

Awasthi, R., Gaur, P., Turner, N. C., et al. (2017). Effects of individual and combined heat and drought stress during seed filling on the oxidative metabolism and yield of chickpea (*Cicer arietinum*) genotypes differing in heat and drought tolerance. *Crop & Pasture Science, 68*, 823–841.

Badhan, S., Ball, A. S., & Mantri, N. (2021). First report of CRISPR/Cas9 mediated DNA-free editing of 4CL and RVE7 genes in chickpea protoplasts. *International Journal of Molecular Sciences, 22*, 396. https://doi.org/10.3390/ijms22010396

Bakht, J., Bano, A., & Dominy, P. (2006). The role of abscisic acid and low temperature in chickpea (*Cicer arietinum* L.) cold tolerance. II. Effects on plasma membrane structure and function. *Journal of Experimental Botany, 57*, 3707–3715. https://doi.org/10.1093/jxb/erl120

Bamji, M. S., Murty, P. V. V. S., & Sudhir, P. D. (2020). Nutritionally sensitive agriculture – An approach to reducing hidden hunger. *European Journal of Clinical Nutrition, 75*, 1001–1009. https://doi.org/10.1038/s41430-020-00760-x

Berger, J., Turner, N. C., & French, R. J. (2003). The role of phenology in adaptation of chickpea to drought. In *Solutions for a better environment. Proceedings of the 11th Australian agronomy conference*, 2–6 February 2003. Australian Society of Agronomy.

Berger, J. D., Buck, R., & Henzell, J. M. (2005). Evolution in the genus *Cicer*-vernalisation response and low temperature pod set in chickpea (*Cicer arietinum* L.) and its annual wild relatives. *Australian Journal of Agricultural Research, 56*, 1191–1200. https://doi.org/10.1071/AR05089

Berger, J. D., Ali, M., Basu, P. S., et al. (2006). Genotype by environment studies demonstrates the critical role of phenology in adaptation of chickpea (*Cicer arietinum* L.) to high and low yielding environments of India. *Field Crops Research, 98*, 230–244.

Berger, J. D., Milroy, S. P., Turner, N. C., et al. (2011). Chickpea evolution has selected for contrasting phenological mechanisms among different habitats. *Euphytica, 180*, 1–15.

Berger, J. D., Kumar, S., Nayyar, H., et al. (2012). Temperature-stratified screening of chickpea (*Cicer arietinum* L.) genetic resource collections reveals very limited reproductive chilling tolerance compared to its annual wild relatives. *Field Crops Research, 126*, 119–129. https://doi.org/10.1016/j.fcr.2011.09.020

Bhavyasree, R. K., Singh, S., & Singh, I. (2018). Advanced backcross strategy for alien introgression for productivity enhancing traits in chickpea (*Cicer arietinum* L.). *Legume Research, 41*, 379–383.

Blum, A. (2011). *Plant breeding for water – Limited environments*. Springer.

Bouis, H. E., Hotz, C., McClafferty, B., et al. (2011). Biofortification: A new tool to reduce micronutrient malnutrition. *Food and Nutrition Bulletin, 32*, S31–S40. https://doi.org/10.1177/15648265110321S105

Breseghello, F., & Coelho, A. S. G. (2013). Traditional and modern plant breeding methods with examples in rice (*Oryza sativa* L.). *Agricultural and Food Chemistry, 61*, 8277–8286.

Canci, H., & Toker, C. (2009a). Evaluation of yield criteria for drought and heat resistance in chickpea (*Cicer arietinum* L.). *Journal of Agronomy and Crop Science, 19*, 47–54. https://doi.org/10.1111/j.1439-037X.2008.00345.x

Canci, H., & Toker, C. (2009b). Evaluation of annual wild *Cicer* species for drought and heat resistance under field conditions. *Genetic Resources and Crop Evolution, 56*, 1.

Chakraborty, U., & Tongden, C. (2005). Evaluation of heat acclimation and salicylic acid treatments as potent inducers of thermotolerance in chickpea (*Cicer arietinum* L.). *Current Science, 89*, 1–6.

Chaturvedi, S. K., Mishra, D. K., Vyas, P., et al. (2009). Breeding for cold tolerance in chickpea. *Trends in Biosciences, 2*, 1–6.

Chidambaranathan, P., Jagannadham, P. T. K., & Satheesh, V. (2018). Genome-wide analysis identifies chickpea (*Cicer arietinum*) heat stress transcription factors (Hsfs) responsive to heat stress at the pod development stage. *Journal of Plant Research, 31*(3), 525–542. https://doi.org/10.1007/s10265-017-0948-y

Clarke, H. J. (2001). Improving tolerance to low temperature in chickpea. In *4th European conference on grain legumes. Towards the sustainable production of healthy food, feed and novel products*, 8–12 July 2001, pp. 34–35.

Clarke, H. J., & Siddique, K. H. M. (1998). Growth and development. In S. Loss, N. Brandon, & K. H. M. Siddique (Eds.), *The chickpea* (Western Australia Agriculture Bulletin 1326). Department of Western Australia Agriculture.

Clarke, H. J., & Siddique, K. H. M. (2004). Response of chickpea genotypes to low temperature stress during reproductive development. *Field Crops Research, 90*, 323–334. https://doi.org/10.1016/j.fcr.2004.04.001

Clarke, H., Khan, T. N., & Siddique, K. H. M. (2004). Pollen selection for chilling tolerance at hybridisation leads to improved chickpea cultivars. *Euphytica, 139*, 65–74. https://doi.org/10.1007/s10681-004-2466-y

Croser, J. S., Clarke, H. J., Siddique, K. H. M., et al. (2003). Low temperature stress: Implications for chickpea (*Cicer arietinum* L.) improvement. *Critical Reviews in Plant Sciences, 22*, 185–219.

Deokar, A. A., Kondawar, V., Jain, P. K., et al. (2011). Comparative analysis of expressed sequence tags (ESTs) between drought-tolerant and -susceptible genotypes of chickpea under terminal drought stress. *BMC Plant Biology, 11*, 70. https://doi.org/10.1186/1471-2229-11-70

Deokar, A. A., Ramsay, L., Sharpe, A. G., et al. (2014). Genome wide SNP identification in chickpea for use in development of a high density genetic map and improvement of chickpea reference genome assembly. *BMC Genomics, 15*, 708. https://doi.org/10.1186/1471-2164-15-708

Devasirvatham, V., & Tan, D. K. Y. (2018). Impact of high temperature and drought on chickpea production. *Agronomy, 8*, 145. https://doi.org/10.3390/agronomy8080145

Devasirvatham, V., Tan, D. K. Y., Gaur, P. M., et al. (2012). High temperature tolerance in chickpea and its implications for plant improvement. *Crop & Pasture Science, 63*, 419. https://doi.org/10.1071/cp11218

Devasirvatham, V., Gaur, P. M., Mallikarjuna, N., et al. (2013). Reproductive biology of chickpea response to heat stress in the field is associated with the performance in controlled environments. *Field Crops Research, 142*, 9–19. https://doi.org/10.1016/j.fcr.2012.11.011

Devasirvatham, V., Gaur, P. M., Raju, T. N., et al. (2015). Field response of chickpea (*Cicer arietinum* L.) to high temperature. *Field Crops Research, 172*, 59–71.

Diapari, M., Sindhu, A., Bett, K., et al. (2014). Genetic diversity and association mapping of iron and zinc concentrations in chickpea (*Cicer arietinum* L.). *Genome, 57*, 459–468.

Doria, E., Galleschi, L., Calucci, L., et al. (2009). Phytic acid prevents oxidative stress in seeds: Evidence from a maize (*Zea mays* L.) low phytic acid mutant. *Journal of Experimental Botany, 60*, 967–978.

Esechie, H. A., Al-Saidi, A., & Al-Khanjari, S. (2002). Effect of sodium chloride salinity on seedling emergence in chickpea. *Journal of Agronomy and Crop Science, 188*, 155–160.

Eyidogan, F., & Oz, M. T. (2007). Effect of salinity on antioxidant responses of chickpea seedlings. *Acta Physiologiae Plantarum, 29*, 485–493.

Fahad, S., Bajwa, A. A., Nazir, U., et al. (2017). Crop production under drought and heat stress: Plant responses and management options. *Frontiers in Plant Science, 8*, 1147. https://doi.org/10.3389/fpls.2017.01147

Fang, X., Turner, N. C., Yan, G., et al. (2009). Flower numbers, pod production, pollen viability, and pistil function are reduced and flower and pod abortion increased in chickpea (*Cicer arietinum* L.) under terminal drought. *Journal of Experimental Botany, 61*, 335–345. https://doi.org/10.1093/jxb/erp307

FAOSTAT. (2021). Food and Agriculture Organization-statistical database (FAO-STAT) of the United Nations Food and Agriculture Organization (FAO) statistical division. http://www.fao.org/faostat/en/#data/QC. Accessed 5 May 2021.

Farooq, M., Hussain, M., Nawaz, A., et al. (2017). Seed priming improves chilling tolerance in chickpea by modulating germination metabolism, trehalose accumulation and carbon assimilation. *Plant Physiology and Biochemistry, 111*, 274–283. https://doi.org/10.1016/j.plaphy.2016.12.012

Farooq, M., Ullah, A., Lee, D. J., et al. (2018). Desi chickpea genotypes tolerate drought stress better than kabuli types by modulating germination metabolism, trehalose accumulation, and carbon assimilation. *Plant Physiology and Biochemistry, 126*, 47–54. https://doi.org/10.1016/j.plaphy.2018.02.020

Flowers, T. J., Gaur, P. M., Gowda, C. L., et al. (2010). Salt sensitivity in chickpea. *Plant, Cell & Environment, 33*, 490–509.

Garg, R., Patel, R. K., Jhanwar, S., et al. (2011). Gene discovery and tissue-specific transcriptome analysis in chickpea with massively parallel pyrosequencing and web resource development. *Plant Physiology, 156*, 1661–1678. https://doi.org/10.1104/pp.111.178616

Garg, M., Sharma, N., Sharma, S., et al. (2018). Biofortified crops generated by breeding, agronomy, and transgenic approaches are improving lives of millions of people around the world. *Frontiers in Nutrition, 5*, 12. https://doi.org/10.3389/fnut.2018.00012

Gaur, P. M., & Gour, V. K. (1999). An induced fasciated mutant of chickpea. *Indian Journal of Genetics, 59*, 325–330.

Gaur, P. M., & Gour, V. K. (2003). Broad-few-leaflets and outwardly curved wings: Two new mutants of chickpea. *Plant Breeding, 122*, 192–197.

Gaur, P. M., Gaur, V. K., & Singh, K. (2004). Induction and inheritance of a variegated leaf and an apical chlorosis mutant in chickpea (*Cicer arietinum* L.). *Indian Journal of Genetics and Plant Breeding, 64*, 208–211.

Gaur, P. M., Kumar, J., Gowda, C. L. L., et al. (2008a). Breeding chickpea for early phenology: Perspectives, progress and porspects. In M. C. Kharkwal (Ed.), *Proceedings of fourth international food legume research conference*, 18–22 October 2005.

Gaur, P. M., Gaur, V. K., & Srinivasan, S. (2008b). An induced brachytic mutant of chickpea and its possible use in ideotype breeding. *Euphytica, 59*, 35–41.

Gaur, P. M., Chaturvedi, S. K., Tripathi, S., et al. (2010). Improving heat tolerance in chickpea to increase its resilience to climate change. In *Paper presented at 5th international food legumes research conference & 7th European conference on grain legumes*, 26–30 April 2010.

Gaur, P. M., Jukanti, A. K., Samineni, S., et al. (2013). Climate change and heat stress tolerance in chickpea. In N. Tuteja & S. S. Gill (Eds.), *Climate change and plant abiotic stress tolerance*. Wiley Blackwell.

Gaur, P. M., Samineni, S., Thudi, M., et al. (2019). Integrated breeding approaches for improving drought and heat adaptation in chickpea (*Cicer arietinum* L.). *Plant Breeding, 138*, 389–400.

Geilfus, C.-M. (2018). Chloride: From nutrient to toxicant. *Plant & Cell Physiology, 59*, 877–886.

Gödecke, T., Stein, A. J., & Qaim, M. (2018). The global burden of chronic and hidden hunger: Trends and determinants. *Global Food Security, 17*, 21–29. https://doi.org/10.1016/j.gfs.2018.03.004

Goyal, S., Wani, M. R., Laskar, R. A., Raina, A., & Khan, S. (2019a). Assessment on cytotoxic and mutagenic potency of gamma rays and EMS in *Vigna mungo* L. Hepper. *Biotecnologia Vegetal, 19*, 193–204.

Goyal, S., Wani, M. R., Laskar, R. A., Raina, A., Amin, R., & Khan, S. (2019b). Induction of morphological mutations and mutant phenotyping in black gram [*Vigna mungo* (L.) Hepper] using gamma rays and EMS. *Vegetos, 32*(4), 464–472.

Grossiord, C., Buckley, T. N., Cernusak, L. A., et al. (2020). Plant responses to rising vapor pressure deficit. *New Phytologist, 226*, 1550–1566.

Gupta, S., Nawaz, K., Parween, S., et al. (2017). Draft genome sequence of *Cicer reticulatum* L., the wild progenitor of chickpea provides a resource for agronomic trait improvement. *DNA Research, 24*, 1–10.

Hamwieh, A., Imtiaz, M., & Malhotra, R. S. (2013). Multi-environment QTL analyses for drought-related traits in a recombinant inbred population of chickpea (*Cicer arietinum* L.). *Theoretical and Applied Genetics, 126*, 1025–1038.

Hasanuzzaman, M., Nahar, K., Alam, M. M., et al. (2013). Physiological, biochemical, and molecular mechanisms of heat stress tolerance in plants. *International Journal of Molecular Sciences, 14*, 9643–9684. https://doi.org/10.3390/ijms14059643

Hidoto, L., Taran, B., Worku, W., et al. (2017). Towards zinc biofortification in chickpea: Performance of chickpea cultivars in response to soil zinc application. *Agronomy, 7*, 11. https://doi.org/10.3390/agronomy7010011

Hiremath, P. J., Farmer, A., Cannon, S. B., et al. (2011). Large-scale transcriptome analysis in chickpea (*Cicer arietinum* L.), an orphan legume crop of the semi-arid tropics of Asia and Africa. *Plant Biotechnology Journal, 9*, 922–931.

Jaganathan, D., Thudi, M., Kale, S., et al. (2015). Genotyping-by-sequencing based intra-specific genetic map refines a "QTL-hotspot" region for drought tolerance in chickpea. *Molecular Genetics and Genomics, 290*, 559–571.

Jain, M., Misra, G., Patel, R. K., et al. (2013). A draft genome sequence of the pulse crop chickpea (*Cicer arietinum* L.). *The Plant Journal, 74*, 715–729.

Jha, U. C., Jha, R., Bohra, A., et al. (2018). Population structure and association analysis of heat stress relevant traits in chickpea (*Cicer arietinum* L.). *3 Biotech, 8*, 43.

Jha, U. C., Kole, P. C., & Singh, N. P. (2019). QTL mapping for heat stress tolerance in chickpea (*Cicer arietinum* L.). *Legume Research, 44*, 382–387.

Jha, U. C., Bohra, A., Pandey, S., et al. (2020). Breeding, genetics, and genomics approaches for improving fusarium wilt resistance in major grain legumes. *Frontiers in Genetics, 11*, 1001. https://doi.org/10.3389/fgene.2020.01001

Johansen, C., Saxena, N. P., & Saxena, M. C. (1996). Comparisons of abiotic constraints to chickpea production in WANA and SAT. In N. P. Saxena, M. C. Saxena, C. Johansen, S. M. Virmani, & H. Harris (Eds.), *Adaptation of chickpea in the West Asia and North Africa Region* (pp. 181–188). International Crops Research Institute for the Semi-Arid Tropics/International Center for Agricultural Research in the Dry Areas.

Joshi-Saha, A., & Reddy, K. S. (2015). Repeat length variation in the 5′UTR of myo-inositol monophosphatase gene is related to phytic acid content and contributes to drought tolerance in chickpea ( *Cicer arietinum* L.). *Journal of Experimental Botany, 66*, 5683–5690. https://doi.org/10.1093/jxb/erv156

Joshi-Saha, A., & Reddy, K. S. (2016). Phytic acid boon or bane? Conflict between animal/human nutrition and plant health. *Journal of Food Legumes, 29*, 163–173.

Joshi-Saha, A., & Reddy, K. S. (2018). The pulse of pulses under climate change: From physiology to phenology. In N. Benkebelia (Ed.), *Climate change and crop production: Foundations for agroecosystem resilience*. CRC Press, Taylor and Francis Group.

Joshi-Saha, A., Reddy, K. S., Petwal, V. C., et al. (2015). Identification of novel mutants through electron beam and gamma irradiation in chickpea (*Cicer arietinum* L.). *Journal of Food Legumes, 28*, 1–6.

Joshi-Saha, A., Mehzabin, P., Reddy, K. S., et al. (2018). Effect of seasonal variation on micronutrient content in chickpea (*Cicer arietinum* L.) and identification of accessions having high iron and zinc. *Journal of Food Legumes, 31*, 1–4.

Joshi-Saha, A., Misra, G., & Reddy, K. S. (2021). Quality improvement in chickpea. In D. S. Gupta, S. Gupta, & J. Kumar (Eds.), *Breeding for enhanced nutrition and bio-active compounds in food legumes*. Springer. https://doi.org/10.1007/978-3-030-59215-8_2

Joshua, D. C. (2000). Guidelines for successful mutation breeding. In *Proceedings of DAE-BRNS symposium on the use of nuclear and molecular techniques in crop improvement* (pp. 21–26).

Jukanti, A. K., Gaur, P. M., Gowda, C. L., et al. (2012). Nutritional quality and health benefits of chickpea (*Cicer arietinum* L.): A review. *The British Journal of Nutrition, 108*, S11–S26. https://doi.org/10.1017/S0007114512000797

Kale, S., Jaganathan, D., Ruperao, P., et al. (2015). Prioritization of candidate genes in "QTL-hotspot" region for drought tolerance in chickpea (*Cicer arietinum* L.). *Scientific Reports, 5*, 15296.

Kaloki, P., Devasirvatham, V., & Tan, D. K. Y. (2019). Chickpea abiotic stresses: Combating drought, heat and cold. In A. B. de Oliviera (Ed.), *Abiotic and biotic stress in plants*. IntechOpen. https://doi.org/10.5772/intechopen.83404

Kamble, G. C., & Patil, A. S. (2014). Comparative mutagenicity of EMS and gamma radiation in wild chickpea. *International Journal of Science Technology, 3*, 166–180.

Kar, S., Garin, V., Kholová, J., et al. (2020). SpaTemHTP: A data analysis pipeline for efficient processing and utilization of temporal high-throughput phenotyping data. *Frontiers in Plant Science, 11*, 552509. https://doi.org/10.3389/fpls.2020.552509

Karami-Moalem, S., Maali-Amiri, R., & Kazemi-Shahandashti, S.-S. (2018). Effect of cold stress on oxidative damage and mitochondrial respiratory properties in chickpea. *Plant Physiology and Biochemistry, 122*, 31–39. https://doi.org/10.1016/j.plaphy.2017.11.011

Kashiwagi, J., Krishnamurthy, L., Upadhyaya, H. D., et al. (2005). Genetic variability of drought-avoidance root traits in the mini-core germplasm collection of chickpea (*Cicer arietinum* L). *Euphytica, 146*, 213–222.

Kashiwagi, J., Krishnamurthy, L., Crouch, J. H., et al. (2006a). Variability of root length density and its contributions to seed yield in chickpea (*Cicer arietinum* L.) under terminal drought stress. *Field Crops Research, 95*, 171–181.

Kashiwagi, J., Krishnamurthy, L., Singh, S., et al. (2006b). Relationships between transpiration efficiency and carbon isotope discrimination in chickpea (*Cicer arietinum* L). *Journal of SAT Agricultural Research, 2*, 1–3.

Kashiwagi, J., Krishnamurthy, L., Singh, S., et al. (2006c). Variation in SPAD chlorophyll meter readings (SCMR) in the minicore germplasm of chickpea. *International Chickpea Pigeonpea Newsletters, 13*, 16–18.

Kashiwagi, J., Krishnamurthy, L., Gaur, P. M., et al. (2013). Traits of relevance to improve yield under terminal drought stress in chickpea (*C. arietinum* L.). *Field Crops Research, 145*, 88–95.

Kashiwagi, J., Krishnamurthy, L., Purushothaman, R., et al. (2015). Scope for improvement of yield under drought through the root traits in chickpea (*Cicer arietinum* L.). *Field Crops Research, 170*, 47–54.

Kaur, G., Kumar, S., Nayyar, H., et al. (2008). Cold stress injury during the pod-filling phase in chickpea (*Cicer arietinum* L.): Effects on quantitative and qualitative components of seeds. *Journal of Agronomy and Crop Science, 194*, 457–464. https://doi.org/10.1111/j.1439-037X.2008.00336.x

Kaur, S., Gupta, A. K., Kaur, N., et al. (2009). Antioxidative enzymes and sucrose synthase contribute to cold stress tolerance in Chickpea. *Journal of Agronomy and Crop Science, 195*, 393–397. https://doi.org/10.1111/j.1439-037x.2009.00383.x

Kaur, D., Grewal, S. K., Kaur, J., et al. (2017). Differential proline metabolism in vegetative and reproductive tissues determines drought tolerance in chickpea. *Biologia Plantarum, 61*, 359–366. https://doi.org/10.1007/s10535-016-0695-2

Kaushal, N., Gupta, K., Bhandhari, K., et al. (2011). Proline induces heat tolerance in chickpea (*Cicer arietinum* L.) plants by protecting vital enzymes of carbon and antioxidative metabolism. *Plant Physiology and Molecular Biology, 17*, 203–213. https://doi.org/10.1007/s12298-011-0078-2

Kaushal, N., Awasthi, R., Gupta, K., et al. (2013). Heat-stress-induced reproductive failures in chickpea (*Cicer arietinum*) are associated with impaired sucrose metabolism in leaves and anthers. *Functional Plant Biology, 40*, 1334–1349. https://doi.org/10.1071/FP13082

Khan, S., Wani, M. R., Bhat, M., et al. (2005). Induced chlorophyll mutations in chickpea (*Cicer arietinum* L.). *International Journal of Agriculture and Biology, 7*, 764–767.

Khan, H. A., Siddique, K. H. M., & Colmer, T. D. (2016a). Salt sensitivity in chickpea is determined by sodium toxicity. *Planta, 244*, 623–637.

Khan, A., Sovero, V., & Gemenet, D. (2016b). Genome-assisted breeding for drought resistance. *Current Genomics, 7*, 330–342. https://doi.org/10.2174/1389202917999160211101417

Khan, H. A., Siddique, K. H. M., & Colmer, T. D. (2017). Vegetative and reproductive growth of salt-stressed chickpea are carbon-limited: Sucrose infusion at the reproductive stage improves salt tolerance. *Journal of Experimental Botany, 68*, 2001–2011.

Kharkwal, M. C. (1998). Induced mutations in chickpea (*Cicer arietinum* L.) II. Frequency and spectrum of chlorophyll mutations. *Indian Journal of Genetics and Plant Breeding, 58*, 465–474.

Kharkwal, M. C. (1999). Induced mutations in chickpea (*Cicer arietinum* L.) II. Frequency and spectrum of viable mutations. *Indian Journal of Genetics and Plant Breeding, 59*, 451–464.

Khursheed, S., Raina, A., Laskar, R. A., & Khan, S. (2018). Effect of gamma radiation and EMS on mutation rate: Their effectiveness and efficiency in faba bean (*Vicia faba* L.). *Caryologia, 71*(4), 397–404.

Kiran, A., Kumar, S., Nayyar, H., et al. (2019). Low temperature induced aberrations in male and female reproductive organ development cause flower abortion in Chickpea. *Plant, Cell & Environment, 42*, 2075–2089. https://doi.org/10.1111/pce.13536

Kotula, L., Clode, P. L., Jimenez Juan, D. L. C., et al. (2019). Salinity tolerance in chickpea is associated with the ability to 'exclude' Na from leaf mesophyll cells. *Journal of Experimental Botany, 70*, 4991–5002.

Kozgar, M. I. (2014). *Mutation breeding in chickpea: Perspective and prospects for food security* (pp. 41–51). De Gruyter Open Ltd.

Krishnamurthy, L., Johansen, C., & Sethi, S. C. (1999). Investigation of factors determining genotypic differences in seed yield of non-irrigated and irrigated chickpea using a physiological model of yield determination. *Journal of Agronomy and Crop Science, 183*, 9–17.

Krishnamurthy, L., Turner, N. C., Gaur, P. M., et al. (2011a). Consistent variation across soil types in salinity resistance of a diverse range of chickpea (*Cicer arietinum* L.) genotypes. *Journal of Agronomy and Crop Science, 197*, 214–227.

Krishnamurthy, L., Gaur, P., Basu, P., et al. (2011b). Large genetic variation for heat tolerance in the reference collection of chickpea (*Cicer arietinum* L.) germplasm. *Plant Genet Resources, 9*, 59–69. https://doi.org/10.1017/S1479262110000407

Krishnamurthy, L., Kashiwagi, J., Tobita, S., et al. (2013). Variation in carbon isotope discrimination and its relationship with harvest index in the reference collection of chickpea germplasm. *Functional Plant Biology, 40*, 1350–1361.

Kumar, S., Malik, J., Thakur, P., et al. (2011). Growth and metabolic responses of contrasting chickpea (*Cicer arietinum* L.) genotypes to chilling stress at reproductive phase. *Acta Physiologiae Plantarum, 33*, 779–787. https://doi.org/10.1007/s11738-010-0602-y

Kumar, N., Nandwal, A. S., Yadav, R., et al. (2012a). Assessment of chickpea genotypes for high temperature tolerance. *Indian Journal of Plant Physiology, 17*, 225–232.

Kumar, S., Kaushal, N., Nayyar, H., et al. (2012b). Abscisic acid induces heat tolerance in chickpea (*Cicer arietinum* L.) seedlings by facilitated accumulation of osmoprotectants. *Acta Physiologiae Plantarum, 34*, 1651–1658. https://doi.org/10.1007/s11738-012-0959-1

Kumar, S., Thakur, P., Kaushal, N., et al. (2013). Effect of varying high temperatures during reproductive growth on reproductive function, oxidative stress and seed yield in chickpea genotypes differing in heat sensitivity. *Archives of Agronomy and Soil Science, 59*, 823–843. https://doi.org/10.1080/03650340.2012.683424

Kumar, P., Shah, D., & Singh, M. P. (2017). Evaluation of chickpea (*Cicer arietinum* L.) genotypes for heat tolerance: A physiological assessment. *Indian Journal of Plant Physiology, 22*, 164–177. https://doi.org/10.1007/s40502-017-0301-4

Ladeiro, B. (2012). Saline agriculture in the 21st century: Using salt contaminated resources to cope food requirements. *Journal of Botany, 2012*, 1–7.

Lalitha, N., Upadhyaya, H. D., Krishnamurthy, L., et al. (2015). Assessing genetic variability for root traits and identification of trait-specific germplasm in chickpea reference set. *Crop Science, 55*, 1–12.

Laskar, R. A., Khan, S., Khursheed, S., Raina, A., & Amin, R. (2015). Quantitative analysis of induced phenotypic diversity in chickpea using physical and chemical mutagenesis. *Journal of Agronomy, 14*(3), 102–111.

Laskar, R. A., Laskar, A. A., Raina, A., & Amin, R. (2018a). Induced mutation analysis with biochemical and molecular characterization of high yielding lentil mutant lines. *International Journal of Biological Macromolecules, 109*, 167–179.

Laskar, R. A., Wani, M. R., Raina, A., Amin, R., & Khan, S. (2018b). Morphological characterization of gamma rays induced multipodding mutant (mp) in lentil cultivar pant L 406. *International Journal of Radiation Biology, 94*(11), 1049–1053.

Lauter, D. J., & Munns, D. N. (1987). Salt sensitivity of chickpea duringvegetative growth and at different humidities. *Australian Journal of Plant Physiology, 14*, 171–180.

Lee, C., Yu, D., Choi, H. K., et al. (2017). Reconstruction of a composite comparative map composed of ten legume genomes. *Genes & Genomics, 39*, 111–119.

Lemtiri-Chlieh, F., MacRobbie, E. A., & Brearley, C. A. (2000). Inositol hexakisphosphate is a physiological signal regulating the K + inward rectifying conductance in guard cells. *Proceedings of the National Academy of Sciences of the United States of America, 97*, 8687–8692.

Lemtiri-Chlieh, F., MacRobbie, E. A., Webb, A. A., et al. (2003). Inositol hexakisphosphate mobilizes an endomembrane store of calcium in guard cells. *Proceedings of the National Academy of Sciences of the United States of America, 100*, 10091–10095.

Leport, L., Turner, N. C., French, R. J., et al. (1999). Physiological responses of chickpea genotypes to terminal drought in a Mediterranean-type environment. *European Journal of Agronomy, 11*, 279–291. https://doi.org/10.1016/s1161-0301(99)00039-8

Levitt, J. (1980). *Response of plants to environmental stresses. Chilling, freezing and high temperature stresses. I*. Academic.

Lichtenzveig, J., Scheuring, C., Dodge, J., et al. (2005). Construction of BAC and BIBAC libraries and their applications for generation of SSR markers for genome analysis of chickpea, *Cicer arietinum* L. *Theoretical and Applied Genetics, 110*, 492–510.

Lucas, L., & Fuller, D. Q. (2014). Chickpea: Origins and development. In C. Smith (Ed.), *Encyclopediaofglobalarchaeology*.Springer.https://doi.org/10.1007/978-1-4419-0465-2_2324

Macar, T. K., & Ekmekci, Y. (2009). Alterations in photochemical and physiological activities of Chickpea (*Cicer arietinum* L.) cultivars under drought stress. *Journal of Agronomy and Crop Science, 195*, 335–346.

Mafakheri, A., Siosemardeh, A., Bahramnejad, B., et al. (2010). Effect of drought stress on yield, proline and chlorophyll contents in three chickpea cultivars. *Australian Journal of Crop Science, 4*, 580–585.

Mafakheri, A., Siosemardeh, A., Bahramnejad, B., et al. (2011). Effect of drought stress and subsequent recovery on protein, carbohydrate contents, catalase and peroxidase activities in three chickpea (*Cicer arietinum*) cultivars. *Australian Journal of Crop Science, 5*, 1255–1260.

Maheswari, M., Sarkar, B., Vanaja, M. et al. (2019). Climate resilient crop varieties for sustainable food production under aberrant weather conditions. In *National innovation in climate resilient agriculture* (p. 47). Central Research Institute for Dryland Agriculture (ICAR).

Makonya, G. M., Ogola, J. B. O., Muasya, A. M., et al. (2019). Chlorophyll fluorescence and carbohydrate concentration as field selection traits for heat tolerant chickpea genotypes. *Plant Physiology and Biochemistry, 141*, 172–182.

Maliro, M. F. A., MacNeil, D., Redden, B., et al. (2008). Sampling strategies and screening of chickpea (*Cicer arietinum* L.) germplasm for salt tolerance. *Genetic Resources and Crop Evolution, 55*, 53–63.

Maqbool, M. A., Aslam, M., & Ali, H. (2017). Breeding for improved drought tolerance in Chickpea (*Cicer arietinum* L.). *Plant Breeding, 136*, 300–318. https://doi.org/10.1111/pbr.12477

McVay, K., Burrows, M., Menalled, F., et al. (2013). *Montana cool-season pulse. Production guide* (pp. 1–28). Montana State University Extension Service.

Meis, S. J., Fehr, W. R., & Schnebly, S. R. (2003). Seed source effect on field emergence of soybean lines with reduced phytate and raffinose saccharides. *Crop Science, 43*, 1336–1339.

Merga, B., & Haji, J. (2019). Economic importance of chickpea: Production, value, and world trade. *Cogent Food Agriculture, 5*, 1. https://doi.org/10.1080/23311932.2019.1615718

Misra, G., Joshi-Saha, A., Salaskar, D., et al. (2020). Baseline status and effect of genotype, environment and genotype × environment interactions on iron and zinc content in Indian chickpeas (*Cicer arietinum* L.). *Euphytica, 216*, 137. https://doi.org/10.1007/s10681-020-02673-z

Moore, C. E., Meacham-Hensold, K., Lemonnier, P., et al. (2021). The effect of increasing temperature on crop photosynthesis: From enzymes to ecosystems. *Journal of Experimental Botany, 72*, 2822–2844. https://doi.org/10.1093/jxb/erab090

Mugabe, D., Coyne, C. J., Piaskowski, J., et al. (2019). Quantitative trait loci for cold tolerance in chickpea. *Crop Science, 2019*, 573–582.

Munns, R., & Tester, M. (2008). Mechanisms of salinity tolerance. *Annual Review of Plant Biology, 59*, 651–681. https://doi.org/10.1146/annurev.arplant.59.032607.092911

Murphy, A. M., Otto, B., Brearley, C. A., et al. (2008). A role for inositol hexakisphosphate in the maintenance of basal resistance to plant pathogens. *The Plant Journal, 56*, 638–665.

Naidoo, R., Tongoona, P., Derera, J., et al. (2012). Combining ability of low phytic acid (lpa1-1) and quality protein maize (QPM) lines for seed germination and vigour under stress and non-stress conditions. *Euphytica, 185*, 529.

Nasr Esfahani, M., Sulieman, S., Schulze, J., et al. (2014). Mechanisms of physiological adjustment of $N_2$ fixation in *Cicer arietinum* L. (chickpea) during early stages of water deficit: Single or multi-factor controls. *The Plant Journal, 79*, 964–980. https://doi.org/10.1111/tpj.12599

Nayyar, H., Bains, T. S., & Kumar, S. (2005a). Chilling stressed chickpea seedlings: Effect of cold acclimation, calcium and abscisic acid on cryoprotective solutes and oxidative damage. *Environmental and Experimental Botany, 54*, 275–285.

Nayyar, H., Bains, T. S., Kumar, S., et al. (2005b). Chilling effects during seed filling on accumulation of seed reserves and yield of chickpea. *Journal of Science and Food Agriculture, 85*, 1925–1930. https://doi.org/10.1002/jsfa.2198

Nezami, A., Bandara, M. S., & Gusta, L. V. (2012). An evaluation of freezing tolerance of winter chickpea (*Cicer arietinum* L.) using controlled freeze tests. *Canadian Journal of Plant Science, 92*, 155–161.

Nguyen, T. T., Taylor, P. W. J., Redden, R. J., et al. (2004). Genetic diversity estimates in Cicer using AFLP analysis. *Plant Breeding, 123*, 173–179.

Oltmans, S. E., Fehr, W. R., Welke, G. A., et al. (2005). Agronomic and seed traits of soybean lines with low–phytate phosphorus. *Crop Science, 45*, 593–598.

Pal, V., Singh, G., & Dhaliwal, S. S. (2019a). Agronomic biofortification of chickpea with zinc and iron through application of zinc and urea. *Communications in Soil Science and Plant Analysis, 50*, 1864–1877.

Pal, V., Singh, G., & Dhaliwal, S. S. (2019b). Yield enhancement and biofortification of chickpea (*Cicer arietinum* L.) grain with iron and zinc through foliar application of ferrous sulfate and urea. *Journal of Plant Nutrition, 42*, 1789–1802.

Pal, V., Singh, G., & Dhaliwal, S. S. (2021). A new approach in agronomic biofortification for improving zinc and iron content in chickpea (*Cicer arietinum* L.) grain with simultaneous foliar application of zinc sulphate, ferrous sulphate and urea. *Journal of Soil Science and Plant Nutrition, 21*, 883–896. https://doi.org/10.1007/s42729-021-00408-0

Pang, J., Turner, N. C., Khan, T., et al. (2017). Response of chickpea (*Cicer arietinum* L.) to terminal drought: Leaf stomatal conductance, pod abscisic acid concentration, and seed set. *Journal of Experimental Botany, 68*, 1973–1985.

Parankusum, S., Bhatnagar-Mathur, P., & Sharma, K. K. (2017). Heat responsive proteome changes reveal molecular mechanisms underlying heat tolerance in chickpea. *Environmental and Experimental Botany, 141*, 132–144.

Pareek, A., Rathi, D., Mishra, D., et al. (2019). Physiological plasticity to high temperature stress in chickpea: Adaptive responses and variable tolerance. *Plant Science, 289*, 110258. https://doi.org/10.1016/j.plantsci.2019.110258

Parida, S. K., Verma, M., Yadav, S. K., et al. (2015). Development of genome-wide informative simple sequence repeat markers for large-scale genotyping applications in chickpea and development of web resource. *Frontiers in Plant Science, 6*, 645. https://doi.org/10.3389/fpls.2015.00645

Paul, P. J., Samineni, S., Thudi, M., et al. (2018). Molecular mapping of QTLs for heat tolerance in chickpea. *International Journal of Molecular Sciences, 19*, 2166.

Prasad, R., Shivay, Y. S., & Kumar, D. (2016). Interactions of Zinc with other nutrients in soils and plants – A review. *Indian Journal of Fertilisers, 12*, 16–26.

Prasad, P. V. V., Bheemanahalli, R., & Jagadish, S. V. K. (2017). Field crops and the fear of heat stress: Opportunities, challenges and future directions. *Field Crops Research, 200*, 114–121.

Pratap, A., Gupta, S., Nair, R. M., et al. (2019). Using plant phenomics to exploit the gains of genomics. *Agronomy, 9*, 126. https://doi.org/10.3390/agronomy9030126

Priya, M., Siddique, K. H. M., Dhankhar, O. P., et al. (2018). Molecular breeding approaches involving physiological and reproductive traits for heat tolerance in food crops. *Indian Journal of Plant Physiology, 23*, 697–720. https://doi.org/10.1007/s40502-018-0427-z

Purushothaman, R., Upadhyaya, H. D., Gaur, P. M., et al. (2014). Kabuli and desi chickpeas differ in their requirement for reproductive duration. *Field Crops Research, 163*, 24–31.

Purushothaman, R., Krishnamuthy, L., Upadhyaya, H. D., et al. (2016). Shoot traits and their relevance in terminal drought tolerance of chickpea (*Cicer arietinum* L.). *Field Crops Research, 197*, 10–27.

Purushothaman, R., Krishnamuthy, L., Upadhyaya, H. D., et al. (2017). Root traits confer grain yield advantages under terminal drought in chickpea (*Cicer arietinum* L.). *Field Crops Research, 201*, 146–161.

Pushpavalli, R., Zaman-Allah, M., Turner, N. C., et al. (2015a). Higher flower and seed number leads to higher yield under water stress conditions imposed during reproduction in chickpea. *Functional Plant Biology, 42*, 162–174.

Pushpavalli, R., Krishnamurthy, L., Thudi, M., et al. (2015b). Two key genomic regions harbour QTLs for salinity tolerance in ICCV 2 × JG 11 derived chickpea (*Cicer arietinum* L.) recombinant inbred lines. *BMC Plant Biology, 15*, 124.

Raboy, V. (2009). Approaches and challenges to engineering seed phytate and total phosphorus. *Plant Science, 177*, 281–296.

Raboy, V., Peterson, K., Jackson, C., et al. (2015). A Substantial fraction of barley (*Hordeum vulgare* L.) low phytic acid mutations have little or no effect on yield across diverse production environments. *Plants, 4*, 225–239.

Rahbarian, R., Khavari-Nejad, R., Ganjeali, A., et al. (2011). Drought stress effects on photosynthesis, chlorophyll fluorescence and water relations in tolerant and susceptible chickpea (*Cicer arietinum* L.) genotypes. *Acta Biologica Cracoviensia Series Botanica, 53*, 47–56.

Raina, A., & Khan, S. (2020). Increasing rice grain yield under biotic stresses: Mutagenesis, transgenics and genomics approaches. In C. Aryadeep (Ed.), *Rice research for quality improvement: Genomics and genetic engineering* (pp. 149–178). Springer. https://doi.org/10.1007/978-981-15-5337-0_8

Raina, A., Laskar, R. A., Khursheed, S., Amin, R., Tantray, Y. R., Parveen, K., & Khan, S. (2016). Role of mutation breeding in crop improvement-past, present and future. *Asian Research Journal of Agriculture, 2*(2), 1–13.

Raina, A., Laskar, R. A., Khursheed, S., Khan, S., Parveen, K., Amin, R., & Khan, S. (2017). Induced physical and chemical mutagenesis for improvement of yield attributing traits and their correlation analysis in chickpea. *International Letters of Natural Sciences, 61*, 14–22.

Raina, A., Khan, S., Laskar, R. A., Wani, M. R., & Mushtaq, W. (2019). Chickpea (*Cicer arietinum* L.) cytogenetics, genetic diversity and breeding. In J. M. Al-Khayri et al. (Eds.), *Advances in plant breeding: Legumes*. Springer. https://doi.org/10.1007/978-3-030-23400-3_3

Raina, A., Laskar, R. A., Tantray, Y. R., Khursheed, S., Wani, M. R., & Khan, S. (2020a). Characterization of induced high yielding cowpea mutant lines using physiological, biochemical and molecular markers. *Scientific Reports, 10*(1), 1–22.

Raina, A., Khan, S., Sahu, P., & Roa, R. (2020b). Increasing rice grain yield under abiotic stresses: Mutagenesis, transgenics and genomics approaches. In C. Aryadeep (Ed.), *Rice research for quality improvement: Genomics and genetic engineering* (pp. 753–777). Springer. https://doi.org/10.1007/978-981-15-4120-9_31

Raina, A., Laskar, R. A., Wani, M. R., Jan, B. L., Ali, S., & Khan, S. (2022a). Gamma rays and sodium azide induced genetic variability in high yielding and biofortified mutant lines in cowpea [*Vigna unguiculata* (L.) Walp.]. *Frontiers in Plant Science, 13*, 911049. https://doi.org/10.3389/fpls.2022.911049

Raina, A., Laskar, R. A., Wani, M. R., Jan, B. L., Ali, S., & Khan, S. (2022b). Comparative mutagenic effectiveness and efficiency of gamma rays and sodium azide in inducing chlorophyll and morphological mutants of cowpea. *Plants, 11*, 1322. https://doi.org/10.3390/plants11101322

Raina, A., Laskar, R. A., Wani, M. R., & Khan, S. (2022c). Chemical mutagenesis: Role in breeding and biofortification of lentil (*Lens culinaris* Medik) mutant lines. *Molecular Biology Reports, 49*(12), 11313–11325. https://doi.org/10.1007/s11033-022-07678-6

Raina, A., Laskar, R. A., Wani, M. R., & Khan, S. (2022d). Plant breeding strategies for abiotic stress tolerance in cereals. In *Omics approach to manage abiotic stress in cereals* (pp. 151–177). Springer. https://doi.org/10.1007/978-981-19-0140-9_8

Randhawa, N., Kaur, J., Singh, S., et al. (2014). Growth and yield in chickpea (*Cicer arietinum* L.) genotypes in response to water stress. *African Journal of Agricultural Research, 9*, 982–992.

Rani, A., Devi, P., Jha, U. C., et al. (2020). Developing climate-resilient chickpea involving physiological and molecular approaches with a focus on temperature and drought stresses. *Frontiers in Plant Science, 10*, 1759. https://doi.org/10.3389/fpls.2019.01759

Rasik, S., Raina, A., Laskar, R. A., Wani, M. R., Reshi, Z., & Khan, S. (2022). Lower doses of Sodium azide and Methyl methanesulphonate improved yield and pigment contents in vegetable cowpea [*Vigna unguiculata* (L.) Walp.]. *South African Journal of Botany, 148*, 727–736. https://doi.org/10.1016/j.sajb.2022.04.034

Rehman, A., Malhotra, R., Bett, K., et al. (2011). Mapping QTL associated with traits affecting grain yield in chickpea (*Cicer arietinum* L.) under terminal drought stress. *Crop Science, 51*, 450–463.

Roorkiwal, M., Jarquin, D., Singh, M. K., et al. (2018). Genomic-enabled prediction models using multi-environment trials to estimate the effect of genotype × environment interaction on prediction accuracy in chickpea. *Scientific Reports, 8*, 11701. https://doi.org/10.1038/s41598-018-30027-2. Published 2018 Aug 3.

Roorkiwal, M., Bharadwaj, C., Barmukh, R., et al. (2020). Integrating genomics for chickpea improvement: Achievements and opportunities. *Theoretical and Applied Genetics, 133*, 1703–1720. https://doi.org/10.1007/s00122-020-03584-2

Ryan, J. (1997). A global perspective on pigeonpea and chickpea sustainable production system: Present status and future potential. In A. Asthana & A. M. Kanpur (Eds.), *Recent advances in pulses research in India* (pp. 1–31). Indian Society for Pulses Research and Development.

Sab, S., Lokesha, R., Mannur, D. M., et al. (2020). Genome-wide SNP discovery and mapping QTLs for seed iron and zinc concentrations in chickpea (*Cicer arietinum* L.). *Frontiers in Nutrition, 7*, 559120. https://doi.org/10.3389/fnut.2020.559120

Sabagpour, S. H., Kumar, J., & Rao, T. N. (2003). Inheritance of growth vigor and its association with other characters in chickpea. *Plant Breeding, 122*, 542–544.

Saghfi, S., & Eivazi, A. R. (2014). Effects of cold stress on proline and soluble carbohydrates in two chickpea cultivars. *International Journal of Current Microbiology and Applied Sciences, 3*, 591–595.

Saltzman, A., Birol, E., Bouis, H. E., et al. (2013). Biofortification: Progress toward a more nourishing future. *Global Food Security, 2*, 9–17. https://doi.org/10.1016/j.gfs.2012.12.003

Saltzman, A., Birol, E., Oparinde, A., et al. (2017). Availability, production, and consumption of crops biofortified by plant breeding: Current evidence and future potential. *Annals of the New York Academy of Sciences, 1390*, 104–114. https://doi.org/10.1111/nyas.13314

Salvi, P., Saxena, S. C., Petla, B. P., et al. (2016). Differentially expressed galactinol synthase(s) in chickpea are implicated in seed vigor and longevity by limiting the age induced ROS accumulation. *Scientific Reports, 6*, 35088.

Salvi, P., Kamble, N. U., & Majee, M. (2018). Stress-inducible galactinol synthase of chickpea (CaGolS) is implicated in heat and oxidative stress tolerance through reducing stress-induced excessive reactive oxygen species accumulation. *Plant & Cell Physiology, 59*, 155–166.

Samineni, S., Siddique, K. H. M., Gaur, P. M., et al. (2011). Salt sensitivity of the vegetative and reproductive stages in chickpea (*Cicer arietinum* L.): Podding is a particularly sensitive stage. *Environmental and Experimental Botany, 71*, 260–268.

Samineni, S., Sen, M., Sajja, S. B., et al. (2020). Rapid generation advance (RGA) in chickpea to produce up to seven generations per year and enable speed breeding. *Crop Journal, 8*, 164–169.

Saxena, M. C. (1990). Problems and potential of chickpea production in the nineties. In H. A. Van Rheenen & M. C. Saxena (Eds.), *Chickpea in the nineties, Proceedings 2nd international workshop chickpea improvement*. ICRISAT.

Schlemmer, U., Frølich, W., Prieto, R. F., et al. (2009). Phytate in foods and significance for humans: Food sources, intake, processing, bioavailability, protective role and analysis. *Molecular Nutrition & Food Research, 53*, S330–S375.

Sellapillai, L., Dhanarajan, A., Raina, A., & Ganesan, A. (2022). Gamma ray induced positive alterations in morphogenetic and yield attributing traits of finger millet (*Eleusine coracana* (L.) Gaertn.) in M2 generation. *Plant Science Today, 9*(4), 939–949.

Sellapillaibanumathi, L., Dhanarajan, A., Raina, A., & Ganesan, A. (2022). Effects of gamma radiations on morphological and physiological traits of finger millet (Eleusine coracana (L.) Gaertn.). *Plant Science Today, 9*(1), 89–95.

Serraj, R., Krishnamurthy, L., & Upadhyaya, H. D. (2004). Screening of chickpea mini-core germplasm for tolerance to soil salinity. *International Chickpea Pigeonpea Newsletters, 11*, 29–32.

Sethy, N. K., Shokeen, B., & Bhatia, S. (2003). Isolation and characterization of sequence tagged microsatellite sites markers in chickpea (*Cicer arietinum* L.). *Molecular Ecology Notes, 3*, 428–430.

Sethy, N. K., Choudhary, S., Shokeen, B., et al. (2006). Identification of microsatellite markers from *Cicer reticulatum*: Molecular variation and phylogenetic analysis. *Theoretical and Applied Genetics, 112*, 347–357.

Shah, T. M., Atta, B. M., & Mirza, J. I. (2009). Screening of Chickpea (*Cicer arietinum*) induced mutants against Fusarium wilt. *Pakistan Journal of Botany, 41*, 1945–1955.

Shah, T. M., Imran, M., Atta, B. M., et al. (2020). Selection and screening of drought tolerant high yielding chickpea genotypes based on physio-biochemical indices and multi-environmental yield trials. *BMC Plant Biology, 20*, 171. https://doi.org/10.1186/s12870-020-02381-9

Sharma, K., & Nayyar, H. (2014). Cold stress alters transcription in meiotic anthers of cold tolerant chickpea (*Cicer arietinum* L.). *BMC Research Notes, 7*, 717. https://doi.org/10.1186/1756-0500-7-717

Sharma, K. D., & Nayyar, H. (2016). Regulatory networks in pollen development under cold stress. *Frontiers in Plant Science, 7*, 402. https://doi.org/10.3389/fpls.2016.00402

Shivay, Y. S., Prasad, R., & Pal, M. (2015). Effects of source and method of zinc application on yield, zinc biofortification of grain, and Zn uptake and use efficiency in chickpea (*Cicer arietinum* L.). *Communications in Soil Science and Plant Analysis, 46*, 2191–2200. https://doi.org/10.1080/00103624.2015.1069320

Siddique, K. H. M., Loss, S. P., Regan, K. L., et al. (1999). Adaptation and seed yield of cool season grain legumes in Mediterranean environments of south-western Australia. *Australian Journal of Agricultural Research, 50*, 375–388. https://doi.org/10.1071/A98096

Simon, C. J., & Muehbauer, F. J. (1997). Construction of a chickpea linkage map and its comparison with maps of pea and lentil. *The Journal of Heredity, 88*, 115–119.

Singh, N. (2017). Pulses: An overview. *Journal of Food Science and Technology, 54*, 853–857. https://doi.org/10.1007/s13197-017-2537-4

Singh, K. B., Saxena, M. C., & Gridley, B. E. (1984). Screening chickpea for cold tolerance and frost resistance. In M. C. Saxena & K. B. Singh (Eds.), *Ascochyta blight and winter sowing of chickpeas*. Martinus Nijhoff/Dr. W. Junk Publishers.

Singh, K. B., Malhotra, R. S., & Saxena, M. C. (1989). Chickpea evaluation for cold tolerance under field conditions. *Crop Science, 29*, 282–285.

Singh, K. B., Malhotra, R. S., & Saxena, M. C. (1990). Sources for tolerance to cold in *Cicer* species. *Crop Science, 30*, 1136.

Singh, K. B., Malhotra, R. S., & Saxena, M. C. (1995). Additional source of tolerance to cold in cultivated and wild *Cicer* species. *Crop Science, 35*, 1491–1497.

Singh, K. B., Omar, M., Saxena, M. C., et al. (1997). Screening for drought resistance in spring chickpea in the Mediterranean region. *Journal of Agronomy and Crop Science, 178*, 227–235.

Singh, V. K., Khan, A. W., Jaganathan, D., et al. (2016). QTL-seq for rapid identification of candidate genes for 100-seed weight and root/total plant dry weight ratio under rainfed conditions in chickpea. *Plant Biotechnology Journal, 14*, 2110–2119.

Singh, J., Singh, V., & Sharma, P. C. (2018). Elucidating the role of osmotic, ionic and major salt responsive transcript components towards salinity tolerance in contrasting chickpea (*Cicer arietinum* L.) genotypes. *Physiology and Molecular Biology of Plants, 24*, 441–453. https://doi.org/10.1007/s12298-018-0517-4

Sivasakthi, K., Tharanya, M., Kholová, J., et al. (2017). Chickpea genotypes contrasting for vigor and canopy conductance also differ in their dependence on different water transport pathways. *Frontiers in Plant Science, 8*, 1663. https://doi.org/10.3389/fpls.2017.01663

Sivasakthi, K., Thudi, M., Tharanya, M., et al. (2018). Plant vigour QTLs co-map with an earlier reported QTL hotspot for drought tolerance while water saving QTLs map in other regions of the chickpea genome. *BMC Plant Biology, 18*, 29.

Snyder, R. L., & de Melo-Abreu, J. P. (2005). *Frost protection: Fundamentals, practice, and economics* (FAO environmental natural resource service series, No. 10). FAO.

Soltani, A., & Sinclair, T. R. (2012). Optimizing chickpea phenology to available water under current and future climate. *European Journal of Agronomy, 38*, 22–31.

Soren, K. R., Madugula, P., Kumar, N., et al. (2020). Genetic dissection and identification of candidate genes for salinity tolerance using Axiom® *Cicer* SNP array in chickpea. *International Journal of Molecular Sciences, 21*, 5058. https://doi.org/10.3390/ijms21145058

Srinivasan, A., Takeda, H., & Senboku, T. (1996). Heat tolerance in food legumes as evaluated by cell membrane thermo stability and chlorophyll fluorescence techniques. *Euphytica, 88*, 35–45. https://doi.org/10.1007/BF00029263

Srinivasan, A., Johansen, C., & Saxena, N. P. (1998). Cold tolerance during early reproductive growth of chickpea (*Cicer arietinum* L.): Characterization of stress and genetic variation in pod set. *Field Crops Research, 57*, 181–193.

Srivastava, R., Bajaj, D., Malik, A., et al. (2016). Transcriptome landscape of perennial wild *Cicer* microphyllum uncovers functionally relevant molecular tags regulating agronomic traits in chickpea. *Scientific Reports, 6*, 33616.

Srivastava, A. K., Chaturvedi, S. K., & Singh, N. P. (2017). Genetic base of Indian chickpea (*Cicer arietinum* L.) varieties revealed by pedigree analysis. *Legume Research-International Journal, 40*, 22–26.

Summerfield, R. J., & Roberts, E. H. (1985). Grain legume species of significant importance in world agriculture. In A. H. Halevy (Ed.), *Handbook of flowering* (pp. 61–73). CRC Press.

Summerfield, R. J., Hadley, P., Roberts, E. H., et al. (1984). Sensitivity of chickpea (*Cicer arietinum* L.) to hot temperatures during the reproductive period. *Experimental Agriculture, 20*, 77–93.

Suzuki, N., & Mittler, R. (2006). Reactive oxygen species and temperature stresses: A delicate balance between signaling and destruction. *Plant Physiology, 126*, 45–51. https://doi.org/10.1111/j.0031-9317.2005.00582

Tan, G. Z. H., Das-Bhowmik, S. S., Hoang, T. M. L., et al. (2018). Investigation of baseline iron levels in Australian chickpea and evaluation of a transgenic biofortification approach. *Frontiers in Plant Science, 9*, 788. https://doi.org/10.3389/fpls.2018.00788

Tanksley, S. D., & Nelson, J. C. (1996). Advanced backcross QTL analysis: A method for the simultaneous discovery and transfer of valuable QTLs from unadapted germplasm into elite breeding lines. *Theoretical and Applied Genetics, 92*, 191–203. https://doi.org/10.1007/BF00223376

Tantray, A. Y., Raina, A., Khursheed, S., Amin, R., & Khan, S. (2017). Chemical mutagen affects pollination and locule formation in capsules of black cumin (*Nigella sativa* L.). *International Journal of Agricultural Science, 8*(1), 108–117.

Tardieu, F. (2012). Any trait or trait-related allele can confer drought tolerance: Just design the right drought scenario. *Journal of Experimental Botany, 63*, 25–31. https://doi.org/10.1093/jxb/err269

Teakle, N. L., & Tyerman, S. D. (2010). Mechanisms of $Cl^-$ transport contributing to salt tolerance. *Plant, Cell & Environment, 33*, 566–589. https://doi.org/10.1111/j.1365-3040.2009.02060.x

Thakur, P., Kumar, S., Malik, J. A., et al. (2010). Cold stress effects on reproductive development in grain crops: An overview. *Environmental and Experimental Botany, 67*, 429–443. https://doi.org/10.1016/j.envexpbot.2009.09.004

Thavarajah, D., & Thavarajah, P. (2012). Evaluation of chickpea (*Cicer areitinum* L.) micronutrient composition: Biofortification opportunities to combat global micronutrient malnutrition. *Food Research International, 49*, 99–104.

Thudi, M., Upadhyaya, H. D., Rathore, A., et al. (2014a). Genetic dissection of drought and heat tolerance in chickpea through genome-wide and candidate gene-based association mapping approaches. *PLoS ONE, 9*, e96758. https://doi.org/10.1371/journal.pone.0096758

Thudi, M., Gaur, P. M., Krishnamurthy, L., et al. (2014b). Genomics-assisted breeding for drought tolerance in chickpea. *Functional Plant Biology, 41*, 1178–1190.

Toker, C. (2005). Preliminary screening and selection for cold tolerance in annual wild *Cicer* species. *Genetic Resources and Crop Evolution, 52*, 1–5. https://doi.org/10.1007/s10722-005-1743-5

Toker, C. (2009). A note on the evolution of kabuli chickpeas as shown by induced mutations in *Cicer reticulatum* Ladizinsky. *Genetic Resources and Crop Evolution, 56*, 7–12. https://doi.org/10.1007/s10722-008-9336-8

Toker, C., Llunch, C., Tejera, N. A., et al. (2007a). Abiotic stresses. In S. S. Yadav, R. J. Redden, W. Chen, & B. Sharma (Eds.), *Chickpea breeding and management* (pp. 474–496). CAB International.

Toker, C., Canci, H., & Yildirim, T. (2007b). Evaluation of perennial wild *Cicer* species for drought resistance. *Genetic Resources and Crop Evolution, 54*, 1781–1786.

Toker, C., Canci, H., Ertoy Inci, N., et al. (2012). Pyramiding of the resistance to Fe-deficiency chlorosis and leaf miner (*Liriomyza cicerina* ROND.) in chickpea (*Cicer arietinum* L.) by mutation breeding. *Turkish Journal of Field Crops, 17*, 41–45.

Toker, C., Berger, J., Eker, T., et al. (2021). *Cicer turcicum*: A new *Cicer* species and its potential to improve chickpea. *Frontiers in Plant Science, 12*, 662891. https://doi.org/10.3389/fpls.2021.662891

Turan, O., & Ekmekci, Y. (2011). Activities of photosystem II and antioxidant enzymes in chickpea (*Cicer arietinum* L.) cultivars exposed to chilling temperatures. *Acta Physiologiae Plantarum, 33*, 67–78. https://doi.org/10.1007/s11738-010-0517-7

Turner, N. C., Colmer, T. D., Quealy, J., et al. (2013). Salinity tolerance and ion accumulation in chickpea (*Cicer arietinum* L.) subjected to salt stress. *Plant and Soil, 365*, 347–361.

Ulemale, C. S., Mate, S. N., & Deshmukh, D. V. (2013). Physiological indices for drought tolerance in chickpea (*Cicer arietinum* L.). *World Journal of Agricultural Sciences, 9*, 123–131.

Upadhyaya, H. D., Salimath, P. M., Gowda, C. L. L., et al. (2007). New early-maturing germplasm lines for utilization in chickpea improvement. *Euphytica, 157*, 195–208.

Upadhyaya, H. D., Dronavalli, N., Gowda, C. L. L., et al. (2011). Identification and evaluation of chickpea germplasm for tolerance to heat stress. *Crop Science, 51*, 2079–2094. https://doi.org/10.2135/cropsci2011.01.0018

Upadhyaya, H. D., Kashiwagi, J., Varshney, R. K., et al. (2012). Phenotyping chickpeas and pigeonpeas for adaptation to drought. *Frontiers in Physiology, 3*, 179. https://doi.org/10.3389/fphys.2012.00179

Upadhyaya, H. D., Bajaj, D., Das, S., et al. (2016). Genetic dissection of seed-iron and zinc concentrations in chickpea. *Scientific Reports, 6*, 24050.

Vadez, V., Krishnamurthy, L., Serraj, R., et al. (2007). Large variation in salinity tolerance in chickpea is explained by differences in sensitivity at the reproductive stage. *Field Crops Research, 104*, 123–129.

Vadez, V., Krishnamurthy, L., Thudi, M., et al. (2012). Assessment of ICCV 2 × JG 62 chickpea progenies shows sensitivity of reproduction to salt stress and reveals QTLs for seed yield and yield components. *Molecular Breeding, 30*, 9–21.

van der Maesen, L. J. G. (1987). Origin, history and taxonomy of the chickpea. In M. C. Saxena & K. B. Singh (Eds.), *The chickpea*. CABI publishing.

Vandemark, G. J., Grusak, M. A., & McGee, R. J. (2018). Mineral concentrations of chickpea and lentil cultivars and breeding lines grown in the U.S. Pacific Northwest. *Crop Journal, 6*, 253–262. https://doi.org/10.1016/j.cj.2017.12.003

Varshney, R. K., Hiremath, P. J., Lekha, P., et al. (2009). A comprehensive resource of drought- and salinity- responsive ESTs for gene discovery and marker development in chickpea (*Cicer arietinum* L.). *BMC Genomics, 10*, 523.

Varshney, R. K., Song, C., Saxena, R. K., et al. (2013). Draft genome sequence of chickpea (*Cicer arietinum*) provides a resource for trait improvement. *Nature Biotechnology, 31*, 240–246.

Varshney, R. K., Thudi, M., Nayak, S. N., et al. (2014). Genetic dissection of drought tolerance in chickpea (*Cicer arietinum* L.). *Theoretical and Applied Genetics, 127*, 445–462.

Varshney, R. K., Thudi, M., Roorkiwal, M., et al. (2019). Resequencing of 429 chickpea accessions from 45 countries provides insights into genome diversity, domestication and agronomic traits. *Nature Genetics, 51*, 857–864.

Veillet, F., Durand, M., Kroj, T., et al. (2020). Precision breeding made real with CRISPR: Illustration through genetic resistance to pathogens. *Plant Communications, 1*, 100102.

Verbruggen, N., & Hermans, C. (2008). Proline accumulation in plants: A review. *Amino Acids, 35*, 753–759.

Vessal, S., Palta, J. A., Atkins, C. A., et al. (2012). Development of an assay to evaluate differences in germination rate among chickpea genotypes under limited water content. *Functional Plant Biology, 39*, 60–70.

Wahid, A., Gelani, S., Ashraf, M., et al. (2007). Heat tolerance in plants: An overview. *Environmental and Experimental Botany, 61*, 199–223. https://doi.org/10.1016/j.envexpbot.2007.05.011

Wakeel, A. (2013). Potassium–sodium interactions in soil and plant under saline-sodic conditions. *Journal of Plant Nutrition and Soil Science, 176*, 344–354.

Wang, J., Gan, Y. T., Clarke, F., et al. (2006). Response of chickpea yield to high temperature stress during reproductive development. *Crop Science, 46*, 2171–2178.

Wang, Y. H., Zou, C. Q., Mirza, Z., et al. (2016). Cost of agronomic biofortification of wheat with zinc in China. *Agronomy for Sustainable Development, 36*, 44. https://doi.org/10.1007/s13593-016-0382-x

Wani, A. A. (2009). Mutagenic effectiveness and efficiency of gamma rays, ethyl. *Journal of Plant Sciences, 8*, 318–321.

Wani, M. R., Dar, A. R., Tak, A., Amin, I., Shah, N. H., Rehman, R., Baba, M. Y., Raina, A., Laskar, R., Kozgar, M. I., & Khan, S. (2017). Chemo-induced pod and seed mutants in mungbean (*Vigna radiata* L Wilczek). *SAARC Journal of Agriculture, 15*(2), 57–67.

Wani, M. R., Laskar, R. A., Raina, A., Khan, S., & Khan, T. U. (2021). Application of chemical mutagenesis for improvement of productivity traits in lentil (*Lens culinaris* Medik). *Annals of Biology, 37*(1), 69–75.

Wery, J. (1990). Adaptation to frost and drought stress in chickpea and implications in plant breed. *Option Mediterraneennes-Series Seminaires, 9*, 77–85.

Winter, P., Pfaff, T., Udupa, S. M., et al. (1999). Characterization and mapping of sequence tagged microsatellite sites in the chickpea (*C. arietinum* L.). *Molecular & General Genetics, 262*, 90–101.

Wood, J. A., Knights, E. J., & Choct, M. (2011). Morphology of Chickpea Seeds (*Cicer arietinum* L.): Comparison of desi and kabuli types. *International Journal of Plant Sciences, 172*, 632–643.

Yang, Y., & Guo, Y. (2017). Elucidating the molecular mechanisms mediating plant salt-stress responses. *New Phytologist, 217*, 523–539.

Yildirim, T., Canci, H., Ertoy Inci, N., et al. (2013). Inheritance of female sterility in induced *Cicer* species. *Turkish Journal of Field Crops, 18*, 78–81.

Yuan, W., Yi, Z., Piao, S., et al. (2019). Increased atmospheric vapor pressure deficit reduces global vegetation growth. *Science Advances, 5*, eaax1396.

Zaman-Allah, M., Jenkinson, D. M., & Vadez, V. (2011). A conservative pattern of water use, rather than deep or profuse rooting, is critical for the terminal drought tolerance of chickpea. *Journal of Experimental Botany, 62*, 4239–4252.

# Application of Molecular Markers on Assessing Genetic Diversity in Faba Bean


**Nurmansyah, Salem S. Alghamdi, Hussein M. Migdadi, and M. Afzal**

**Abstract** With the advancement of sequencing technologies, the identification of molecular approaches has been used for the preservation of gene pool sources and for improving breeding programs in recent years. At the molecular level, diversity within and between genotypes or accessions is commonly assessed using various laboratory-based techniques, such as allozyme or DNA analysis, which directly determine diversity levels. Population size, heterozygosity, and quantification of genetic diversity are employed as fitness indicators and positively correlated with population fitness. The current study provides an overview and application of the molecular marker techniques used for the analysis of genetic diversity in faba beans (*Vicia faba* L.). Different markers were used to identify specific genes, gene actions and construct gene maps that helped in the development of gene transfer. Furthermore, these markers have also played a significant role to determine the species evolution and phylogeny analysis and help to a better understanding of the distribution and breadth of the genetic diversity within and among the species. The current chapter discussed the application of different molecular markers, i.e., non-PCR-based markers (RFLP), PCR-based markers (RAPD, ISSR, SSR, SSAP, AFLP, SRAP), and sequence-based markers (SNP) that were used for the improvement of faba bean. The advanced marker technique has an advantage over the basic technique and enhanced resolution and sensitivity to detect the genetic gap and differences among the genotypes.

**Keywords** Genetic mutation · Mutation detection techniques · Restriction fragment length polymorphism · Denaturing high-performance liquid chromatography · Targeting induced local lesion in genomes

Nurmansyah (✉)
Department of Agronomy, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta, Indonesia
e-mail: nurmansyah@ugm.ac.id

S. S. Alghamdi · M. Afzal
Production Department, Faculty of Food and Agricultural Sciences, King Saud University, Riyadh, Saudi Arabia

H. M. Migdadi
National Agricultural Research Center, Baqa, Jordan



A. Raina et al. (eds.), *Advanced Crop Improvement, Volume 2*,
https://doi.org/10.1007/978-3-031-26669-0_11

## 1 Introduction

Genetic diversity can be assessed by using three types of markers: (i) morphological, (ii) biochemical, and (iii) molecular markers. The simplicity and relatively low cost of the morphological marker make it a widely used tool to study the genetic variation of germplasm. However, a limited number of traits that can be evaluated and most of the economical traits influenced by the environment became drawbacks of this technique. While the biochemical marker has some negatives due to the limited number of markers and is influenced by the developmental stages of plants, the molecular or DNA-based marker has unlimited markers and is not affected by the developmental stages and the environment. Therefore, DNA-based markers provide a strong tool for the characterization of diversity in various crop plants (Alghamdi et al., 2012a). Furthermore, molecular markers can be utilized to figure out the generated mutants in pedigree and tag significant mutations. In addition, closely connected markers can also be employed for marker-assisted selection (MAS), mutation, and gene pyramiding used in cloning (Suprasanna et al., 2015). The various applications of molecular markers are shown in Fig. 1.

This chapter will focus on the use of molecular markers for analyzing genetic diversity in faba beans. Genetic diversity assessment using molecular markers is a critical step in most plant breeding programs to improve the efficiency and efficacy

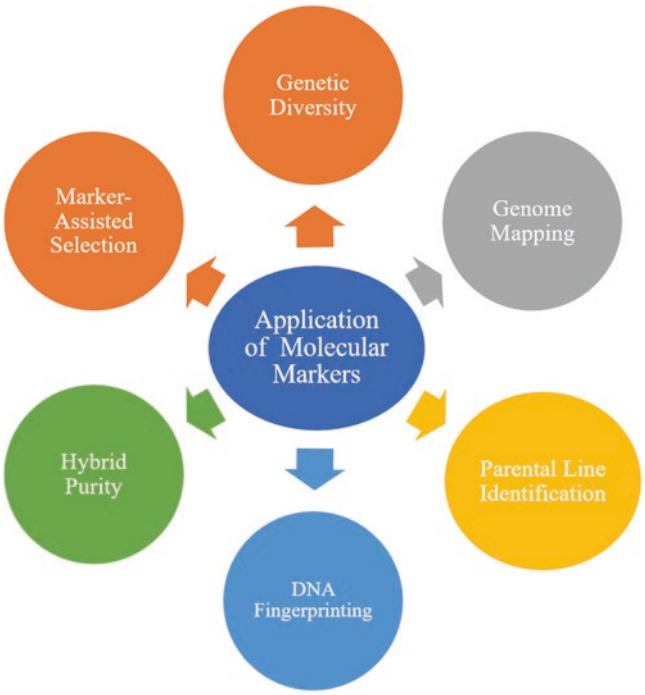



**Fig. 1** The application of molecular markers

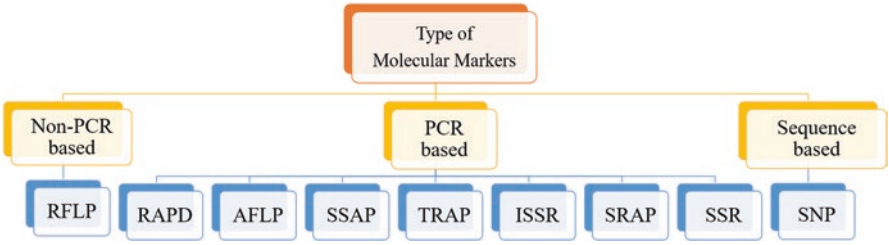



**Fig. 2** Classification of different molecular markers

of the program. Therefore, it is crucial to select a molecular marker that is suitable for a particular use. Many types of molecular markers were utilized to analyze the diversity in faba bean including Restriction Fragment Length Polymorphism (RFLP) (Van de Ven et al., 1990), Random Amplified Polymorphic DNA (RAPD) (Alghamdi, 2009), Amplified Fragment Length (AFLP) (Zeid et al., 2003; Zong et al., 2010; Ammar et al., 2015; Nurmansyah et al., 2020), Sequence-Specific Amplification Polymorphism (SSAP) (Sanz et al., 2007), Target Region Amplification Polymorphism (TRAP) (Kwon et al., 2010), Inter Simple Sequence Repeats (ISSRs) (Alghamdi et al., 2011), Sequence-related Amplified Polymorphism (SRAP) (Alghamdi et al., 2012b), Single Nucleotide Polymorphism (SNP) (Kaur et al., 2014) and Simple Sequence Repeats (SSRs) (Rebaa et al., 2017; Raina et al., 2020; Laskar et al., 2018). The molecular markers could be classified according to the technology used (Fig. 2). The application of numerous molecular markers to evaluate differences based on molecular analysis among and within the faba bean population is discussed in Table 1.

## 2 Non-PCR-Based Markers

### *2.1 Restriction Fragment Length Polymorphism (RFLP)*

Van de Ven et al. (1990) used RFLP markers on 16 faba bean accessions and its four wild relatives, namely, *V. narbonensis*, *V. johannis*, *V. galilaea*, and *V. hyaeniscyamus*, to evaluate the degree of polymorphism at the DNA level as well as the identification of appropriate faba bean parents in order to create a physical map. Most of the polymorphisms discovered were related to wild species; however, since interspecific crosses of faba bean and its wild relatives cannot be produced, they developed a three-way hybrid, A × B × C, which was responsible for most of the polymorphisms detected in the *V. faba* gene pool sampled. Knowledge of no successful interspecific crosses between *V. faba* and other *Vicia* species led researchers to exploit heterosis to develop a better faba bean cultivar. However, genetic structure and identification of germplasm groups are required for optimizing the crossing plans and selection procedures (Link et al., 1995).

**Table 1** Application of molecular markers in the genetic diversity assessment of faba bean

| No. | Marker type | No. of samples | Sample origin | Objective | Reference |
|---|---|---|---|---|---|
| 1. | RFLP | 20 | 16 modern cultivar accessions of faba bean and 4 wild relatives of *Vicia* species | Identification of suitable faba bean parents for creating a linkage map | Van de Ven et al. (1990) |
| 2. | RAPD | 28 | 13 European minor, 6 European major, and 9 Mediterranean accessions | Genetic diversity analysis within and between European and Mediterranean accessions | Link et al. (1995) |
| | | 26 | Palestinian faba bean landraces | Genetic diversity and relatedness assessment among Palestinian landraces | Basheer-Salimia et al. (2013) |
| 3. | SSAP | 9 | Accessions from Tunisia with two subspecies (minor and major) | Genetic relationship assessment among Tunisian faba bean accessions | Ouji et al. (2012) |
| | | 76 | 56 *V. narbonensis* accessions and 20 *V. faba* accessions | Genetic diversity assessment within the *Vicia* species | Sanz et al. (2007) |
| 4. | TRAP | 151 | 137 accessions (107 from China, 15 from Afghanistan, 7 from Germany, 6 from Bulgaria, 4 from Nepal, 3 from France, 2 from each of Finland, Hungary, and the United Kingdom, and 1 from Poland), 12 cultivars from Germany, and 2 commercial varieties. | Genetic diversity and relationship assessment among faba bean accessions | Kwon et al. (2010) |
| 5. | ISSR | 34 | 8 from ICARDA, 9 from Saudi Arabia, 10 from Egypt, 2 from Sudan, 3 from Spain, and 1 from Pakistan and Yemen accessions | Genetic diversity assessment among faba bean accessions | Alghamdi et al. (2011) |
| | | 802 | 538 accessions from China and 264 from outside China | Assessment of diversity among faba bean genotypes collected from different areas of China and outside China | Wang et al. (2012) |
| | | 39 | 39 Mexican faba bean accessions | Genetic diversity and relatedness assessment among Mexican genotypes | Salazar-Laureles et al. (2015) |

(continued)

**Table 1** (continued)

| No. | Marker type | No. of samples | Sample origin | Objective | Reference |
|---|---|---|---|---|---|
| 6. | SRAP | 58 | 18 accessions from Saudi Arabia, 25 from Egypt, 6 from ICARDA, 4 from Sudan, 3 from Spain, and 1 from Pakistan and Yemen accessions | Genetic diversity assessment among faba bean accessions | Alghamdi et al. (2012b) |
| | | 40 | 5 accessions from the United Kingdom, 28 from ICARDA, 2 from each of Saudi Arabia, Egypt, and Sudan, and 1 from Spain | Genetic diversity assessment among faba bean accessions | Ammar et al. (2015) |
| 7. | SNP | 45 | 45 accessions from several different geographical regions (North Africa, China, Ecuador, Europe, and Australia) | Genetic diversity and pedigree relationship assessment among faba bean accessions from diverse localities | Kaur et al. (2014) |
| 8. | AFLP | 79 | 63 cultivars from Europe, 8 from Asia, and 8 from North Africa | Genetic structure assessment among faba bean cultivars | Zeid et al. (2003) |
| | | 243 | 204 Chinese landraces and 39 faba bean winter accessions from outside of China | The genetic diversity of the Chinese and their association with the global winter faba bean | Zong et al. (2009) |
| | | 175 | 39 Chinese spring faba bean landraces and 136 non-Chinese spring faba bean accessions | Genetic diversity and relationship between Chinese and global spring faba bean accessions | Zong et al. (2010) |
| | | 40 | 5 accessions from the United Kingdom, 28 from ICARDA, 2 from each of Saudi Arabia, Egypt, and Sudan, and 1 from Spain | Genetic diversity assessment among faba bean accessions | Ammar et al. (2015) |
| 9. | SSR | 35 | 15 accessions from Northern Africa, 12 from Eastern Africa, and 8 from the Near East | Assessment of genetic diversity and population organization in three different geographical locations | El-Esawi (2017) |
| | | 21 | 21 Tunisian faba bean accessions from five geographical areas | Genetic variability assessment of Tunisian faba bean accessions | Rebaa et al. (2017) |
| | | 255 | 151 landraces and 104 cultivars from 30 different countries | Genetic diversity and relationship assessment among different geographical origins and seed sizes | Göl et al. (2017) |

## 3 PCR-Based Markers

### *3.1 Random Amplified Polymorphic DNA (RAPD)*

Link et al. (1995) evaluated the diversity among three different regions of faba bean inbred lines (European small- and large-seeded and Mediterranean germplasms) using RAPD markers. The RAPD results revealed that European small-seeded and Mediterranean lines are distinct groups, with European large-seeded lines located in between. Their RAPD results were also consistent with field data, which showed that the crosses (heterosis) between dissimilar lines were greater than those of similar lines. Therefore, they concluded that RAPD markers effectively classify germplasm and identify different heterotic groups in faba beans. Similarly, Basheer-Salimia et al. (2013) tested 26 Palestinian faba bean landraces for genetic diversity and relatedness. Genetic distances (GD) based on the Jaccard similarity index ranged from 0.069 to 0.358 with a mean of 0.213, which were lower than those obtained by Link et al. (1995), where GD ranged from 0.306 to 0.646. It indicated that Palestinian landraces are less diverse than European and Mediterranean germplasms. This consistency of RAPD markers in assessing the genetic diversity makes it suitable for faba bean fingerprinting.

### *3.2 Sequence-Specific Amplification Polymorphism (SSAP)*

Sanz et al. (2007) have successfully developed long terminal repeat retrotransposon-based SSAP markers to construct a phylogenetic tree within a collection of 56 *V. narbonensis* and 20 *V. faba* accessions. The SSAP results demonstrated extremely limited diversity based on geographical origin and clustering among accessions, and no evident link was recorded between diversity and morphology-based taxonomy groups. Ouji et al. (2012) also used SSAP to study the nine Tunisian faba bean populations for population structure and genetic diversity between local genotypes and commercial varieties with different botanical classes (large- and small-seeded). The study found high genetic variation within the population due to the high outcrossing rate. The unweighted pair group method with arithmetic averaging (UPGMA) revealed no clear separation based on botanical class, as is in line with Sanz et al. (2007).

### *3.3 Amplified Fragment Length (AFLP)*

Although in some crops, the SSAP markers were chosen over AFLP markers due to the diverse nature of polymorphism (Waugh et al., 1997; Ellis et al., 1998; Tam et al., 2005), no comparative study has been done between these markers in faba

bean. However, it seems that the AFLP marker was preferred over SSAP in assessing the genetic diversity in faba beans because an unlimited number of AFLP primers can be generated by simply varying the restriction enzymes and altering supplementary bases. Moreover, AFLP markers have the capacity of generating strong polymorphism bands or high repeatability without prior knowledge of the DNA sequence. Additionally, using the sequencer (fragment analysis) along with the software to analyze the comparison among banding patterns facilitates faster analysis (Sorkheh et al., 2007).

Zeid et al. (2003) reported the genetic diversity of 79 faba bean genotypes consisting of six different groups (Asian, North African, South European, European winter bean, European spring minor, and European spring major) using AFLP markers. The UPGMA clustering group of 79 genotypes is based on the mean of genetic similarity and their results revealed that there was no distinct grouping, neither based on seed size (European minor and major) nor geographic origin. Only six of the eight Asian cultivars clustered together. However, cluster analysis of the six groups convincingly separated Asian cultivars from the other groups. Moreover, their findings revealed a higher level of similarity (genetic) among the European winter bean, European spring minor, and European spring major.

Zong et al. (2009) assessed the genetic diversity of 243 Chinese and worldwide winter faba bean germplasms with varying seed sizes (major, equina, and minor types) using ten AFLP primer combinations. The results suggested that 204 Chinese faba bean accessions were segregated from 39 accessions from outside China. They came to the conclusion that the Chinese winter faba bean germplasm was distinct from the rest of the world's faba bean germplasm. The results were also in line with Kwon et al. (2010) who studied genetic diversity among 151 accessions consisting of 107 accessions from China, 15 from Afghanistan, 4 from Nepal, 23 from European countries, and 2 vegetable-type commercial varieties. Using TRAP markers, they divided the 151 entries into five clusters. The findings revealed a strong link between geographic origin and molecular diversity among the entries studied. The striking example is that 101 out of 107 China accessions were grouped in one cluster.

### *3.4 Sequence-Related Amplified Polymorphism (SRAP)*

Ammar et al. (2015) evaluated 40 accessions consisting of 33 ICARDA accessions and 7 local and exotic faba bean accessions using four AFLP and six SRAP primer combinations. Four AFLP primers generated 202 alleles with a mean of 50.5 alleles, while six SRAP primers generated 183 alleles with a mean of 30.5 alleles per primer combination. Seven native and exotic faba bean accessions were isolated from 33 ICARDA accessions based on the UPGMA cluster analysis constructed using the AFLP marker. The SRAP marker also separated six out of seven local and exotic faba bean accessions from the ICARDA accessions. These results revealed that AFLP generated more polymorphic alleles or had higher reproducibility than SRAP markers.

### 3.5 Inter Simple Sequence Repeats (ISSRs)

ISSR primers were used by Alghamdi et al. (2011) to evaluate the diversity of 34 newly introduced local faba beans in Saudi Arabia. Twelve of the 24 ISSR primers showed an unambiguous band pattern that generated 71 polymorphic alleles throughout the samples. The cluster analysis revealed a high number of subclusters that indicated high genetic variability in the sample tested and should be utilized in faba bean improvement. Wang et al. (2012) used ISSR to determine the genetic diversity and interrelationships using 802 faba bean accessions and genotypes collected from different origins, i.e., China ($n$ = 538) and globally ($n$ = 264). Eleven ISSR primers were used and produced 209 diverse bands. The results revealed that the faba bean was present in the same group of origins from North Africa and Europe, and the genotypes were grouped according to the origin. Furthermore, it was also suggested that China has the second-highest genetic diversity of the faba bean due to being more reproductively isolated from the rest of the Asian, African, and European gene pools.

### 3.6 Simple Sequence Repeat (SSR)

Large numbers of SSR markers with expressed sequence tags (ESTs) (Gong et al., 2010; Ma et al., 2011) and cDNA (Pozarkova et al., 2002; Suresh et al., 2013) have been produced and described from the faba bean genomes. Rebaa et al. (2017) used eight EST-SSR primers to evaluate 21 Tunisian faba beans. There were 53 alleles, with an average of 6.62 alleles per locus. The UPGMA cluster analysis based on Nei & Li's similarity coefficients divided the genotypes into clusters and subdivided them into three subclusters based on localities. Furthermore, AMOVA results revealed that more genetic variation was recorded within populations, which they suggested was most likely due to the partially allogamous reproductive system of the faba bean. El-Esawi (2017) used 15 cDNA-SSR primers to estimate the genetic diversity of 35 accessions of faba beans representing three geographical regions (Northern Africa, East Africa, and the Near East). A total of 100 alleles with an average of 6.67 were obtained. The faba bean genotypes were grouped into two clusters based on Nei genetic distance and population structure. The cluster (I) was composed of faba bean accession from the Near East region; however, the cluster (II) was composed of Northern and Eastern African faba bean genotypes and suggested that Eastern and Northern African genotypes were closely related.

Göl et al. (2017) also used SSR markers to determine the diversity among 255 faba bean accessions that originated from 30 different countries with different seed sizes (large-, medium-, and small-seeded). A total of 305 polymorphic loci and an average of eight alleles per locus were obtained from 32 SSR primers. Based on unweighted neighbor-joining and population structure analyses, the 255 genotypes were divided into two groups; however, the genotypes were also subgrouped based

on geographic origins as well as seed size. Interestingly, with a large coverage of different countries, the AMOVA analysis revealed that the majority of genetic variation (90%) was obtained within the population, while 10% diversity was present among the populations.

## 4 Sequence-Based Marker

### *4.1 Single Nucleotide Polymorphism (SNP)*

Recent technological advancements in genotyping and sequencing allow for the generation of a large set of single nucleotide polymorphism (SNP) markers for many crops, including faba beans. Kaur et al. (2014) used a subset of 768 SNPs to determine the genetic diversity of 45 faba bean accessions collected from China, Europe, Ecuador, North Africa, and Australia. According to pedigree analysis and geographical origin, faba bean lines displayed a clear clustering pattern based on the neighbor-joining (NJ) dendrogram and principal coordinate analysis (PCoA). Most of the studied faba bean lines have a high amount of heterozygosity, which is likely related to the species' largely allogamous character. They concluded that SNP markers are considered a useful technique to find out the genetic variation among and within the faba bean genotypes and for varietal identification and discrimination due to the sufficient number of varietal-specific alleles. Moreover, Mulugeta et al. (2021) used 37 SNPs based on Kompetitive Allele-Specific PCR (KASP) SNP markers in 48 Ethiopian faba bean genotypes. Out of 37 SNPs, 36 SNPs recorded polymorphic alleles among the total faba bean genotypes and recorded a 95.6 PIC value. The diversity was 0.16–0.50 with an average of 0.42 was recorded.

### *4.2 Expressed Sequence Tags (EST)-SSR*

The creation of simple sequence repeat (SSR) markers from expressed sequence tags (EST) provided a good technique for investigating *Vicia* species genetic diversity. Gong et al. (2011) utilized 5031 faba bean EST sequences from the NCBI database and identified 107 SSR primers. Eleven of them were used to evaluate the genetic diversity of 29 faba bean accessions from China and Europe. With an average PIC of 0.29, it indicated that European accessions had wider genetic diversity than China accessions. A similar study conducted by Akash and Myers (2012) was designed to determine the diversity among 20 Jordanian faba bean genotypes. Thirty-one out of 349 SSR primers successfully amplified 20 Jordanian faba bean accessions with a PIC value of 0.54. Moreover, EST-SSR markers could also be used to generate a linkage map in faba beans. A total of 128 SSR markers generated from 5090 faba bean nonredundant EST sequences were developed to study the

linkage map of 109 F2 plants. A dense linkage map developed in this study, representing a total length of 684.7 cM with 552 loci, is expected to accelerate marker-assisted selection in faba bean breeding (El-Rodeny et al., 2014).

## 5 Conclusions

Molecular markers correspond to a significant tool that is susceptible to new genome-based discoveries and technological breakthroughs and, hence, is constantly evolving. In the assessment of genetic diversity and the development of physical and genetic maps, most molecular marker techniques are used. The results of DNA fingerprinting from the results of wild ancestors revealed a long-term evolutionary alteration among the allelic interactions. The information presently accessible is also critical in developing appropriate conservation plans. However, it is exceedingly doubtful that these data and DNA sample collections will be able to replace the preservation of whole organisms' germplasm. Polygenes code for the majority of agronomically essential traits, and it would be very difficult to reconstruct all the implied gene blocks with their regulatory components based on existing knowledge.

## References

Akash, M., & Myers, G. (2012). The development of faba bean expressed sequence tag-simple sequence repeats (EST-SSRs) and their validity in diversity analysis. *Plant Breeding, 131*, 522–530. https://doi.org/10.1111/j.1439-0523.2012.01969.x

Alghamdi, S. S. (2009). Varietal identification and genetic purity assessment of F1 hybrid seeds using RAPD markers in faba bean (*Vicia faba* L.). *Acta Horticulturae, 829*, 269–274. https://doi.org/10.17660/ActaHortic.2009.829.39

Alghamdi, S. S., Al-Faifi, S. A., Migdadi, H. M., Ammar, M. H., & Siddique, K. M. (2011). Inter-simple sequence repeat (ISSR)-based diversity assessment among faba bean genotypes. *Crop & Pasture Science, 62*(9), 755–760. https://doi.org/10.1071/CP11045

Alghamdi, S. S., Migdadi, H. M., Ammar, M. H., Paull, J. G., & Siddique, K. H. M. (2012a). Faba bean genomics, current status and future prospects. *Euphytica, 186*(3), 609–624. https://doi.org/10.1007/s10681-012-0658-4

Alghamdi, S. S., Al-Faifi, S. A., Migdadi, H. M., Khan, M. A., El-Harty, E. H., & Ammar, M. H. (2012b). Molecular diversity assessment using Sequence Related Amplified Polymorphism (SRAP) markers in *Vicia faba* L. *International Journal of Molecular Sciences, 1(12)3*, 16457–16471. https://doi.org/10.3390/ijms131216457

Ammar, M. H., Alghamdi, S. S., Migdadi, H. M., Khan, M. A., El-Harty, E. H., & Al-Faifi, S. A. (2015). Assessment of genetic diversity among faba bean genotypes using agro-morphological and molecular markers. *Saudi Journal of Biological Sciences, 22*(3), 340–350. https://doi.org/10.1016/j.sjbs.2015.02.005

Basheer-Salimia, R., Shtaya, M., Awad, M., Abdallah, J., & Hamdan, Y. (2013). Genetic diversity of Palestine landraces of faba bean (*Vicia faba*) based on RAPD markers. *Genetics and Molecular Research, 12*(3), 3314–3323.

El-Esawi, M. A. (2017). SSR analysis of genetic diversity and structure of the germplasm of faba bean (*Vicia faba* L.). *Comptes Rendus Biologies, 340*, 474–480. https://doi.org/10.1016/j.crvi.2017.09.008

Ellis, T. H. N., Poyser, S. J., Knox, M. R., Vershinin, A. V., & Ambrose, M. J. (1998). Polymorphism of the insertion site of *Ty1-copia* class retrotransposon and its use for linkage and diversity analysis in pea. *Molecular and General Genetics, 260*, 9–19. https://doi.org/10.1007/PL00008630

El-Rodeny, W., Kimura, M., Hirakawa, H., Sabah, A., Shirasawa, K., Sato, S., Tabata, S., Sasamoto, S., Watanabe, A., Kawashima, K., Kato, M., Wada, T., Tsuruoka, H., Takahashi, C., Minami, C., Nanri, K., Nakayama, S., Kohara, M., Yamada, M., & Isobe, S. (2014). Development of EST-SSR markers and construction of a linkage map in faba bean (*Vicia faba*). *Breeding Science, 64*, 252–263.

Göl, S., Doganlar, S., & Frary, A. (2017). Relationship between geographical origin, seed size and genetic diversity in faba bean (*Vicia faba* L.) as revealed by SSR markers. *Molecular Genetics and Genomics, 292*(5), 991–999. https://doi.org/10.1007/s00438-017-1326-0

Gong, Y. M., Xu, S. C., Mao, W. H., Hu, Q. Z., Zhang, G. W., Ding, J., & Li, Z. Y. (2010). Generation and characterization of 11 novel EST derived microsatellites from *Vicia faba* (Fabaceae). *American Journal of Botany, 97*, 69–71. https://doi.org/10.3732/ajb.1000166

Gong, Y. M., Xu, S. C., Mao, W. H., Li, Z. Y., Hu, Q. Z., Zhang, G. W., & Ju, D. (2011). Genetic diversity analysis of faba bean (*Vicia faba* L.) based on EST-SSR markers. *Agricultural Sciences in China, 10*(6), 838–844.

Kaur, S., Cogan, N. O. I., Forster, J. W., & Paull, J. G. (2014). Assessment of genetic diversity in faba bean based on single nucleotide polymorphism. *Diversity, 6*(1), 88–101. https://doi.org/10.3390/d6010088

Kwon, S. J., Hu, J., & Coyne, C. J. (2010). Genetic diversity and relationship among faba bean (*Vicia faba* L.) germplasm entries as revealed by TRAP markers. *Plant Genetic Resources: Characterization and Utilization, 8*(3), 204–213. https://doi.org/10.1017/S1479262110000201

Laskar, R. A., Laskar, A. A., Raina, A., & Amin, R. (2018). Induced mutation analysis with biochemical and molecular characterization of high yielding lentil mutant lines. *International Journal of Biological Macromolecules, 109*, 167–179.

Link, W., Dixkens, C., Singh, M., Schwall, M., & Melchinger, A. E. (1995). Genetic diversity in European and Mediterranean faba bean germplasm revealed by RAPD markers. *Theoretical and Applied Genetics, 90*(1), 27–32. https://doi.org/10.1007/BF00220992

Ma, Y., Yang, T., Guan, J., Wang, S., Wang, H., Sun, X., & Zong, X. (2011). Development and characterization of 21 EST-derived microsatellite markers in *Vicia faba* (faba bean). *American Journal of Botany, 98*, 22–24. https://doi.org/10.3732/ajb.1000407

Mulugeta, B., Tesfaye, K., Keneni, G., & Ahmed, S. (2021). Genetic diversity in spring faba bean (*Vicia faba* L.) genotypes as revealed by high-throughput KASP SNP markers. *Genetic Resources and Crop Evolution, 68*(5), 1971–1986.

Nurmansyah, Alghamdi, S. S., Migdadi, H. M., Khan, M. A., & Afzal, M. (2020). AFLP-based analysis of variation and population structure in mutagenesis induced faba bean. *Diversity, 12*(8), 1–14.

Ouji, A., El Bok, S., Syed, N. H., Abdellaoui, R., Rouaissi, M., Flavell, A. J., & El Gazzah, M. (2012). Genetic diversity of faba bean (*Vicia faba* L.) populations revealed by sequence specific amplified polymorphism (SSAP) markers. *African Journal of Biotechnology, 11*(9), 2162–2168. https://doi.org/10.5897/AJB11.2991

Pozarkova, D., Koblizkova, A., Roman, B., Torres, A. M., Lucretti, S., Lysak, M., Dolezel, J., & Macas, J. (2002). Development and characterization of microsatellite markers from chromosome 1-specifi cDNA libraries of *Vicia faba*. *Biologia Plantarum, 45*, 337–345. https://doi.org/10.1023/A:1016253214182

Raina, A., Laskar, R. A., Tantray, Y. R., Khursheed, S., Wani, M. R., & Khan, S. (2020). Characterization of induced high yielding cowpea mutant lines using physiological, biochemical and molecular markers. *Scientific Reports, 10*(1), 1–22.

Rebaa, F., Abid, G., Aouida, M., Abdelkarim, S., Aroua, I., Muhovski, Y., Baudoin, J., M'hamdi, M., Sassi, K., & Jebara, M. (2017). Genetic variability in Tunisian populations of faba bean (*Vicia faba* L. var. major) assessed by morphological and SSR markers. *Physiology and Molecular Biology of Plants, 23*(2), 397–409. https://doi.org/10.1007/s12298-017-0419-x

Salazar-Laureles, M. E., Pérez-López, D. J., González-Huerta, A., Vázquez-García, L. M., & Valadez-Moctezuma, E. (2015). Genetic variability analysis of faba bean accessions using inter-simple sequence repeat (ISSR) markers. *Chilean Journal of Agricultural Research, 75*(1), 122–130. https://doi.org/10.4067/S0718-58392015000100017

Sanz, A. M., Gonzalez, S. G., Syed, N. H., Suso, M. J., Saldaña, C. C., & Flavell, A. J. (2007). Genetic diversity analysis in *Vicia* species using retrotransposon-based SSAP markers. *Molecular Genetics and Genomics, 278*(4), 433–441. https://doi.org/10.1007/s00438-007-0261-x

Sorkheh, K., Shiran, B., Aranzana, M. J., Mohammadi, S. A., & Martinez-Gomez, P. (2007). Application of amplified fragment length polymorphism (AFLPs) analysis to plant breeding and genetics, procedures, applications and prospects. *Journal of Food, Agriculture and Environment, 5*(1), 197–204.

Suprasanna, P., Mirajkar, S. J., & Bhagwat, S. G. (2015). Induced mutations and crop improvement. In B. Bahadur, R. M. Venkat, L. Sahijram, & K. Krishnamurthy (Eds.), *Plant biology and biotechnology* (pp. 593–617). Springer. https://doi.org/10.1007/978-81-322-2286-6_23

Suresh, S., Park, J. H., Cho, G. T., Lee, H. S., Baek, H. J., Lee, S. Y., & Chung, J. W. (2013). Development and molecular characterization of 55 novel polymorphic cDNA-SSR markers in faba bean (*Vicia faba* L.) using 454 pyrosequencing. *Molecules, 18*(2), 1844–1856. https://doi.org/10.3390/molecules18021844

Tam, S. M., Mhiri, C., Vogelaar, A., Kerkveld, M., Pearce, S. R., & Grandbastien, M. A. (2005). Comparative analyses of genetic diversities within tomato and pepper collections detected by retrotransposon-based SSAP, AFLP and SSR. *Theoretical and Applied Genetics, 110*, 819–831. https://doi.org/10.1007/s00122-004-1837-z

Van de Ven, M., Powell, W., Ramsay, G., & Waugh, R. (1990). Restriction fragment length polymorphisms as genetic markers in Vicia. *Heredity, 65*, 329–342. https://doi.org/10.1038/hdy.1990.102

Wang, H., Zong, X., Guan, J., Yang, T., Sun, X., Ma, Y., & Redden, R. (2012). Genetic diversity and relationship of global faba bean (*Vicia faba* L.) germplasm revealed by ISSR markers. *Theoretical and Applied Genetics, 124*(5), 789–797. https://doi.org/10.1007/s00122-011-1750-1

Waugh, R., McLean, K., Flavell, A. J., Pearce, S. R., Kumar, A., Thomas, B. B., & Powell, W. (1997). Genetic distribution of Bare-1-like retrotransposable elements in the barley genome revealed by sequence-specific amplification polymorphisms (S-SAP). *Molecular and General Genetics, 253*, 687–694. https://doi.org/10.1007/s004380050372

Zeid, M., Schon, C. C., & Link, W. (2003). Genetic diversity in recent elite faba bean lines using AFLP markers. *Theoretical and Applied Genetics, 107*(7), 1304–1314. https://doi.org/10.1007/s00122-003-1350-9

Zong, X., Liu, X., Guan, J., Wang, S., Liu, Q., Paull, J. G., & Redden, R. (2009). Molecular variation among Chinese and global winter faba bean germplasm. *Theoretical and Applied Genetics, 118*(5), 971–978. https://doi.org/10.1007/s00122-008-0954-5

Zong, X., Ren, J., Guan, J., Wang, S., Liu, Q., Paull, J. G., & Redden, R. (2010). Molecular variation among Chinese and global germplasm in spring faba bean areas. *Plant Breeding, 129*(5), 508–513. https://doi.org/10.1111/j.1439-0523.2009.01718.x

# Conventional and Molecular Breeding for Genetic Improvement of Maize (*Zea mays* L.)

**Mukesh Choudhary, Jeetram Choudhary, Pawan Kumar, Pardeep Kumar, Bahadur Singh Jat, Vishal Singh, and Manoj Choudhary**

**Abstract** After rice and wheat, maize is the third most important cereal crop globally. It has diversified uses in the form of food, feed, fodder, fuel, and industrial raw materials. Maize has witnessed great success in terms of evolution and improvement and is the most explored crop for which most breeding strategies have been devised. Maize, being a cross-pollinated crop, explores the phenomenon of heterosis. The yield improvement in maize has been achieved mainly due to the adoption of single-cross hybrids through conventional breeding. Conventional breeding approaches like hybrid development and population improvement contributed immensely to maize improvement, mainly yield and oil content. However, considering the huge time and resource investment in conventional breeding and the availability of molecular markers, breeders gradually started to shift to molecular breeding. In addition, the complex nature of abiotic and biotic stresses also made breeders integrate conventional breeding approaches with genomics to give rise to genomics-assisted breeding. Genomics-assisted breeding helped identify important major quantitative trait loci (QTLs) for yield, quality, and biotic and abiotic stresses via QTL mapping and genome-wide association studies. Hence, genomics-assisted breeding offers an excellent opportunity to speed up

M. Choudhary (✉)
ICAR-Indian Institute of Maize Research, Ludhiana, Punjab, India

School of Agriculture and Environment, The University of Western Australia, Perth, WA, Australia

J. Choudhary
ICAR-Indian Agricultural Research Institute, New Delhi, India

P. Kumar
ICAR- Central Institute for Arid Horticulture, Bikaner, Rajasthan, India

P. Kumar · B. S. Jat · V. Singh
ICAR-Indian Institute of Maize Research, Ludhiana, Punjab, India

M. Choudhary
ICAR-National Centre for Integrated Pest Management, New Delhi, India

University of Florida, Gainesville, FL, USA



A. Raina et al. (eds.), *Advanced Crop Improvement, Volume 2*,
https://doi.org/10.1007/978-3-031-26669-0_12

maize improvement programs globally. This chapter focuses on the conventional and molecular breeding-based approaches to maize improvement for various important traits.

**Keywords** Maize · Conventional · Quantitative trait loci · Heterosis · Genomics

## 1 Introduction

Maize is a cereal crop with wide adaptability to diverse climatic conditions (Choudhary et al., 2019). Maize is globally cultivated on 197 million hectares with 1148 million tonnes of annual production (FAOSTAT, 2019). It is utilized in diverse forms like food, feed, industrial products, fodder, and biofuel (Choudhary et al., 2020; Yadav et al., 2015). In addition to being a rich source of calories, maize is a good source of micronutrients and other phytochemicals, such as carotenoids, phenolics, and anthocyanins. Therefore, the maize history witnessed a greater emphasis on grain yield in the early stages but later also emphasized quality improvement and biotic and abiotic stress resilience. The breeding efforts resulted in improvements of all the traits, like continuous improvement in yields, but in the last few decades, quality improvement has been remarkable. The reason for the greater success with quality improvement is due to the fact that qualitative traits are governed by fewer genes and hence easier to improve upon as compared to quantitative traits (governed by many genes), but the most economically important traits are quantitative in nature (Kearsey & Pooni, 1998).

Conventional breeding relies mainly on the extensive screening or characterization of the germplasm for target traits and the selection of the best lines, followed by crossing to look at genetic gain over repeated cycles of selection. Maize holds special importance in shaping the breeding methods over the years, as witnessed by the historical importance of concepts such as single cross hybrids, double cross hybrids, synthetics, and composites. Being a cross-pollinated crop, exploitation of heterosis remained the most effective approach for yield improvement in maize (Reif et al., 2005). However, for wide adaptation and complex traits (biotic and abiotic stresses), the population improvement approach via recurrent selection (RS) proved effective and is still practiced (Tomlekova et al., 2014a, b). The wild relatives are the best source to mine for abiotic and biotic stress tolerance in maize (Choudhary et al., 2017). The inbred line development via repeated selfing followed by a heterotic grouping of the lines tested for per se performance is the basic platform of hybrid breeding programs. Although, maize witnessed success for different traits like yield improvement, biotic and abiotic stress tolerance, and quality improvement, it did so at a slower pace. However, with the availability of molecular markers, the pace of improvement for these traits improved significantly. This resulted from the integrated approach of conventional breeding and molecular markers, known as molecular breeding.

Several quantitative trait loci (QTLs) were identified during the last decade for different traits in maize, and as a resultant molecular breeding boosted the improvement of maize for different traits, however, quality improvement witnessed greater success relative to others. The reason is that quality traits are characterized by less complexity and high heritability (Kumar et al., 2019). This chapter is aimed at covering the contribution of conventional breeding approaches, recent progress in QTL mapping, and molecular breeding for different traits in maize, however, with special emphasis on quality traits, which most benefited from molecular breeding.

## 2 Conventional Breeding Approaches: Success and Limitations

The conventional methods of maize breeding majorly involve selection, population improvement (synthetics and composites), and hybrid breeding. RS schemes were used to enhance the frequency of desirable alleles in the population, mainly for quantitative traits. For example, historical oil improvement experiments in Illinois improved the oil content by up to 30% over existing lines (Jugenheimer, 1961). The hybrids were developed to exploit the heterosis (superiority of the offspring over its parents) at its fullest; however, it depends upon the genetic diversity of parental lines, the more divergent the parents, the better the heterosis (Reif et al., 2005). However, initially, the breeders involved in hybrid breeding programs faced two major challenges: improving at least two populations for adaptation while maintaining the genetic diversity among them, and establishing an efficient seed production system for hybrids. The former was addressed by the concept of heterotic grouping (a process of maintaining the genetic diversity and identity of populations by classifying the elite gene pool into subsets followed by separate breeding in each subset). The latter problem was addressed by the development of elite parental lines for attaining better yields (seed production); in addition, the use of male sterility genes proved effective (Kauffmann et al., 1982).

### *2.1 Grain Yield*

Yield holds the utmost importance for improvement for the plant breeders. Conventional breeding has resulted in the release of many varieties annually to improve food and nutritional security (Evenson & Gollin, 2002; Glenn et al., 2017). Landraces and wild relatives contribute to the foundation of maize improvement through introduction or as source material for developing populations or inbred lines (Choudhary et al., 2017). For example, introductions, namely, Laxmi and Suwan 1, were released directly as cultivars (composites) in India, whereas Tuxpeno, Antigua Gr 1, Suwan 2, Eto, Stiff Stalk Synthetic, and Lancaster Surecrop were

used as sources to derive the inbred lines and establish heterotic groups (Dhillon & Prasanna, 2001). Ganga 1, Ganga 101, Ranjit, and Deccan were the first double-cross hybrids released in India in 1961. Later, composite cultivars, Amber, Jawahar, Kisan, Vijay, Vikram, and Sona were released in 1967, of which Vijay and Kisan were widely adopted. Later, shifting back to hybrids, Trishulata (first three-way hybrid) and Paras (first single cross hybrid) were released in India. The availability and adoption of high-yielding hybrids resulted in a rapid increase in maize cultivation under single cross hybrids. More than 20% more yield was obtained over the OPVs with the adoption of CIMMYT-based hybrids under optimal conditions, while an increase of 30–60% was observed under abiotic and biotic stress conditions (Masuka et al., 2017). Furthermore, the genetic gain increased from 0.5 to 1.94 quintal/hectare by adopting the single cross hybrids of eight countries yield data (Smith et al., 2014). International Institute of Tropical Agriculture (IITA) researchers have also utilized the conventional methods successfully for improving quantitative traits, such as yield, biotic and abiotic stresses (Badu-Apraku et al., 2018; Menkir & Meseka, 2019).

## *2.2 Biotic and Abiotic Stresses*

High-yielding cultivars contributed immensely to achieving higher production over time, but various biotic and abiotic stresses affected the progress significantly (Choudhary et al., 2019). For abiotic stresses, conventional breeding through selection resulted in achieving tangible disease resistance and genetic gains. Maize is affected by various diseases like maize lethal necrosis (MLN), Maize ear rot (*Fusarium* spp.), Maize southern leaf blight (MLSB), *Turcicum* or Northern corn leaf blight (TLB or NCLB), Maize streak virus, gray leaf spot (GLS), Maize rough dwarf disease and fungal aflatoxin. Menkir et al. (2008) used aflatoxin-resistant tropical elite to develop the resistant line TZAR101–106 (in the background of temperate inbred lines) through backcrossing. Hung and Holland (2012) used diallel crossing to develop hybrids that exhibited a reduction of ear rot and fumonisin content by 27% and 30%, respectively, over the parental lines. Similarly, half-diallel approach-based $F_1$ hybrid "CKLTI0227 × CML550," "CKDHL120918 × CKLTI0138," and "CKDHL120918 × CKLTI0136 exhibited better resistance against MLN in Africa (Beyene et al., 2017). The resistant line (CML191) is a good product of conventional breeding that exhibits partial resistance against NCLB in Africa (Welz & Geiger, 2000). Furthermore, Longe1 and Longe4 (MSV resistant varieties) have also been developed through conventional breeding in sub-Saharan Africa.

Stem borers are major pests that reduce maize yields by damaging the leaves, stems, ears, and kernels. However, stem borer resistance, being governed by polygenes with low heritability, is difficult to breed (Sharma et al., 2007). But Mihm (1985) and Mugo et al. (2001) used population improvement approaches to develop multiple borer resistance populations. Similarly, Klenke et al. (1986) used BSSCO (Iowa Stiff Stalk Synthetic) as the base population and employed RS to develop the

European corn borer (ECB) resistant synthetic variety, BS9. Sandoya et al. (2008) also employed RS to develop Mediterranean corn borer (MCB) and ECB-resistant inbred lines. Maize weevil (*Sitophilus zeamais*) resistance breeding is also important to avoid storage losses (Zunjare et al., 2015). Garcia-Lara and Bergvinson (2014) employed 3 cycles of intrapopulation RS to increase the resistance against *S. zeamais* by three-fold. Sometimes, hybrids exhibit tolerance to insect-pests, as is evident from the tolerance of Basto/*Enano levantixo* (stem resistance) × Longfellow (positive variety effects for grain yield) heterotic pattern against *S. nonagrioides* infestation (Soengas et al., 2004).

Conventional breeding has not much progressed in developing the abiotic stress-tolerant genotype due to the variable and complex intensity of abiotic stress, linkage of undesirable genes with desirable traits, and limited gene pool barriers to transferring the desired gene from various sources (Gazal et al., 2018). Maize breeders select for the best genotypes under favorable conditions, followed by extensive multi-environment testing for different abiotic stresses; however, managed stress screening is the best approach to keep the heritability high by reducing chances of experimental errors (Bänziger et al., 2006). The abiotic stress tolerance attributing traits include anthesis-silking interval (ASI), cell membrane thermostability, leaf firing, kernels per ear, 100-grain weight, and grain yield (Choudhary et al., 2019). Low ASI helps in better synchronization of male and female flowering for better seed set under drought, waterlogging, and heat stress (Choudhary et al., 2019). Further, reduced kernel abortion under high temperatures is also a good selection trait (Rattalino & Otegui, 2012). The reproductive stages are the most sensitive stages for drought and heat stress, whereas the early seedling (V2) and knee-high (V7) stages are the most sensitive stages for waterlogging stress (Zaidi et al., 2004; Anjos e Silva et al., 2007). Zaidi et al. (2007) identified Pop 3121 and Pop 3118 as relatively better yielders under waterlogged conditions. Maize hybrids have been found to exhibit better stress tolerance than parental lines (Dass et al., 1997; Tollenaar et al., 1994; Zaidi et al., 2010). Sometimes, selection for tolerance can confer tolerance against other abiotic stresses as is evident from the performance of tropical maize selected for drought tolerance, which exhibited tolerance to low nitrogen as well (Bänziger et al., 2002). Considering the additive and nonadditive nature of most abiotic stress-associated traits, reciprocal RS selection can be employed to develop tolerant synthetics and hybrids (Zaidi et al., 2010). Although, conventional approaches resulted in significant yield improvement under drought and heat (Meseka et al., 2014), repeatability was found to be low for yields under drought stress (Meseka et al., 2018).

## *2.3 Quality Traits*

The quality trait improvement through indirect selections proved ineffective due to the lack of correlation between quality traits (biochemical) and morphological characteristics. Most of the quality traits in maize are governed by recessive genes

(Mertz et al., 1964). In the case of quality traits, the strict control over pollination and need to self after every backcross to obtain desirable segregants (homozygous state) makes breeding difficult. However, remarkable success has been achieved by conventional and molecular maize breeders in improving maize quality (Fig. 1). Maize scientists succeeded in developing quality enriched maize like lysine and tryptophan-rich quality protein maize (QPM) (Atlin et al., 2011; Prasanna et al., 2001), provitamin A-enriched orange colored maize (Pixley et al., 2013), and high-Zn-maize (Andersson et al., 2017).

QPM can be defined as nutritionally superior to normal maize with higher lysine and tryptophan contents (Maqbool et al., 2021). The concept of QPM given by Vasal and Villegas resulted in the adoption of QPM breeding and the release of QPM varieties (composites and hybrids). In Asia, conventional breeding efforts have resulted in the release of >40 QPM varieties. Initially, QPM breeding relied on phenotypic selection and biochemical analysis (Pixley et al., 2013). QPM breeding involves the tweaking of three distinct genetic systems: the simple recessive allele

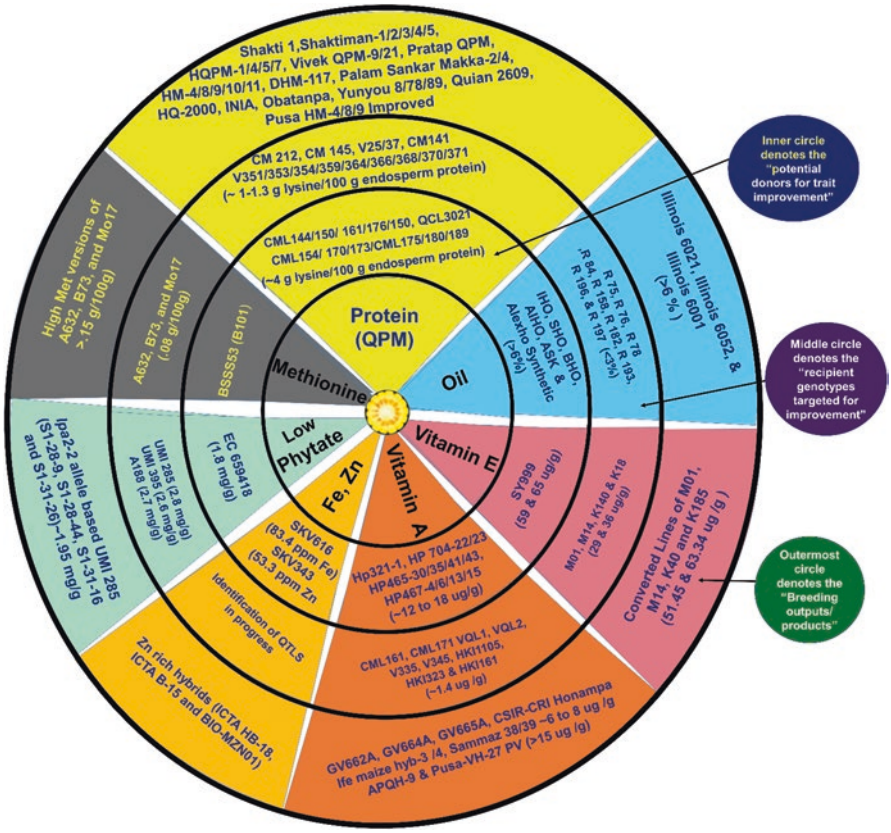



**Fig. 1** Illustration of successful examples of the utilization of conventional or molecular breeding for development of biofortified maize for different quality traits

of the *opaque2* (*o2*) gene in homozygous condition; modifiers/enhancers of the *o2o2*-containing endosperm conferring higher lysine and tryptophan; and genes modifying *opaque2*-induced soft endosperm to hard endosperm (Bjarnason & Vasal, 1992; Prasanna et al., 2001). Several commercial varieties have been developed and released for QPM in different countries through conventional breeding (Chaudhary et al., 2014). For example, India (Composites-Protina, Shakti, Ratan; Hybrids-Shaktiman 1, HQPM-1, HQPM-4, HQPM-5, and HQPM-7), Nicaragua (Composite-NB-Nutrinta; Hybrid-Hg INTA-993), Guatemala (Hybrid-HB-PROTIOTA), China (Hybrids-Zhongdan 9409, Zhongdan 385D, QUIAN2@9), Mali (Composite-Obatampa) and Uganda (Composite-Obangaina).

Yellow maize can synthesize provitamin A (PVA) carotenoids naturally, especially β-carotene (BC) and β-cryptoxanthin (BCX), but only contains <2 mg/g of provitamin A (Ortiz-Monasterio et al., 2007; Pixley et al., 2013). Therefore, a target level of 15 ppm of β-carotene equivalents was set for PVA improvement (through conventional breeding) in maize (Hotz & McClafferty, 2007; Menkir et al., 2017; Pixley et al., 2013). The profiling of carotenoids in large germplasm (tropical and temperate) led to the identification of a few temperate lines with PVA ≥15 ppm (Menkir et al., 2008; Ortiz-Monasterio et al., 2007). The high heritability and additive gene action of PVA, along with the lack of correlation between PVA and agronomic performance, indicated the possibility to improve the PVA content without any compensatory effect on yield (Menkir et al., 2018; Ortiz-Covarrubias et al., 2019; Suwarno et al., 2014). Temperate lines (with high PVA) were used to enhance the PVA content in tropical maize inbred lines at CIMMYT. Initially, BC was targeted for PVA content improvement (Muthusamy et al., 2014), but later realizing the lower stability and bioavailability of BC, the focus shifted to BCX improvement (Dhliwayo et al., 2014; Ortiz et al., 2016; Sowa et al., 2017).

Tropical maize germplasm (landraces, inbreds, hybrids, and open-pollinated varieties) exhibits enormous genetic variation for kernel-Zn (up to 96 ppm) (Bänziger & Long, 2000; Hindu et al., 2018; Ortiz-Monasterio et al., 2007). Unlike wheat and rice (Guzmán et al., 2014), maize does not have a significant correlation between Zn and Fe content, as evident from high Zn maize (which contain 18–20 ppm Fe, equivalent to the average content in maize kernels). The target level of 33 ppm kernel-Zn on a dry weight basis was set for the breeding programs initiated by IITA and CIMMYT, especially for white maize and QPM (Bouis et al., 2011). CIMMYT employed intrapopulations RS (using Population 62-white flint QPM and Population 63-white dent QPM), to develop high Zn tropical maize lines, CML176, CML491, and CML492, that have been utilized to a greater extent as parental lines in pedigree-based selection (CIMMYT, 1998). Zn rich varieties and hybrids (ICTA HB-18, ICTA B-15, and BIO-MZN01) have been released in Guatemala and Colombia with the efforts of CIMMYT collaborations. Mallikarjuna et al. (2014) identified two lines, SKV616 (83.4 ppm Fe) and SKV343 (53.3 ppm Zn) that can be utilized as potential donors in breeding high Fe and Zn rich maize. Although QPM germplasm was found to contain an above-average concentration of Zn relative to normal maize, this was not true for all QPM varieties (Chakraborti et al., 2011). The availability of Zn is also affected by the presence of phytic acid/

phytate due to the chelation of Zn by the negatively charged phytate (Raboy, 2001). The screening of maize germplasm led to the identification of low phytic acid (lpa) mutants (normal amount of total phosphorus but reduced phytic acid phosphorus) in maize (Pilu et al., 2003; Raboy et al., 2000). Mutants, lpa1–1 and lpa2–1, reduce phytic acid up to 55–65% and 50%, respectively (Raboy et al., 2000). Further efforts have been made to develop low phytate genotypes and populations by the backcross method (Ertl et al., 1998) and RS (Beavers et al., 2015).

Maize kernels generally constitutes 4–5% oil (Boyer & Hannah, 2001). Oil and protein improvement in maize is the classic example of conventional breeding, as evident from the Illinois long-term selection experiment with Burr's White Variety (Hopkins, 1899). The resultant of selection, Illinois High Oil (oil concentration >6%) lines, were also found to have higher protein concentrations, indicating a correlated response (Dudley & Lambert, 2004). Later in 1982, a similar long-term selection experiment in China resulted in the development of high-oil maize populations, such as Beijing High Oil (BHO), with an enhancement of oil content from 4.71–15.5% through cycle-0 to cycle-18 (Song et al., 1999; Song & Chen, 2004). RS (27 selection cycles) resulted in 21.2% oil in an improved population (Alexender, 1988). Similarly, Song et al. (2004) applied 18 cycles of RS to the synthetic variety Zhongzong No. 2 (4.71%) and developed high-oil maize (15.55%). However, the negative correlation of oil yield has been found to hamper the grain yield potential of high-oil lines (yield of high-oil hybrids is 5–10% lower than normal maize), as oil synthesis consumes a lot of energy (Moose et al., 2004). But, the University of Illinois succeeded in developing high oil populations IHO, SHO, DHO, ALHO, ASK, ALEX synthetic, and KYHO, through cyclic selection in high-oil lines (Hopkins, 1899) and three maize hybrids, Illinois 6021, Illinois 6052, and Illinois 6001 (30% more oil, and 10% more protein than existing commercial hybrids) without grain yield penalty (Jugenheimer, 1961).

However, conventional breeding has many limitations. First is the linkage drag, for example, the use of intrapopulation RS by Carena et al. (1998) for greater prolificacy and Hallauer et al. (2004) for increased ear lengths resulted in decreased grain yields. Similarly, with interpopulation RS, it is difficult to decide on an optimum number of individuals for intermating to maintain genetic variation for continued response to selection (Rawlings, 1970). In addition, the conventional breeding approach is relatively constrained by the cost, time involved, and throughput of phenotyping for quality traits.

## 3 Molecular Breeding

The limitations of conventional breeding can be addressed through the utilization of molecular markers. Molecular breeding is molecular marker-based rapid crop improvement. QTL mapping is the first step of molecular breeding and provides the major and minor QTLs. The next step in molecular breeding is to introgress the major QTLs. Several QTLs have been reported during the last decade for different

traits in maize. Molecular breeding relies on the availability of major QTLs for target traits. In this section, the information on the mapped QTLs for different traits will be discussed, followed by the success studies of trait improvement via introgression of major QTLs.

## *3.1 QTL Mapping for Different Traits*

QTL mapping is carried out using the mapping population derived from parents that are contrasting for target traits. This helps to map the genomic regions governing the target traits through the establishment of linkage using molecular markers.

### 3.1.1 Grain Yield

Yield improvement is the primary target of improvement in any crop, however, it is a complex trait, highly influenced by the environment. Yield can be improved by increasing the grain weight and grain number or kernel row number (KRN) per ear. Lu et al. (2011) used $F_{2:3}$ families (Ye478 × Dan340) to map a consistent major QTL for KRN. Later, a major QTL, *KRN4,* was mapped on chromosome 4 using linkage and association mapping (Brown et al., 2011) and later, cloned by Liu et al. (2015). Yang et al. (2015) mapped three consistent QTLs (*qERN2–1*, *qERN8–1*, and *qERN8–2*) using B73 and SICAU1212-based RILs. Chen et al. (2016) identified two QTL for kernel length (*qKL3–1* and *qKL7–1*) and one QTL for kernel width (*qKW5–1*), which can be utilized to increase the kernel size. Calderón et al. (2016) used W22 and teosinte (*Zea mays* ssp. *parviglumis*)-based population to map a major QTL with ~50% phenotypic variance. Later, Liu et al., 2016 used abe2- and B73-based $F_2$ populations to map four QTLs, namely, *qKRN1*, *qKRN2, qKRN5*, and *qKRN8–1*. In the recent past, Wang et al. (2019) used a Brazilian landrace and tropical inbred line-based population to map a major QTL, *KRN1*.

### 3.1.2 Biotic and Abiotic Stress

Disease resistance in maize is qualitative (R gene and R gene analogs (RGA)) and quantitative in nature. The qualitative nature was easy to explore and impart biotic stress resistance through conventional breeding; however, identification and better exploitation of QTL required the use of molecular breeding. In maize, over 50 R genes and 228 RGA have been identified (Wisser et al., 2006). Some of the important genes like *Hm1*, *Htn1*, *Pan1*, *qMCMV3–108*/*qMLN3–108* have been well characterized in maize (Table 1). Balint-Kurti et al. (2007) mapped a major QTL for MLSB (*Cochliobolus heterostrophus* race O) in B73- and Mo17-based RILs. Later, a major QTL, *Ht2,* was mapped on chromosome 8 for NCLB resistance in maize (Poland et al., 2011). For GLS resistance, Zhang et al. (2012) mapped *qRgls1*

**Table 1** List of some major disease resistance genes in maize

| Gene name | Disease | Predicted features | Gene identification method | Chromosomal Location | References |
|---|---|---|---|---|---|
| *Hm1* | Maize leaf blight and ear mold | NADPH-dependent HC-toxin reductase | Transposon tagging | 1 | Johal and Briggs (1992) |
| *Rp1-D* | Common rust | NB-LRR | Transposon tagging | 10 | Collins et al. (1999) |
| *Htn1* | Northern leaf blight | Wall-associated receptor-like protein | Fine-mapping followed by analysis of mutants generated using TILLING | 8 | Hurni et al. (2015) |
| *pan1* | Northern leaf blight and Stewart's wilt | Receptor-like kinase | Fine-mapping and mutant analysis | 1 | Jamann et al. (2014) |
| *GST* | Northern leaf blight, southern leaf blight, and grey leaf spot | Glutathione *S*-transferase | Association analysis | 7 | Wisser et al. (2011) |
| *qMCMV3–108/ qMLN3–108* | Maize lethal necrosis virus | | Genome-wide association study | 3 | Sitonik et al. (2019) |

(chromosome 8) and *qRgls2* (chromosome 5) in Q11 × Y32-based $F_{2:3}$ population, whereas Zhang et al. (2017) mapped *Qgls8* on chromosome 8. Liu et al. (2016) also mapped a major QTL, *qMrdd8* on chromosome 8 for maize rough dwarf disease resistance. Hence, chromosome 8 can be a putative chromosome for multiple disease resistance in maize (Table 2).

For insect resistance in maize, Bohn et al. (2000) first mapped six and five QTLs for tunnel length and stalk damage resistance against the ECB in the D06 and D408 derived $F_3$ population. Later, Cardinal et al. (2001) and Krakowsky et al. (2004) mapped nine and 10 QTLs for ECB stalk tunneling (resistance) using B73 × B52 and B73× De8-based RIL populations, respectively. A total of 21 QTLs for maize weevil resistance were identified using CML290- and Muneng-8128C0HC1-18-2-1-1-based $F_{2:3}$ population (García-Lara et al., 2009). Meihls et al. (2013) mapped a major QTL, *HDMBOAGlc,* for corn leaf aphid resistance on chromosome 1 in B73- and CML322-based RILs. Later, Betsiashvili et al. (2015) also mapped a major QTL for corn leaf aphid resistance using B73 × Mo17-based RILs. Hessel (2014) and Bohn et al. (2018) mapped major QTLs for western corn rootworms on different chromosomes. The list of mapped QTLs on resistance against different insect pests is provided in Table 2. For detailed insights for genetic resistance and transgenic approaches for insect resistance breeding, an excellent article by Karjagi et al. (2017) can be referred.

**Table 2** QTLs identified for traits associated with biotic and abiotic stress tolerance in maize

| QTL/Loci | Mapping Population | Cross(s) | Marker | Chromosomal location | Stress | References |
|---|---|---|---|---|---|---|
| *qRfg1* and *qRfg2* | $F_2$, $BC_4F_2$ and DH lines | Y331 × 1145 | SSR | 10,1 | *Gibberella* stalk rot | Yang et al. (2010) |
| *Qgls8* | $F_{2:3}$ and $F_4$ | Teosinte×B73 | SNP/InDel and SSR | 8 | Grey leaf spot | Zhang et al. (2017) |
| *qRgls1* and *qRgls2* | $F_{2:3}$ | Q11 × Y32 | SSR | 5,8 | Grey leaf spot | Zhang et al. (2012) |
| *Ht2* | NAM RILs | | SNPs | 8 | Northern corn leaf blight | Poland et al. (2011) |
| *qMrdd8* | $F_2$ | B73 × NL203 | SSR and Indels | 8 | Maize rough dwarf disease | Liu et al. (2016) |
| 62 SNP-trait associations | Mapping panel | 341 tropical maize lines | DArTseq markers | All | Maize weevil and FAW | Badji et al. (2020) |
| 4 major QTLs for root damage, regrowth, and root size traits | RIL | IBM-Intermated B73 × Mo17 | SSR and SNP (*umc1395*, *umc1321*; *bnlg1598*, *umc1123*; *csu3*, *mmp61*; *bnlg1867*, *mmp13*) | 1 and 6 | Western corn rootworm | Brkić et al. (2020) |
| 4 and 3 putative QTLs for root damage, regrowth, and root size | DHLs | (NGSDCRW1 × AG1 and LH51 × CRW8–1) topcrossed with PHZ51 | SNP | 7, 9, 10, and 6, 8, respectively | Western corn rootworm | Bohn et al. (2018) |
| 6 (3 for tunnel length, 1 each for kernel resistance, stalk damage, and yield) | RIL | A637 × A509 | SNP | 5, 8, 9, and 10 | Mediterranean corn borer | Jiménez-Galindo et al. (2017) |

(continued)

**Table 2** (continued)

| QTL/Loci | Mapping Population | Cross(s) | Marker | Chromosomal location | Stress | References |
|---|---|---|---|---|---|---|
| 8 QTLs for resistance traits like tunnel length, stalk damage, stalk lodging, kernel resistance, and grain yield | RILs | B73 × CML103 | SNP (*30460922-73132746, 30460922-73132746, 9498146-88522572)* | 1, 5, and 6 | Mediterranean corn borer | Samayoa et al. (2015) |
| *Aphid resistance QTL* | RILs | B73 × Mo17 | AC213878 and AC204415 | 4 | Corn leaf aphid | Betsiashvili et al. (2015) |
| 15 QTLs for resistance traits like grain weight losses, adult progeny, and flour production | RILs | Population 84 × Kilima | SSR (*bnlg1909-umc1884*, *bnlg1909-umc1884*, *Phi094-umc2189*) | 1, 2, 3, 4, 8, and 10 | Maize weevil | Castro–Álvarez et al. (2015) |
| *c3 NI (q03.165)* | $F_2$, BC, and DH | FS8B × B86 UR2 × Mo47 | SNP (*MAGI_14202* and *MAGI_72398*) | 3 | Western corn rootworm | Hessel ( 2014) |
| *HDMBOAGlc QTL* | RILs | B73 × CML322 | *PZA03189.4* and *PMH5098.25* | 1 | Corn leaf aphid | Meihls et al. (2013) |
| 17 QTLs for leaf chlorophyll, senescence, and electric root capacitance | RIL | CML444 × SC-Malawi | SSR | 1, 2, 4, 5, 6, and 10 | Drought | Messmer et al. (2011) |
| 22 QTLs for root architecture associated traits, sugar concentration, biomass, relative water content, leaf ABA | $F_{2:3}$ | DTP79 × B73 | RFLP | 1, 3, 5, 6, 7, and 9 | Drought | Rahman et al. (2011) |
| 25 QTLs for ASI, plant height, grain yield, ear height, and ear setting | $F_{2:3}$ | D5 × 7924 | SSR | 1,2,3,4,6,8,9, and 10 | Drought | Zhu et al. (2011) |

| | | | | | | |
|---|---|---|---|---|---|---|
| 9 QTLs for leaf temperature and seedling dry matter | RILs | Zong3 × 87–1 | SSR | 1,2,9, and10 | Drought | Liu et al. (2011) |
| 64 QTLs for grain yield associated traits, relative water content, osmotic potential, and relative sugar content | $F_{2:3}$ | DTP79 × B73 | RFLP, SSR, AFLP | 1, 2, 3, 4, 5, 7, 8, and 10 | Drought | Nikolic et al. (2012) |
| 45 QTLs for grain yield per plant and yield components | $F_{2:3}$ | B73 × DTP79 | SSR | 1, 2, 3, 4, 5, 6, 7, 8, and 10 | Drought | Nikolic et al. (2013) |
| 83 QTLs for grain yield and 62 QTLs for ASI | RILs<br>$F_{2:3}$<br>$F_{2:3}$ | CML44 × MALAWI<br>CML440 × CML504<br>CML444 × CML441 | SNP | 1,2,3,4,5,6,7,8, and 10 | Drought | Almeida et al. (2013) |
| 15 QTLs for root associated traits | RILs | | SNP | 1,2,3,4,5,7,8, and 9 | Drought | Burton et al. (2014) |
| 17 QTLs for stomatal conductance, ASI, grain yield, and leaf relative water content | $BC_1F_{2:3}$ | DTPWC9F1 × LPSC7F64 | SNP | 1,2,3,4,6,7,9, and 10 | Drought | Trachsel et al. (2016) |
| 169 QTLs for grain yield, ear length, ear weight, kernel number per row, and hundred kernel weight | Nested association mapping panel | 11 parents | SNP | 1, 3, and 10 | Drought | Li et al. (2016) |

Almeida et al. (2013) mapped six constitutively expressed meta-QTLs on chromosomes 1, 4, 5, and 10 for grain yield under well-watered and drought conditions. Two mQTLs, one on chromosome 7 for GY and another on chromosome 3 for ASI, were found to be "adaptive" to drought conditions. Later, Almeida et al. (2014) identified 203 QTLs for different drought-related morpho-physiological traits. Li et al. (2016) conducted SNP markers-based association mapping and identified 354 candidate genes, of which 52 genes exhibited significant differential expression in B73 under optimum and drought stress (Li et al., 2016). Zaidi et al. (2015) mapped 18 QTLs in RILs derived from a waterlogging-tolerant line (CAWL-46-3-1) and a sensitive line (CML311-2-1-3) for brace roots, chlorophyll content, % stem, and root lodging. Allam et al. (2016) mapped 27 QTLs on different chromosomes for germination and early growth under field conditions in two RILs derived from independent crosses, B73 × P39 and B73 × IL14h. Hoque et al. (2015) mapped 15 QTLs for salt tolerance associated traits in B73- and CZ-7-based $F_{2:3}$ population.

### 3.1.3 Quality Traits

The molecular markers have been most successful in mapping the key quality traits associated with genes in maize. For example, protein quality (high lysine content) associated genes, namely, *o2* (gene-specific markers: *umc1066*, *Phi057,* and *Phi112*) and *o16,* were mapped using molecular markers by Babu et al. (2005) and Yang et al. (2005), respectively. The markers for *o16-umc1141* and *umc1149* were mapped in the $F_2$ mapping population derived from a cross of QCL3024 (o16) and QCL3010 (wild type). Later, Liu et al. (2016) developed the functional marker *qg27* for endosperm modification. The quality traits have been well explored for the identification of key genes involved in the pathways of particular traits like carotenoids biosynthesis pathways. The provitamin A biosynthesis pathway has been well-characterized, and markers have been developed for the favorable alleles. The main two genes contributing to high levels of provitamin A are *β-carotene hydroxylase1* (*crtRB1*) and lycopene epsilon cyclase (*lcyE*) (Harjes et al., 2008; Yan et al., 2010). The favorable allele of the former gene (crtRB1–3′TE) was found to be more effective than the later (Babu et al., 2013). Similarly, the vitamin-E biosynthesis pathway is also well-characterized (DellaPenna & Pogson, 2006). Several earlier studies (Diepenbrock et al., 2017; Feng et al., 2013; Lipka et al., 2013; Shutu et al., 2012) reported QTLs for higher levels of tocopherols in maize. Among the major genes for tocopherol biosynthesis such as *homogentisate phytyltransferase* (*VTE2*), *homogentisate geranylgeranyl transferase* (*HGGT*), *methyltransferase* (*VTE3*), *tocopherol cyclase* (*VTE1*), *phytol kinase* (*VTE5*), and *γ-tocopherol methyltransferase* (*VTE4*), *VTE4* was identified as most important for enhancing the α-tocopherol content (Li et al., 2012; Lipka et al., 2013). The markers (SNPs and InDels) have already been developed for the *VTE4* that facilitates the selection of desirable genotypes. CIMMYT is focused on developing a low-cost trait-specific-SNP marker

panel (automated high-throughput assay) for the selection of favorable *crtRB1–3′TE* and *MSV1* in sub-Saharan African maize breeding programs (Nair et al., 2015).

Extensive efforts have been made in the last decade to map the regions for Fe and Zn concentrations through QTL mapping in maize. Qin et al. (2012) mapped three stable QTLs for kernel-Zn concentration in multiple populations and different environments. Similarly, Šimić et al. (2012) mapped two minor QTLs on chromosomes 3 and 4. Lunga'ho et al. (2011) mapped 3 and 10 QTLs for kernel Fe concentration and bioavailability, respectively, on three different chromosomes. Later, Jin et al. (2013) executed a meta-QTL approach to identify nine meta-QTLs across the different chromosomes with a potential role in enhancing Zn content. Hindu et al. (2018) first conducted a multilocation GWAS study using a panel of 923 CIMMYT inbreds and identified 20 SNPs to be associated with kernel-Zn. Of these, 11 SNPs were found to be confirmed in independent biparental populations. These SNPs have also been utilized for developing SNP haplotypes for high Zn content.

Many QTLs have been identified for oil synthesis and content in maize (Table 3). Berke and Rocheford (1995) first used 80 markers in an IHO × ILO-based population to detect 16, 31, and 28 markers for association with protein, oil, and starch concentrations, respectively. Later, Song et al. (2004) mapped 20 QTLs for oil concentration in By804 × B73-based $F_2$ and $F_3$ populations. Similarly, Laurie et al. (2004) mapped over 50 QTLs for oil content in IHO and ILO-based populations using SNP markers. Several QTLs have been mapped for oil and protein content in the recent past (Li et al., 2012; Yang et al., 2012; Yang et al., 2014; Yang et al., 2016).

## *3.2 Molecular Breeding-Based Trait Improvement: Achievements*

The effectiveness of molecular breeding over conventional breeding is well documented. Abalo et al. (2009) observed superior resistance (79%) for MSV with MAS than conventional breeding (62%). Samayoa et al. (2015) and Foiada et al. (2015) also reported MAS as a feasible strategy for imparting insect resistance without any yield penalty. As per recent updates, breeding programs at CIMMYT and IITA are already undertaking marker-assisted backcrossing (MABC) and forward breeding to develop high-yielding MLN resistance hybrids (Prasanna et al., 2020b). Zhao et al. (2012) introgressed the head smut resistance QTL, *qHSR1* (*ZmWAK* gene), through MABC into 10 diverse inbred lines that exhibited improved head smut resistance and yield (Zuo et al., 2015). The introgression of ZmWAK into the Chinese maize line Tongsipingtou led to the development of the head smut resistant variety, Jidan558. Marker-assisted gene pyramiding of two genes, *Scmv1* and *Scmv2,* into the background of the maize line, F7 resulted in a completely tolerant line (nearly isogenic line) against the sugarcane mosaic virus (Xing et al., 2006). Yang et al. (2017) also developed a multiple disease resistant line (against SCLB and GLS) by introgression of *qMdr9.02*. Genomic selection (GS) for MLN

**Table 3** List of QTLs identified for nutritional quality associated traits in maize

| QTL/Gene | Mapping population (size) | Parentage | Linked markers | Chromosomal location | PVE (%) | References |
|---|---|---|---|---|---|---|
| 21 QTLs for kernel oil (10) and protein concentration (11) | $F_{2:4}$ | Zheng58 × B73 | *umc1904- phi100175*<br>*umc1272- bnlg1839*<br>*umc1019-umc2038*<br>*umc219-umc2243* | 1, 2, 3, 4, 5, 6, 7, 8, and 10 | 4.6 to 11.1 (oil)<br>4.2 to 11.4 (protein) | Yang et al. (2016) |
| 16 single-population and 19 joint-population QTLs for protein and 21 QTLs for protein-oil | RILs<br>RILs | GY220 × 8984<br>GY220 × 8622 | *umc2075-bnlg2046*<br>*bnlg1325-bnlg1523* | 3, 5, 6, 7, 8, and 9 | 4.4–13.4 | Yang et al. (2014) |
| 12 and 14 QTLs for oil in two populations, respectively | RILs<br>RILs | GY220 × 8984<br>GY220 × 8622 | *umc2316–umc1979* | 1, 3, 4, 5, 6, 8, and 10 | 3.93–14.47 | Yang et al. (2012) |
| 74 SNP-trait associations for oil biosynthesis | Regular and high-oil lines | Diverse panel | *SNPG/T*<br>*Indel_8*<br>*Indel_146/472*<br>*Indel_2000*<br>*Indel_20* | 1,2,4,6,8, and 9 | 83 (total of 26 loci for high oil) | Li et al. (2012) |
| 58 QTLs (kernel oil content, embryo quality-related traits) | RILs | By804 × B73 | *Q8–umc1979* | 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 | 1.1–20.5 | Yang et al. (2012) |
| 3 major QTLs for carotenoids (β-carotene, β-cryptoxanthin) | $F_{2:3}$<br>Testcross families | W64a × A632<br>F2:3 families×AE335 | *y1SSR–bnlg249*<br>*zdsRFLP-phi034*<br>*bmc1176-bmc1599* | 6, 7, and 8 | 11.8–25.4 | Wong et al. (2003) |
| 31 QTLs for carotenoids | RILs | By804 × B73 | *YIssr–umc1595*<br>*umc2313–YIssr* | 1, 3, 5, 6, 7, 8, and 10 | 6.6–27.2 | Chander et al. (2008) |
| 30 QTLs with 3 major QTLs (qd1–1, qc5–1/qd5–1, and qc5–2) | $F_{2:4}$<br>$F_{2:4}$ | K22 × CI7<br>K22 × Dan340 | *PZA02117.1-PHM4926.16*<br>*PZA00352.23-PZA02060.1* | 1 and 5 | 53 (αT) 30 (αT)<br>25 (αT) | Shutu et al. (2012) |

| | | | | | | |
|---|---|---|---|---|---|---|
| Major QTL *ZmVTE4* (α-tocopherol variation)<br>2 inDels and 1 SNP | Association panel | Diverse panel | *InDel7, InDel118, SNP25815 bnlg1237 & phi085* | 5 | 33 | Li et al. (2012) |
| Major QTL *ZmVTE2* | $F_{2:3}$ | N6 × NC296 | SNPs | 9 | 22 | Fenton et al. (2018) |
| 27 QTLs for different minerals | RILs | B73 × Mo17 | *mmp 144, rz87 ay110452, ay110625, and mmp 125* | 9 and 10 | 4–46 | Baxter et al. (2013) |
| 3 and 10 QTLS for grain Fe concentration and bioavailability, respectively | RILs | B73 × Mo17 | *mmp 144, rz87, and sh1 (Fe content) psr754b, php20528, and umc 2134* | 3, 6, and 9 | 26 and 54, respectively | Lunga'ho et al. (2011) |
| 3 co-localized QTLs for Fe, Zn, Mg, and P | $F_4$ | B84 × OS6–2 | *bnlg1456* | 3 | 24.10 (Fe) and 22.40 (Zn) | Šimić et al. (2012) |

disease severity and MLN area under the disease progress curve exhibited prediction accuracy in the range of 0.46–0.86 and 0.46–0.87, respectively (Sitonik et al., 2019). Similarly, Holland et al. (2020) obtained moderate-to-high prediction accuracy for *Fusarium* ear rot (0.46), and fumonisin (0.67). Marker-assisted recurrent selection for grain yield improvement under well-watered and drought-stress conditions in biparental mapping populations resulted in higher grain yield than traditional breeding alone, with greater differences observed under drought stress (Beyene et al., 2016). Similarly, GS also exhibited superiority over traditional breeding through comparatively higher yield gains (two to four folds) in drought-stress environments (Beyene et al., 2015). Zhang et al. (2015) also employed the GS to reveal low to medium prediction accuracy under drought stress, advocating the use of high-density SNP markers for better prediction accuracy of complex traits.

Quality traits, being majorly governed by major genes in biosynthesis pathways, have witnessed greater success in quality improvement via MABC or marker-assisted gene pyramiding (Table 4). For quality traits, introgression of o2 was carried out through MABC to develop India's first MAS-based single-cross QPM hybrid, "Vivek QPM-9" in 2008, which had 41% more tryptophan and 30% more lysine than the original hybrid, Vivek Hybrid-9 (Gupta et al., 2012). Later, Hossain et al. (2018) also developed lysine- and tryptophan-rich "Pusa HM-8 Improved," "Pusa HM-4 Improved," and "Pusa HM-9 Improved" by introgressing the *o2* allele into the respective parental genes. Interestingly, these hybrids (with a flint background), did not exhibit any yield penalty. Similarly, the inbreds introgressed with the *o2* allele at CIMMYT, namely, CML244Q, CML246Q, CML349Q, and CML354Q, did not exhibit any yield penalty (Hossain et al., 2019). MABC has been utilized to a large extent to develop diverse QPM inbred lines (Jiang et al., 2005; Tian et al., 2004). Later after the discovery of *o16*, Zhang et al. (2010) pyramided *o2* and *o16* to develop double mutant inbred (*o2o2*/*o16o16*) with 23% more lysine than *o2o2* inbred alone. Similarly, *o2* and *o16* were pyramided by Zhang et al. (2013) in a waxy genetic background, and higher accumulation of lysine (0.616% in flour) was reported in the pyramided lines. Yang et al. (2013) introgressed *o16* in waxy inbreds to develop *o16o16*-based waxy inbreds with 16–28% higher lysine content than parental lines. Later, Sarika et al. (2018) transferred *o16* through MABC into the parental lines of four popular QPM hybrids (HQPM-1, HQPM-4, HQPM-5, and HQPM-7). Reconstituted hybrids exhibited an average increase of 49 and 60% for lysine and tryptophan, respectively, over the original hybrids.

MABC has also been successfully utilized for the introgression of favorable alleles for carotenoid synthesis. Muthusamy et al. (2014) developed India's first provitamin A maize hybrid, Pusa Vivek QPM9 Improved (APQH9), with provitamin A ~8.15ppm through introgression of crtRB1 in the parental lines of Vivek QPM 9. Recently, Maqbool et al. (2021) introgressed the crtRB1 gene from UMI285β + into the NEHR maize landrace, Yairipok Chujak (CAUM66) to develop four β-carotene-rich maize lines (7.5–8.7 ppm), i.e., CAUM66-54-9-12-2, CAUM66-54-9-12-11, CAUM66-54-9-12–13, and CAUM66-54-9-12–24. Later, Zunjare et al. (2018) pyramided two favorable alleles, *crtRB1* and *lcyE,* to develop

**Table 4** Details of biofortified varieties developed through molecular breeding in maize

| Hybrids/inbreds | Donor parent | Recipient parent | Approach | Target genes | Trait | Value improved | Country | References |
|---|---|---|---|---|---|---|---|---|
| Vivek QPM-9 (hybrid) | CML170/CML180 (tryptophan-0.9%) | CM145/CM212 (0.42–0.55%) | Marker-assisted backcrossing-(MABC) | *opaque2* | Lysine and tryptophan | 41% more tryptophan and 30% more lysine | India | Gupta et al. (2012) |
| Improved lines S1-28-9, S1-28-44, S1-31-16 and S1-31-26 | EC 659418 (1.8 mg/g) | UMI 285 (2.8 mg/g) | *MABC* | *lpa2–2* | Low phytic acid | 1.93 mg/g | India | Tamilkumar et al. (2014) |
| Improved line UMI395 | EC 659418 (1.8 mg/g) | UMI 285 (2.8 mg/g) | *MABC)* | *lpa2–2* | Low phytic acid | 30% reduction | India | Sureshkumar et al. (2014) |
| Improved lines M01, M14, K140 and K185 | SY999 | M01, M14, K140 and K185 (γ-tocopherol 29.11 and total tocopherols 36.63 lg/g) | *MABC* | *VTE4* | Vitamin E (tocopherols) | 51.45 and 63.34 μg/g | China | Feng et al. (2015) |
| Hybrids (HQPM1, HQPM4, HQPM5, and HQPM7) | *HP704–22 and HP704–23* (ProA *1.65–2.04 μg/g and lysine: 0.340% tryptophan 0.083%)* | *HKI161, HKI163, HKI193–1, and HKI193–2* | *Marker-assisted gene pyramiding (MAGP)* | *crtRB1, lcyE & opaque2* | ProA | proA (9.25–12.88 μg/g), and lysine (0.334%), and tryptophan (0.080%) | India | Zunjare et al. (2018) |
| Improved lines CML161 and CML171 | *Hp321–1* | *CML161* (1.60 $\mu g.g^{-1}$) *and CML171* (*1.80 μg* $g^{-1}$) | *MABC* | *crtRB1* | ProA | 5.25 μg $g^{-1}$ and 8.14 μg $g^{-1}$ | China | Liu et al. (2015) |

(continued)

**Table 4** (continued)

| Hybrids/inbreds | Donor parent | Recipient parent | Approach | Target genes | Trait | Value improved | Country | References |
|---|---|---|---|---|---|---|---|---|
| Hybrids-improved Vivek QPM-9 (APQH9), and Vivek Hybrid-27 | *HP465–43, HP465–41, HP465–35, HP467–6, HP-467-4, and HP467–13* | VQL1, VQL2, V335, V345, HKI1105, HKI323, and HKI161 (*1.4 µg/g)* | *MABC* | *crtRB1* | ProA | 8.6–17.5 µg/g inbred and up to 21.7 µg/g in hybrids | India | Muthusamy et al. (2014) |
| Improved inbred lines | TAIXI19 (*o2o2,* 0.43%) and QCL3021 (*o16o16,* 0.32%) | Waxy line QCL5019 (0.28%) | *MAGP* | *Opaque-2 and opaque-16* | Lysine and tryptophan | 0.38–0.62% lysine in improved inbreds | China | Zhang et al. (2013) |
| Improved inbreds lysine (0.41–0.56%) and tryptophan (0.09–0.14%) and reconstituted hybrids | QCL3024 lysine (0.247%) and tryptophan (0.072%) | HKI161, HKI193–1, HKI193–2, and HKI163 (lysine: 0.30–0.35%, tryptophan: 0.06–0.08%) | *MAGP* | *Opaque-2 and opaque-16* | Lysine and tryptophan | Lysine (0.47–0.54%) and tryptophan (0.12–0.14%) in reconstituted hybrids | India | Sarika et al. (2018) |
| Improved inbred lines | *o16* line QCL3024 (0.35% lysine and 60.7% amylopectin and waxy lines, QCL5019 (0.29% lysine and 97.9% amylopectin); and QCL5008 (0.29% lysine and amylopectin, 97.6%) | QCL5008 | *MAGP* | *Wx and opaque16* | Amylopectin and lysine and tryptophan | Increased lysine (16–27%) and amylopectin (18–28%) | China | Yang et al. (2013) |
| Improved inbred lines | QCL3021 | Taixi 19 | *MABC* | *opaque16* | Lysine and tryptophan | 0.47%- 0.60% lysine | China | Zhang et al. (2010) |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Improved inbred lines | CML 144 (0.100% tryptophan and 4.00% lysine) and CML 168 (0.099% tryptophan and 4.00% lysine) | UPYL 101 (yellow) and SMWL 10–8 (white) | *MABC* | *opaque2* | Lysine and tryptophan | 1.0% tryptophan and 4% lysine | Philippines | Magulama and Sales (2009) |
| NILs | CML 144 (0.093%) | ZPL 3 and ZPL 5 (~0.052) | *MABC* | *opaque2* | Lysine and tryptophan | 30% higher tryptophan | Serbia | Kostadinovic et al. (2014) |
| Hybrids (HM4, HM8, and HM9) | HKI161 CML161 HKI193–1 lysine (3.32–3.80% in protein) and tryptophan (0.74–0.85% in protein) | HKI323, HKI1105, and HKI1128 lysine (1.76–2.08% in protein) and tryptophan (0.35–0.52% in protein) | *MABC* | *opaque2* | Lysine and tryptophan | Lysine (48–74%) and tryptophan (55–100%) | India | Hossain et al. (2018) |

provitamin A enriched versions of QPM hybrids, HQPM-1, HQPM-4, HQPM-5, and HQPM-7. For provitamin E enrichment in maize, Feng et al. (2015) first employed MABC and introgressed the favorable allele of *VTE4* from SY999 to four Chinese *shrunken2*-based sweet corn lines (M01, M14, K140, and K185) to develop α-tocopherol rich lines (up to 15.99 ppm). Similarly, Das et al. (2019) also used the MABC approach to develop α-tocopherol rich hybrids like MHVTE-2, MHVTE-18, MHVTE-28, MHVTE-10, and MHVTE-3 (possessed ≥50% α-tocopherol of the total tocopherol). Furthermore, *VTE4* has also been introgressed into provitamin-A-rich QPM elite inbreds to develop multi-nutrient maize hybrids (Prasanna et al., 2020a).

To reduce the phytic acid content for better bioavailability of Zn, the *lpa2–2* allele has been introgressed through MABC into inbreds, viz., UMI-285 and UMI-395 (Sureshkumar et al., 2014; TamilKumar et al., 2014). Furthermore, efforts have also been made to stack *lpa1–1* and *lpa2–1* with high provitamin-A and QPM traits (Bhatt et al., 2018). Hindu et al. (2018) conducted GWAS using 923 lines and identified 31 to be of QPM type, of which 33.3% were Zn rich (>30 μg /g). Of the remaining 892 non-QPM lines, 19.9% were found to be Zn rich (>30 μg /g). Of these identified high Zn lines, only 6% met the breeding target for Zn (33 μg /g). Genomic selection is advocated as an effective approach to achieve better genetic gains for complex traits (Cao et al., 2017; Yuan et al., 2019). Guo et al. (2020) studied the genomic prediction accuracy for kernel Zn content in maize and reported low to moderate prediction accuracy across different populations. Furthermore, the study revealed the superiority of genomic selection over MAS in predicting the kernel Zn content.

## 4 Conclusion and Future Perspectives

Conventional breeding approaches contributed immensely to maize improvement for all traits, especially yield as it is the prime target of the maize breeding program. However, considering the huge time and resource investment in the conventional breeding approach and the increasing affordability of molecular markers, breeders gradually started to shift to molecular breeding. In addition, the complex nature of abiotic and biotic stresses also made breeders integrate conventional breeding approaches with genomics to give rise to genomics-assisted breeding. Genomics-assisted breeding helped to map major QTLs for yield, quality, and biotic and abiotic stresses. Hence, genomics-assisted breeding offers an excellent opportunity to speed up maize improvement programs globally. However, conventional breeding is still and will surely be an integral part of any crop breeding program. For example, CIMMYT and IITA have employed conventional breeding and molecular tools with national partners in developing countries to develop and release a number of maize varieties for different ecologies. Furthermore, the availability and use of phenomics approaches, genome editing approaches, and bioinformatics will further augment the pace of molecular breeding programs in maize (Choudhary et al., 2019;

Choudhary et al., 2020; Kumar et al., 2020; Kumar et al., 2022). The future is also likely to witness the better understanding and exploitation of stress memory genes for developing multiple stress resilient maize hybrids (Choudhary et al., 2021). Although, maize witnessed remarkable success in quality improvement, the adoption of multi-nutrient rich maize can become big success with the support of better extension approaches and the better formulation and execution of policies ensuring the profitability of the farmers.

## References


Abalo, G., Pangirayi, T., John, D., & Richard, E. (2009). A comparative analysis of conventional and marker-assisted selection methods in breeding maize streak virus resistance in maize. *Crop Science, 49*(2), 509–520. https://doi.org/10.2135/cropsci2008.03.0162

Alexender, D. E. (1988). Breeding special nutritional and industrial types. In G. F. Sprague & J. W. Dudley (Eds.), *Corn and corn improvement* (3rd ed., pp. 869–880). Crop Science Society of America.

Allam, M., Revilla, P., Djemel, A., et al. (2016). Identification of QTLs involved in cold tolerance in sweet x field corn. *Euphytica, 208*, 353. https://doi.org/10.1007/s10681-015-1609-7

Almeida, G. D., Makumbi, D., Magorokosho, C., et al. (2013). QTL mapping in three tropical maize populations reveals a set of constitutive and adaptive genomic regions for drought tolerance. *Theoretical and Applied Genetics, 126*, 583. https://doi.org/10.1007/s00122-012-2003-7

Almeida, G. D., Nair, S., Bore'm, A., Cairns, J., Trachsel, S., Ribaut, J. M, et al. (2014). Molecular mapping across three populations reveals a QTL hotspot region on chromosome 3 for secondary traits associated with drought tolerance in tropical maize. *Molecular Breeding, 34*, 701–715.

Andersson, M. S., Saltzman, A., Virk, P. S., & Pfeiffer, W. H. (2017). Progress update: Crop development of biofortified staple food crops under Harvestplus. *African Journal of Food Agriculture Nutrition and Development, 17*(2), 11905–11935. https://doi.org/10.18697/ajfand.78.HarvestPlus05

Anjos e Silva, S. D., Maria, J., Claudia, F. L., Antonio, C. O., & Jose, F. (2007). Inheritance of tolerance to flooded soils in maize. *Crop Breeding Applied Biotechnology, 7*, 165–172.

Atlin, G. N., Palacios, N., Babu, R., Das, B., Twumasi-Afriyie, S., Friesen, D. K., et al. (2011). Quality protein maize: Progress and prospects. *Plant Breeding Reviews, 34*, 83–130. https://doi.org/10.1002/9780470880579.ch3

Babu, R., Nair, S. K., Kumar, A., et al. (2005). Two-generation marker-aided backcrossing for rapid conversion of normal maize lines to quality protein maize (QPM). *Theoretical and Applied Genetics, 111*, 888–897.

Babu, R., Rojas, N. P., Gao, S., Yan, J., & Pixley, K. (2013). Validation of the effects of molecular marker polymorphisms in *LcyE* and *CrtRB1* on provitamin A concentration for 26 tropical maize populations. *Theoretical and Applied Genetics, 126*, 389–399. https://doi.org/10.1007/s00122-012-1987-3

Badji, A., Machida, L., Kwemoi, D., Kumi, F., Okii, D., Mwila, N., Ibanda PA, S. A., Bararyenya, A., Nghituwamhata, S., Odong, T., Wasswa, P., Ochwo-Ssemakula, M., Talwana, H., Otim, M., Kyamanywa, S., & Rubaihayo, P. (2020). *Genomic prediction of tropical maize resistance to fall armyworm and weevils: Genomic selection should focus on effective training set determination*. https://doi.org/10.20944/preprints202007.0336.v1

Badu-Apraku, B., Talabi, A. O., Ifie, B. E., Chabi, Y. C., Obeng-Antwi, K., Haruna, A., et al. (2018). Gains in grain yield of extra-early maize during three breeding periods under drought and rainfed conditions. *Crop Science, 58*, 2399–2412. https://doi.org/10.2135/cropsci2018.03.0168

Balint-Kurti, P. J., Zwonitzer, J. C., Wisser, R. J., Carson, M. L., Oropeza-Rosas, M. A., Holland, J. B., & Szalma, S. J. (2007). Precise mapping of quantitative trait loci for resistance to Southern leaf blight, caused by *Cochliobolus heterostrophus* race O, and flowering time using advanced intercross maize lines. *Genetics, 176*(1), 645–657. https://doi.org/10.1534/genetics.106.067892

Bänziger, M., Setimela, P. S., Hodson, D., & Bindiganavile, V. (2006). Breeding for improved abiotic stress tolerance in maize adapted to southern Africa. *Agricultural Water Management, 80*(1–3), 212–224.

Bänziger, M., Edmeades, G. O., & Lafitte, H. R. (2002). Physiological mechanisms contributing to the increased N stress tolerance of tropical maize selected for drought tolerance. *Field Crops Research, 75*, 223–233. https://doi.org/10.1016/S0378-4290(02)00028-X

Bänziger, M., & Long, J. (2000). The potential for increasing the iron and zinc density of maize through plant-breeding. *Food and Nutrition Bulletin, 21*, 397–400. https://doi.org/10.1177/156482650002100410

Baxter, I. R., Gustin, J. L., Settles, A. M., & Hoekenga, O. A. (2013). Ionomic characterization of maize kernels in the intermated B73 Mo17 population. *Crop Science, 53*, 208–220. https://doi.org/10.2135/cropsci2012.02.0135

Beavers, A. W., Goggi, A. S., Reddy, M. B., Lauter, A. M., & Scott, M. P. (2015). Recurrent selection to alter grain phytic acid concentration and iron bioavailability. *Crop Science, 55*, 2244–2251. https://doi.org/10.2135/cropsci2014.12.0807

Berke, T., & Rocheford, T. R. (1995). Quantitative trait loci for flowering, plant and ear height, and kernel traits in maize. *Crop Science, 35*, 1542–1549.

Betsiashvili, M., Ahern, K. R., & Jander, G. (2015). Additive effects of two quantitative trait loci that confer *Rhopalosiphum maidis* (corn leaf aphid) resistance in maize inbred line Mo17. *Journal of Experimental Botany, 66*(2), 571–578.

Beyene, Y., Semagn, K., Mugo, S., Tarekegne, A., Babu, R., Meisel, B., Sehabiague, P., Makumbi, D., Magorokosho, C., Oikeh, S., & Gakunga, J. (2015). Genetic gains in grain yield through genomic selection in eight bi-parental maize populations under drought stress. *Crop Science, 55*(1), 154–163.

Beyene, Y., Semagn, K., Crossa, J., Mugo, S., Atlin, G. N., Tarekegne, A., & Alvarado, G. (2016). Improving maize grain yield under drought stress and non- stress environments in sub Saharan Africa using marker-assisted recurrent selection. *Crop Science, 56*(1), 344–353.

Beyene, Y., Gowda, M., Suresh, L. M., Mugo, S., Michael, O., Oikeh, S. O., Juma, C., Tarekegne, A., & Prasanna, B. M. (2017). Genetic analysis of tropical maize inbred lines for resistance to maize lethal necrosis disease. *Euphytica, 213*(9), 224. https://doi.org/10.1007/s10681-017-2012-3

Bhatt, V., Muthusamy, V., Jha, S., Zunjare, R. U., Baveja, A., Dosad, S., et al. (2018). Development of low phytic acid maize through marker assisted introgression of lpa1-1 and lpa2-1 genes. In *Abstracts: 13th Asian Maize Conference on and Expert Consultation on Maize for Food, Feed, Nutrition and Environmental Security, Ludhiana, India, October 8–10, 2018* (pp. 143–144). CIMMYT.

Bjarnason, M., & Vasal, S. K. (1992). Breeding for quality protein maize (QPM). *Plant Breeding Reviews, 9*, 181–216. https://doi.org/10.1002/9780470650363.ch7

Bohn, M., Schulz, B., Kreps, R., Klein, D., & Melchinger, A. E. (2000). QTL mapping for resistance against the European corn borer (Os- trinia Nubilalis H.) in early maturing European dent germplasm. *Theoretical and Applied Genetics, 101*, 907–917.

Bohn, M. O., Marroquin, J. J., Garcia, F. S., Dashiell, K., Willmot, D. B., & Hibbard, B. E. (2018). Quantitative trait loci mapping of western corn rootworm (Coleoptera: *Chrysomelidae*) host plant resistance in two populations of doubled haploid lines in maize (*Zea mays* L.). *Journal of Economic Entomology, 111*, 435–444.

Bouis, H. E., Hotz, C. H., McClafferty, B., Meenakshi, J. V., & Pfeiffer, W. (2011). Biofortification: A new tool to reduce micronutrient malnutrition. *Food and Nutrition Bulletin, 32*, S31–S40. https://doi.org/10.1177/15648265110321S105

Boyer, C., & Hannah, L. (2001). Kernel mutants of Corn. In A. R. Hallauer (Ed.), *Specialty Corns* (2nd ed., pp. 1–32). CRC Press.

Brki'c, A., Šimi'c, D., Jambrovi'c, A., Zduni'c, Z., Leden˘can, T., Raspudi'c, E., Brmež, M., Brki'c, J., Mazur, M., & Gali'c, V. (2020). QTL analysis of western corn rootworm resistance traits in maize *IBM* population grown in continuous maize. *Genetik, 52*, 137–148.

Brown, P. J., Upadyayula, N., Mahone, G. S., Tian, F., Bradbury, P. J., Myles, S., Holland, J. B., Flint-Garcia, S., McMullen, M. D., Buckler, E. S., et al. (2011). Distinct genetic architectures for male and female inflorescence traits of maize. *PLoS Genetics, 7*, e1002383.

Burton, A. L., Johnson, J. M., Foerster, J. M., Hirsch, C. N., Buell, C. R., Hanlon, M. T., Kaeppler, S. M., Brown, K. M., & Lynch, J. P. (2014). QTL mapping and phenotypic variation for root architectural traits in maize (Zea mays L.). *Theoretical and Applied Genetics, 127*(11), 2293–2311.

Calderón, C. I., Yandell, B. S., & Doebley, J. F. (2016). Fine mapping of a QTL associated with kernel row number on chromosome 1 of maize. *PLoS One, 11*, e0150276.

Cao, S., Loladze, A., Yuan, Y., Wu, Y., Zhang, A., Chen, J., et al. (2017). Genome wide analysis of Tar Spot Complex resistance in maize using genotyping-bysequencing SNPs and whole-genome prediction. *Plant Genome, 10*(2), 1–14. https://doi.org/10.3835/plantgenome2016.10.0099

Cardinal, A. J., Lee, M., Sharopova, N., Woodman-Clikeman, W. L., & Long, M. J. (2001). Genetic mapping and analysis of quantitative trait loci for resistance to stalk tunneling by the European corn borer in maize. *Crop Science, 41*, 835–845.

Carena, M. J., Santiago, I., & Ordas, A. (1998). Direct and correlated response to recurrent selection for prolificacy in maize at two planting densities. *Maydica, 43*, 95–102.

Castro-Álvarez, F. F., William, M., Bergvinson, D. J., & GarcíaLara, S. (2015). Genetic mapping of QTL for maize weevil resistance in a RIL population of tropical maize. *Theoretical and Applied Genetics, 128*, 411–419. https://doi.org/10.1007/s00122-014-2440-6

Chakraborti, M., Prasanna, B. M., Hossain, F., & Singh, A. M. (2011). Evaluation of single cross quality protein maize (QPM) hybrids for kernel iron and zinc concentrations. *Indian Journal of Genetics, 71*, 312–319.

Chander, S., Guo, Y. Q., Yang, X., Zhang, J., Lu, X., Yan, J., Song, T., Rocheford, T., & Li, J. (2008). Using molecular markers to identify two major loci controlling carotenoid contents in maize grain. *Theoretical and Applied Genetics, 116*, 223–233. https://doi.org/10.1007/s00122-007-0661-7

Chaudhary, D. P., Kumar, S., & Yadav, O. P. (2014). Nutritive value of maize: Improvements, applications and constraints. In *Maize: Nutrition dynamics and novel uses*. (pp. 3–17). Springer.

Chen, J., Zhang, L., Liu, S., Li, Z., Huang, R., Li, Y., Cheng, H., Li, X., Zhou, B., & Wu, S. (2016). The genetic basis of natural variation in kernel size and related traits using a four-way cross population in maize. *PLoS One, 11*, e0153428.

Choudhary, M., Singh, A., Gupta, M., & Rakshit, S. (2020). Enabling technologies for utilization of maize as a bioenergy feedstock. *Biofuels, Bioproducts and Biorefini, 14*, 402–416. https://doi.org/10.1002/bbb.2060

Choudhary, M., Singh, V., Muthusamy, V., & Wani, S. H. (2017). Harnessing crop wild relatives for crop improvement. *International Journal of Life Science, 6*(2), 73–85.

Choudhary, M., Wani, S. H., Kumar, P., Bagaria, P. K., Rakshit, S., Roorkiwal, M., & Varshney, R. K. (2019). QTLian breeding for climate resilience in cereals: Progress and prospects. *Functional & Integrative Genomics, 19*, 685–701.

Choudhary, M., Singh, A., & Rakshit, S. (2021). Coping with low moisture stress: Remembering and responding. *Physiologia Plantarum, 1–8*. https://doi.org/10.1111/ppl.133438

CIMMYT. (1998). *A complete listing of improved maize germplasm from CIMMYT* (Maize program special report). CIMMYT.

Collins, N., Jeff, D., Michael, A., Qing, S., Jeff, E., Scot, H., & Tony, P. (1999). Molecular characterization of the maize *Rp*1-D rust resistance haplotype and its mutants. *Plant Cell, 11*(7), 1365–1376. https://doi.org/10.1105/tpc.11.7.1365

Das, A. K., Muthusamy, V., Zunjare, R. U., Chauhan, H. S., Sharma, P. K., Bhat, J. S., et al. (2019). Genetic variability, genotype × environment interactions and combining ability analyses of kernel tocopherols among maize genotypes possessing novel allele of γ-tocopherol methyl transferase (*ZmVTE4*). *Journal of Cereal Science, 86*, 1–8. https://doi.org/10.1016/j.jcs.2018.12.018

Dass, S., Singh, N. N., Dang, Y. P., & Dhawan, A. K. (1997). Morphophysiological basis of breeding drought and low-N tolerant maize genotypes in India. In *Developing drought and low-N tolerant maize. Edmeades GO, Bänziger M, Mickelson HR, Pena-Valdivia CB eds. Proc Symposium, Match 25–29, 1996* (pp. 107–111). CIMMYT.

DellaPenna, D., & Pogson, B. J. (2006). Vitamin synthesis in plants: Tocopherols and carotenoids. *Annual Review of Plant Biology, 57*, 711–738. https://doi.org/10.1146/annurev.arplant.56.032604.144301

Dhillon, B. S., & Prasanna, B. M. (2001). Maize. In V. L. Chopra (Ed.), *Breeding field crops* (pp. 147–185). Oxford and IBH Publishing Co.

Dhliwayo, T., Palacios-Rojas, N., Crossa, J., & Pixley, K. V. (2014). Effects of S1 recurrent selection for provitamin A carotenoid content for three open-pollinated maize cultivars. *Crop Science, 54*, 2449–2460. https://doi.org/10.2135/cropsci2013.11.0764

Diepenbrock, C. H., Kandianis, C. B., Lipka, A. E., Magallanes-Lundback, M., Vaillancourt, B., Góngora-Castillo, E., et al. (2017). Novel loci underlie natural variation in vitamin E levels in maize grain. *Plant Cell, 29*, 2374–2392. https://doi.org/10.1105/tpc.17.00475

Dudley, J. W., & Lambert, R. J. (2004). 100 generations of selection for oil and protein in corn. In J. Janick (Ed.), *Plant breeding reviews* (Vol. 24, pp. 79–110). Wiley.

Ertl, D. S., Young, K. A., & Raboy, V. (1998). Plant genetic approaches to phosphorus management in agricultural production. *Journal of Environmental Quality, 272*, 299–304. https://doi.org/10.2134/jeq1998.00472425002700020008x

Evenson, R. E., & Gollin, D. (2002). *Crop variety improvement and its effect on productivity*. CABI Publishers.

FAOSTAT. (2019). *Food and Agriculture Organization [FAO] of the United Nations. FAOSTAT Data: Crops*. Available at: http://www.fao.org/faostat/en/#data/QC

Feng, F., Deng, F., Zhou, P., Yan, J., Wang, Q., Yang, R., et al. (2013). QTL mapping for the tocopherols at milk stage of kernel development in sweet corn. *Euphytica, 193*, 409–417. https://doi.org/10.1007/s10681-013-0948-5

Feng, F., Wang, Q., Liang, C., Yang, R., & Li, X. (2015). Enhancement of tocopherols in sweet corn by marker-assisted backcrossing of ZmVTE4. *Euphytica, 206*, 513–521. https://doi.org/10.1007/s10681-015-1519-8

Fenton, M. E., Owens, B. F., Lipka, A. E., et al. (2018). High-density linkage mapping of vitamin E content in maize grain. *Molecular Breeding, 38*, 31. https://doi.org/10.1007/s11032-018-0780-7

Foiada, F., Westermeier, P., Kessel, B., Ouzunova, M., Wimmer, V., Mayerhofer, W., et al. (2015). Improving resistance to the European corn borer: A comprehensive study in elite maize using QTL mapping and genome-wide prediction. *Theoretical and Applied Genetics, 128*, 875–891.

Garcia-Lara, S., & Bergvinson, D. J. (2014). Phytochemical changes during recurrent selection for storage pest resistance in tropical maize. *Crop Science, 54*, 1–10.

García-Lara, S., Khairallah, M. M., Vargas, M., & Bergvinson, D. J. (2009). Mapping of QTL associated with maize weevil resistance in tropical maize. *Crop Science, 49*, 139–149.

Gazal, A., Dar, Z. A., & Lone, A. A. (2018, Mat 9). Molecular breeding for abiotic stresses in maize (*Zea mays* L.). In *Maize germplasm-characterization and genetic approaches for crop improvement 2018*. IntechOpen.

Glenn, K. C., Alsop, B., Bell, E., Goley, M., Jenkinson, J., Liu, B., et al. (2017). Bringing new plant varieties to market: Plant breeding and selection practices advance beneficial characteristics while minimizing unintended changes. *Crop Science, 57*, 2906–2921. https://doi.org/10.2135/cropsci2017.03.0199

Guo, R., Dhliwayo, T., Mageto, E. K., Palacios-Rojas, N., Lee, M., Yu, D., Ruan, Y., Zhang, A., San, V. F., Olsen, M., & Crossa, J. (2020). Genomic prediction of kernel zinc concentration

in multiple maize populations using genotyping-by-sequencing and repeat amplification sequencing markers. *Frontiers in Plant Science, 11*, 534.

Gupta, H. S., Raman, B., Agrawal, P. K., Mahajan, V., Hossain, F., & Thirunavukkarasu, N. (2012). Accelerated development of quality protein maize hybrid through marker-assisted introgression of opaque-2 allele. *Plant Breeding, 13*, 277–282.

Guzmán, C., Medina-Larqué, A. S., Velu, G., González-Santoyo, H., Singh, R. P., Huerta-Espino, J., et al. (2014). Use of wheat genetic resources to develop biofortified wheat with enhanced grain zinc and iron concentrations and desirable processing quality. *Journal of Cereal Science, 60*, 617–622. https://doi.org/10.1016/j.jcs.2014.07.006

Hallauer, A. R., Ross, A. J., & Lee, M. (2004). Long-term divergent selection for ear length in maize. In J. Janick (Ed.), *Plant Breed. Rev* (Vol. 24 (2), pp. 153–168). Wiley.

Harjes, C. E., Rocheford, T. R., Bai, L., Brutnell, T. P., Kandianis, C. B., Sowinski, S. G., et al. (2008). Natural genetic variation in *lycopene epsilon cyclase* tapped for maize biofortification. *Science, 319*, 330–333. https://doi.org/10.1126/science.1150255

Hessel, D. A. (2014). *Deciphering the genetic architecture of native resistance and tolerance to western corn rootworm larval feeding*. Graduate Theses and Dissertations 13874. https://lib.dr.iastate.edu/etd/13874

Hindu, V., Palacios-Rojas, N., Babu, R., Suwarno, W. B., Rashid, Z., Usha, R., et al. (2018). Identification and validation of genomic regions influencing kernel zinc and iron in maize. *Theoretical and Applied Genetics, 131*, 1443–1457. https://doi.org/10.1007/s00122-018-3089-3

Holland, J. B., Marino, T. P., Manching, H. C., & Wisser, R. J. (2020). Genomic prediction for resistance to Fusarium ear rot and fumonisin contamination in maize. *Crop Science, 60*(4), 1863–1875. https://doi.org/10.1002/csc2.20163

Hopkins, C. G. (1899). Improvement in the chemical composition of the corn kernel. *Illinois Agricultural Experiment Station Bulletin, 55*.

Hoque, M. M. I., Jun, Z., & Guoying, W. (2015). Evaluation of salinity tolerance in maize (*Zea mays* L.) genotypes at seedling stage. *Journal of BioScience and Biotechnology, 4*, 39–49.

Hossain, F., Muthusamy, V., Pandey, N., Vishwakarma, A. K., Baveja, A., Zunjare, R. U., & Gupta, H. S. (2018). Marker-assisted introgression of opaque2 allele for rapid conversion of elite hybrids into quality protein maize. *The Journal of Genetics and Genomics, 97*(1), 287–298.

Hossain, F., Sarika, K., Muthusamy, V., Zunjare, R. U., & Gupta, H. S. (2019). Quality protein maize for nutritional security. In A. M. I. Qureshi, Z. A. Dar, & S. H. Wani (Eds.), *Quality breeding in field crops* (pp. 217–237). Springer. https://doi.org/10.1007/978-3-030-04609-5_11

Hotz, C., & McClafferty, B. (2007). From harvest to health: Challenges for developing biofortified staple foods and determining their impact on micronutrient status. *Food and Nutrition Bulletin, 28*, S271–S279. https://doi.org/10.1177/15648265070282s206

Hung, H. Y., & James, B. H. (2012). Diallel analysis of resistance to *Fusarium* ear rot and Fumonisin contamination in maize. *Crop Science, 52*(5), 2173–2181. https://doi.org/10.2135/cropsci2012.03.0154

Hurni, S., Daniela, S., Simon, G. K., Bettina, K., Thomas, W., Gerhard, H., Mirjam, N. F., et al. (2015). The maize disease resistance gene Htn1 against northern corn leaf blight encodes a wall-associated receptor-like kinase. *Proceedings of the National Academy of Sciences USA, 112*(28), 8780–8785. https://doi.org/10.1073/pnas.1502522112

Jamann, T. M., Jesse, A. P., Judith, M. K., Laurie, G. S., & Rebecca, J. N. (2014). Unravelling genomic complexity at a quantitative disease resistance locus in maize. *Genetics, 198*(1), 333–344. https://doi.org/10.1534/genetics.114.167486

Jiang, T., Christian, P., Khatry, S. K., Wu, L., & West, K. P. J. (2005). Micronutrient deficiencies in early pregnancy are common, concurrent, and vary by season among rural Nepali pregnant women. *The Journal of Nutrition, 135*, 1106–1112. https://doi.org/10.1093/jn/135.5.1106

Jiménez-Galindo, J. C., Ordás, B., Butrón, A., Samayoa, L. F., & Malvar, R. A. (2017). QTL mapping for yield and resistance against mediterranean corn borer in maize. *Frontiers in Plant Science, 8*, 698. https://doi.org/10.3389/fpls.2017.00698

Jin, T., Zhou, J., Chen, J., Zhu, L., Zhao, Y., & Huang, Y. (2013). The genetic architecture of zinc and iron content in maize grains as revealed by QTL mapping and meta-analysis. *Breeding Science, 63*, 317–324. https://doi.org/10.1270/2Fjsbbs.63.317

Johal, G. S., & Steven, P. B. (1992). Reductase activity encoded by the *HM1* disease resistance gene in maize. *Science, 258*(5084), 985–987. https://doi.org/10.1126/science.1359642

Jugenheimer, R. W. (1961). Breeding for oil and protein content in maize. *Euphytica, 10*, 152–156. https://doi.org/10.1007/BF00022206

Karjagi, C. G., Sekhar, J. C., Lakshmi, S. P., Suby, S. B., Kaur, J., Mallikarjuna, M. G., & Kumar, P. (2017) Breeding for resistance to insect pests in maize. In: *Breeding insect resistant crops for sustainable agriculture* (pp. 201–229). Springer.

Kauffmann, K. D., Crum, C. W., & Lindsey, M. E. (1982). Exotic germplasm in a corn breeding program. *III. Corn Breeder's School, 18*, 6–39.

Kearsey, M. J., & Pooni, H. S. (1998). *The genetical analysis of quantitative traits*. Taylor and Francis (Publishers) Ltd.

Klenke, J. R., Russell, W. A., & Guthrie, W. D. (1986). Grain yield reduction caused by second-generation European corn borer in BS9 corn synthetic. *Crop Science, 26*, 859–863.

Kostadinovic, M., Ignjatovic-Micic, D., Stankovic, G., Vancetovic, J., Ristic, D., & Drinic, S. M. (2014). Marker assisted backcrossing for incorporation of the opaque2 gene into a standard maize inbred line. *The Journal of the Science of Food and Agriculture, 2*, 199–205.

Krakowsky, M. D., Lee, M., Woodman-Clikeman, W. L., Long, M. J., & Sharopova, N. (2004). QTL mapping of resistance to stalk tunneling by the European corn borer in RILs of maize population B73 × De8. *Crop Science, 44*(1), 274. https://doi.org/10.2135/cropsci2004.2740

Kumar, P., Choudhary, M., Hossain, F., Singh, N., Choudhary, P., Gupta, M., Singh, V., et al. (2019). Nutritional quality improvement in maize (*Zea mays*): Progress and challenges. *Indian Journal of Agricultural Sciences, 89*(6), 895–911.

Kumar, K., Gambhir, G., Dass, A., Tripathi, A. K., Singh, A., Jha, A. K., Yadava, P., Choudhary, M., & Rakshit, S. (2020). Genetically modified crops: Current status and future prospects. *Planta, 251*(4), 1–27.

Kumar, B., Rakshit, S., Kumar, S., Singh, B. K., Lahkar, C., Jha, A. K., Kumar, K., Kumar, P., Choudhary, M., Singh, S. B., & Amalraj, J. J. (2022). Genetic diversity, population structure and linkage disequilibrium analyses in tropical maize using genotyping by sequencing. *Plants, 11*(6), 799. https://doi.org/10.3390/plants11060799

Laurie, C. C., Chasalow, S. D., Ledeaux, J. R., McCarroll, R., Bush, D., Hauge, B., Lai, C. Q., Clark, D., Rocheford, T. R., & Dudley, J. W. (2004). The genetic architecture of response to long-term artificial selection for oil concentration in the maize kernel. *Genetics, 168*, 2141–2155.

Li, C., Sun, B., Li, Y., Liu, C., Wu, X., Zhang, D., & Wang, T. (2016). Numerous genetic loci identified for drought tolerance in the maize nested association mapping populations. *BMC Genomics, 17*(1), 894.

Li, Q., Yang, X., Xu, S., Cai, Y., Zhang, D., Han, Y., et al. (2012). Genome-wide association studies identified three independent polymorphisms associated with a-tocopherol content in maize kernels. *PLoS One, 7*, e36807. https://doi.org/10.1371/journal.pone.0036807

Lipka, A. E., Gore, M. A., Magallanes-Lundback, M., Mesberg, A., Lin, H., Tiede, T., et al. (2013). Genome-wide association study and pathway level analysis of tocochromanol levels in maize grain. *G3 (Bethesda), 3*, 1287–1299. https://doi.org/10.1534/g3.113.006148

Liu, L., Jeffers, D., Zhang, Y., Ding, M., Chen, W., Kang, M. S., & Fan, X. (2015). Introgression of the crtRB1 gene into quality protein maize inbred lines using molecular markers. *Molecular Breeding, 35*(8), 154.

Liu, H., Shi, J., Sun, C., Gong, H., Fan, X., Qiu, F., et al. (2016). Gene duplication confers enhanced expression of 27-kDa g-zein for endosperm modification in quality protein maize. *Proceedings of the National Academy of Sciences, 113*, 4964–4969. https://doi.org/10.1073/pnas.1601352113

Liu, L., Du, Y., Shen, X., Li, M., Sun, W., Huang, J., Liu, Z., Tao, Y., Zheng, Y., Yan, J. & Zhang, Z. (2015). KRN4 controls quantitative variation in maize kernel row number. *PLoS genetics, 11*(11), p.e1005670.

Liu, Y., Subhash, C., Yan, J., Song, C., Zhao, J., & Li, J. (2011). Maize leaf temperature responses to drought: Thermal imaging and quantitative trait loci (QTL) mapping. *Environmental and Experimental Botany, 71*(2), 158–165.

Lu, M., Xie, C. X., Li, X. H., Hao, Z. F., Li, M. S., Weng, J. F., Zhang, D. G., Bai, L., & Zhang, S. H. (2011). Mapping of quantitative trait loci for kernel row number in maize across seven environments. *Mol Breeding, 28*, 143–152.

Lung'aho, M. G., Mwaniki, A. M., Szalma, S. J., Hart, J. J., Rutzke, M. A., et al. (2011). Genetic and physiological analysis of iron biofortification in maize kernels. *PLoS One, 6*(6), e20429.

Magulama, E. E., & Sales, E. K. (2009). Marker-assisted introgression of opaque 2 gene into elite maize inbred lines. *USM R & D, 17*, 131–135.

Mallikarjuna, M. G., Nepolean, T., Hossain, F., Manjaiah, K. M., Singh, A. M., & Gupta, H. S. (2014). Genetic variability and correlation of kernel micronutrients among exotic quality protein maize inbreds and their utility in breeding programme. *The Indian Journal of Genetics and Plant Breeding, 74*, 166–173.

Maqbool, M. A., Beshir Issa, A., & Khokhar, E. S. (2021). Quality protein maize (QPM): Importance, genetics, timeline of different events, breeding strategies and varietal adoption. *Plant Breeding, 140*, 375–399. https://doi.org/10.1111/pbr.12923

Masuka, B., Atlin, G. N., Olsen, M., Magorokosho, C., Labuschagne, M., Crossa, J., Bänziger, M., Pixley, K. V., Vivek, B., Biljon, A., MacRobert, J. F., Alvarado, G., Prasanna, B. M., Makumbi, D., Makumbi, D., Tarekegne, A. T., Das, B., Zaman-Allah, M., & Cairns, J. E. (2017). Gains in maize genetic improvement in Eastern and Southern Africa: I. CIMMYT hybrid breeding pipeline. *Crop Science, 57*, 168–179.

Meihls, L. N., Handrick, V., Glauser, G., et al. (2013). Natural variation in maize aphid resistance is associated with DIMBOA-Glc methyltransferase activity. *The Plant Cell, 25*, 2341–2355. https://doi.org/10.1371/journal.pone.0020429

Menkir, A., Maziya-Dixon, B., Mengesha, W., Rocheford, T., & Alamu, E. O. (2017). Accruing genetic gain in pro-vitamin A enrichment from harnessing diverse maize germplasm. *Euphytica, 213*, 105. https://doi.org/10.1007/s10681-017-1890-8

Menkir, A., & Meseka, S. (2019). Genetic improvement in resistance to *Striga* in tropical maize hybrids. *Crop Science, 59*, 1–14. https://doi.org/10.2135/cropsci2018.12.0749

Menkir, A., Palacios-Rojas, N., Alamu, O., Dias, P., Maria, C., Dhliwayo, T., et al. (2018). *Vitamin a biofortified maize. Exploiting native genetic variation for nutrient enrichment. No. 2187-2019-667*. CIMMYT, IITA, EMBRAPA, HarvestPlus and Crop Trust.

Menkir, A., Robert, L. B., Ranajit, B., & Thomas, E. C. (2008). Registration of six tropical maize germplasm lines with resistance to aflatoxin contamination. *Journal of Plant Registrations, 2*(3), 246–250.

Mertz, E. T., Bates, L. S., & Nelson, O. E. (1964). Mutant gene that changes protein composition and increases lysine content of maize endosperm. *Science, 145*, 279–280.

Meseka, S., Menkir, A., Bossey, B., & Mengesha, W. (2018). Performance assessment of drought tolerant maize hybrids under combined drought and heat stress. *Agronomy, 8*, 274.

Meseka, S., Menkir, A., & Obeng-Antwi, K. (2014). Exploitation of beneficial alleles from maize (*Zea mays L.*) landraces to enhance performance of an elite variety in water stress environments. *Euphytica, 201*, 149–160. https://doi.org/10.1007/s10681-014-1214-1

Messmer, R., Yvan, F., Bänziger, M., Stamp, P., & Ribaut, J. M. (2011). Drought stress and tropical maize: QTLs for leaf greenness, plant senescence, and root capacitance. *Field Crops Research, 124*, 93–103.

Mihm, J. A. (1985). Breeding for host plant resistance to maize stem- borers. *Insect Science and Application, 6*, 369–377.

Moose, S. P., Dudley, J. W., & Rocheford, T. R. (2004). Maize selection passes the century mark: A unique resource for 21st century genomics. *Trends in Plant Science, 9*, 358–364.

Mugo, S. N., Bergvinson, D., & Hoisington, D. (2001). Options in developing stem borer-resistant maize: CIMMYT's approaches and experiences. *Insect Science and Application, 21*, 409–415.

Muthusamy, V., Hossain, F., Thirunavukkarasu, N., Choudhary, M., Saha, S., Bhat, J. S., & Gupta, H. S. (2014). Development of β-Carotene rich maize hybrids through marker-assisted introgression of β-carotene hydroxylase allele. *PLoS One, 9*(12), e113583. https://doi.org/10.1371/journal.pone.0113583

Nair, S. K., Babu, R., Magorokosho, C., Mahuku, G., Semagn, K., Beyene, Y., et al. (2015). Fine mapping of Msv1, a major QTL for resistance to maize streak virus leads to development of production markers for breeding pipelines. *Theoretical and Applied Genetics, 128*, 1839–1854. https://doi.org/10.1007/s00122-015-2551-8

Nikolic, A., Andelkovic, V., Dodig, D., Mladenovic, D. S., Kravic, N., & Ignjatovic, M. D. (2013). Identification of Qtl-s for drought tolerance in maize, II: Yield and yield components. *Genetika, 45*(2), 341–350.

Nikolić, A., Dragana, I. M., Dejan, D., Violeta, A., & Vesna, L. J. (2012). Identification of QTLs for yield and drought- related traits in maize: Assessment of their causal relationships. *Biotechnology & Biotechnological Equipment, 26*(3), 2952–2960.

Ortiz, D., Rocheford, T., & Ferruzzi, M. G. (2016). Influence of temperature and humidity on the stability of carotenoids in biofortified maize (*Zea mays* L.) genotypes during controlled post-harvest storage. *Journal of Agricultural and Food Chemistry, 64*, 2727–2736. https://doi.org/10.1021/acs.jafc.5b05698

Ortiz-Covarrubias, Y., Dhliwayo, T., Palacios-Roja, N., Ndhlela, T., Magorokosho, C., Aguilar-Rincon, V. H., et al. (2019). Effects of drought and low nitrogen stress on provitamin A carotenoid content of biofortified maize hybrids. *Crop Science, 59*, 2521–2532. https://doi.org/10.2135/cropsci2019.02.0100

Ortiz-Monasterio, J. I., Palacios-Rojas, N., Meng, E., Pixley, K., Trethowan, R., & Pena, R. J. (2007). Enhancing the mineral and vitamin content of wheat and maize through plant breeding. *Journal of Cereal Science, 46*, 293–307. https://doi.org/10.1016/j.jcs.2007.06.005

Pilu, R., Panzeri, D., Gavazzi, G., Rasmussen, S. K., Consonni, G., & Nielsen, E. (2003). Phenotypic, genetic and molecular characterization of a maize low phytic acid mutant (lpa241). *Theoretical and Applied Genetics, 107*, 980–987. https://doi.org/10.1007/s00122-003-1316-y

Pixley, K., Palacios-Rojas, N., Babu, R., Mutale, R., Surles, R., & Simpungwe, E. (2013). Biofortification of maize with provitamin a carotenoids. In S. Tanumihardjo (Ed.), *Carotenoids and human health* (pp. 271–292). Springer.

Poland, J. A., Bradbury, P. J., Buckler, E. S., & Nelson, R. J. (2011). Genome-wide nested association mapping of quantitative resistance to northern leaf blight in maize. *The Proceedings of the National Academy of Sciences USA, 108*, 6893–6898.

Prasanna, B. M., Vasal, S. K., Kassahun, B., & Singh, N. N. (2001). Quality protein maize. *Current Science, 81*, 1308–1319.

Prasanna, B., Suresh, L. M., Mwatuni, F., Beyene, Y., Makumbi, D., Gowda, M., Olsen, M., et al. (2020a). *Maize lethal necrosis (MLN): Efforts toward containing the spread and impact of a devastating transboundary disease in sub-Saharan Africa*. Virus Research Elsevier B V. https://doi.org/10.1016/j.virusres.2020.197943

Prasanna, B. M., Palacios-Rojas, N., Hossain, F., Muthusamy, V., Menkir, A., Dhliwayo, T., Ndhlela, T., San Vicente, F., Nair, S. K., Vivek, B. S., Zhang, X., Olsen, M., & Fan, X. (2020b). Molecular breeding for nutritionally enriched maize: Status and prospects. *Frontiers in Genetics, 10*, 1392. https://doi.org/10.3389/fgene.2019.01392

Qin, H., Cai, Y., Liu, Z., Wang, G., Wang, J., Guo, Y., et al. (2012). Identification of QTL for zinc and iron concentration in maize kernel and cob. *Euphytica, 187*, 345–358. https://doi.org/10.1007/s10681-012-0692-2

Raboy, V. (2001). Genetics and breeding of seed phosphorus and phytic acid. *Journal of Plant Physiology, 158*, 489–497. https://doi.org/10.1078/0176-1617-00361

Raboy, V., Gerbasi, P. F., Young, K. A., Stoneberg, S. D., Pickett, S. G., Bauman, A. T., et al. (2000). Origin and seed phenotype of maize low phytic acid 1-1 and low phytic acid 2-1. *Plant Physiology, 124*, 355–368. https://doi.org/10.1104/pp.124.1.355

Rahman, H., Pekic, S., Lazic-Jancic, V., Quarri, S. A., Shah, S. M. A., Pervez, A., & Shah, M. M. (2011). Molecular mapping of quantitative trait loci for drought tolerance in maize plants. *Genetics and Molecular Research, 10*(2), 889–901.

Rattalino Edreira J. I., & Otegui M. E. (2012). Heat Stress in Temperate and Tropical maize Hybrids: Differences in Crop Growth, Biomass Partitioning and Reserves Use. *Field Crops Research, 130*, 87–98. https://doi.org/10.1016/j.fcr.2012.02.009

Rawlings, J. O. (1970). Present status of research on long and short-term recurrent selection in finite populations – Choice of population size, p. 1–15. In Papers presented at the second meeting of the Working Group on Quantitative Genetics, Section 22, lUFRO, 1969. USDA For. Serv. South. For. Exp. Stn., New Orleans, La.

Reif, J. C., Hallauer, A. R., & Melchinger, A. E. (2005). Heterosis and heterotic patterns in maize. *Maydica, 50*, 215.

Samayoa, L. F., Malvar, R. A., McMullen, M. D., & Butrón, A. (2015). Identification of QTL for resistance to Mediterranean corn borer in a maize tropical line to improve temperate germplasm. *BMC Plant Biology, 15*(1), 215. https://doi.org/10.1186/s12870-015-0652-9

Sandoya, G., Butron, A., Alvarez, A., et al. (2008). Direct response of a maize synthetic to recurrent selection for resistance to stem borers. *Crop Science, 48*, 113–118.

Sarika, K., Hossain, F., Muthusamy, V., Zunjare, R. U., Baveja, A., Goswami, R., et al. (2018). Marker-assisted pyramiding of opaque2 and novel opaque16 genes for further enrichment of lysine and tryptophan in sub-tropical maize. *Plant Science, 272*, 142–152. https://doi.org/10.1016/j.plantsci.2018.04.014

Sharma, H., Dhillon, M., Pampapathy, G., et al. (2007). Inheritance of resistance to spotted stem borer *Chilo partellus* in *Sorghum bicolor*. *Euphytica, 156*, 117–128.

Shutu, X., Dalong, Z., Ye, C., Yi, Z., Shah, T., Ali, F., et al. (2012). Dissecting tocopherols content in maize (*Zea mays* L.) using two segregating populations and high-density single nucleotide polymorphism markers. *BMC Plant Biology, 12*(1), 201. http://www.biomedcentral.com/1471-2229/12/201

Šimić, D., Drinić, S. M., Zdunić, Z., Jambrović, A., Ledenčan, T., Brkić, J., et al. (2012). Quantitative trait loci for biofortification traits in maize grain. *The Journal of Heredity, 103*, 47–54. https://doi.org/10.1093/jhered/esr122

Sitonik, C., Suresh, L. M., Beyene, Y., et al. (2019). Genetic architecture of maize chlorotic mottle virus and maize lethal necrosis through GWAS, linkage analysis and genomic prediction in tropical maize germplasm. *Theoretical and Applied Genetics, 132*, 2381–2399. https://doi.org/10.1007/s00122-019-03360-x

Smith, S., Cooper, M., Gogerty, J., Löfer, C., Borcherding, D., & Wright, K. (2014). Maize. In Smith et al. (Eds.), *Yield gains in major U.S. feld crops* (CSSA Spec. Publ. 33) (pp. 125–171). ASA, CSSA, and SSSA.

Soengas, P., Butrón, A., Revilla, P., Ordás, A., & Malvar, R. A. (2004). Performance of crosses among flint maize populations under infestation by *Sesamia nonagrioides* (Lepidoptera: Noctuidae). *Journal of Economic Entomology, 97*, 1438–1443.

Song, T. M., & Chen, S. J. (2004). Long term selection for oil concentration in five maize populations. *Maydica, 49*(1), 9–14.

Song, T. M., Kong, F., Li, C. J., & Song, G. H. (1999). Eleven cycles of single kernel phenotypic recurrent selection for percent oil in Zhongzong No.2 maize synthetic. *Journal of Genetics and Breeding, 53*, 31–35.

Song, X. F., Song, T. M., Dai, J. R., Rocheford, T. R., & Li, J. S. (2004). QTL mapping of kernel oil concentration with high-oil maize by SSR markers. *Maydica, 49*, 41–48.

Sowa, M., Yu, J., Palacios-Rojas, N., Goltz, S. R., Howe, J. A., Davis, C. R., et al. (2017). Retention of carotenoids in biofortified maize flour and bcryptoxanthin-enhanced eggs after household cooking. *ACS Omega, 10*, 7320–7328. https://doi.org/10.1021/acsomega.7b01202

Sureshkumar, S., Tamilkumar, P., Thangavelu, A. U., Senthil, N., Nagarajan, P., Vellaikumar, S., & Raveendran, M. (2014). Marker-assisted introgression of *lpa2* locus responsible for low-phytic acid trait into an elite tropical maize inbred (*Zea mays* L.). *Plant Breeding, 133*(5), 566–578.

Suwarno, W. B., Pixley, K. V., Palacios-Rojas, N., Kaeppler, S. M., & Babu, R. (2014). Formation of heterotic groups and understanding genetic effects in a provitamin A biofortified maize breeding program. *Crop Science, 54*, 14–24. https://doi.org/10.2135/cropsci2013.02.0096

Tamilkumar, P., Senthil, N., Sureshkumar, S., Thangavelu, A. U., Nagarajan, P., Vellaikumar, S., & Raveendran, M. (2014). Introgression of low phytic acid locus ('*lpa2-2′*) into an elite maize (*Zea mays* L.) inbred through marker assisted backcross breeding. Aust. *Journal of Crop Science, 8*(8), 1224.

Tian, Q. Z., Li, X. H., Li, M. S., Jiang, W., & Zhang, S. H. (2004). Molecular markers assisted selection to quality protein maize. *Journal of Maize Sciences, 12*, 108–110.

Tollenaar, M., McCullough, D. E., & Dwyer, L. M. (1994). Physiological basis of the genetic improvement of corn. In G. A. Slafer (Ed.), *Genetic improvement of field crops* (pp. 183–236). Marcel Dekker.

Tomlekova, N. B., Kozgar, M. I., & Wani, M. R. (Eds.) (2014a). *Mutagenesis: Exploring novel genes and pathways* (475 p). Wageningen Academic Publishers. ISBN 978-90-8686-234-4, https://doi.org/10.3920/978-90-8686-787-7_5

Tomlekova, N. B., Kozgar, M. I., & Wani, M. R. (Eds.) (2014b). *Mutagenesis: Exploring genetic diversity of crops* (394 p). Wageningen Academic Publishers. ISBN 978-90-8686-244-3, https://doi.org/10.3920/978-90-8686-796-7.

Trachsel, S., Sun, D., Felix, S. V., Zheng, H., Atlin, G., SuarezE, R. B., & Zhang, X. (2016). Identification of QTL for early vigor and stay-green conferring tolerance to drought in two connected advanced backcross populations in tropical maize (*Zea mays* L.). *PLoS One, 11*(3), e0149636. https://doi.org/10.1371/journal.pone.0149636

Wang, J., Lin, Z., Zhang, X., Liu, H., Zhou, L., Zhong, S., & Lin, Z. (2019). *krn*1, a major quantitative trait locus for kernel row number in maize. *The New Phytologist, 223*(3), 1634–1646. https://doi.org/10.1111/nph.15890

Welz, H. G., & Geiger, H. H. (2000). Genes for resistance to northern corn leaf blight in diverse maize populations. *Plant Breeding, 119*, 1–14. https://doi.org/10.1046/j.1439-0523.2000.00462.x

Wisser, R. J., Balint-Kurti, P. J., & Nelson, R. J. (2006). The genetic architecture of disease resistance in maize: A synthesis of published studies. *Phytopathology, 96*, 120–129.

Wisser, R. J., Kolkman, J. M., Patzoldt, M. E., Holland, J. B., Yu, J., Krakowsky, M., Nelson, R. J., & Balint-Kurti, P. J. (2011). Multivariate analysis of maize disease resistances suggests a pleiotropic genetic basis and implicates a GST gene. *The Proceedings of the National Academy of Sciences USA, 108*, 7339–7344.

Wong, J. C., Lambert, R. J., Tadmor, Y., & Rocheford, T. R. (2003). QTL associated with accumulation of tocopherols in maize. *Crop Science, 43*, 2257–2266. https://doi.org/10.2135/cropsci2003.2257

Xing, Y., Ingvardsen, C., Salomon, R., & Lübberstedt, T. (2006). Analysis of sugarcane mosaic virus resistance in maize in an isogenic dihybrid crossing scheme and implications for breeding potyvirus-resistant maize hybrids. *Genome, 49*(10), 1274–1282. https://doi.org/10.1139/g06-070

Yadav, O. P., Hossain, F., Karjagi, C. G., Kumar, B., Zaidi, P. H., Jat, S. L., Chawla, J. S., Kaul, J., Hood, K. S., Kumar, P., Yadava, P., & Dhillon, B. S. (2015). Genetic improvement of maize in India: Retrospect and prospects. *Agriculture Research, 4*(4), 325–338. https://doi.org/10.1007/s40003-015-0180-8

Yan, J., Kandianis, C. B., Harjes, C. E., Bai, L., Kim, E. H., Yang, X., et al. (2010). Rare genetic variation at *Zea mays crtRB1* increases β-carotene in maize grain. *Nature Genetics, 42*, 322–327. https://doi.org/10.1038/ng.551

Yang, C., Liu, J., & Rong, T. Z. (2015). Detection of quantitative trait loci for ear row number in $F_2$ populations of maize. *Genetics and Molecular Research, 14*(4), 14229–14238.

Yang, G., Li, Y., Wang, Q., Zhou, Y., Zhou, Q., Shen, B., Zhanf, F., & Liang, X. (2012). Detection and integration of quantitative trait loci for grain yield components and oil content in two connected recombinant inbred line populations of high-oil maize. *Molecular Breeding, 29*(2), 313–333.

Yang, G. H., Dong, Y. B., Li, Y. L., Wang, Q. L., Shi, Q. L., & Zhou, Q. (2014). QTL verification of grain protein content and its correlation with oil content by using connected RIL populations of high-oil maize. *Genetics and Molecular Research, 13*, 881–894.

Yang, L., Wang, W., Yang, W., & Wang, M. (2013). Marker-assisted selection for pyramiding the waxy and opaque16 genes in maize using cross and backcross schemes. *Molecular Breeding, 31*, 767–775. https://doi.org/10.1007/s11032-012-9830-8

Yang, Q., He, Y., Kabahuma, M., Chaya, T., Kelly, A., Borrego, E., Bian, Y., El Kasmi, F., Yang, L., Teixeira, P., Kolkman, J., Nelson, R. J., Kolomiets, M., Dang, J. L., Wisser, R., Caplan, J., Li, X., Lauter, N., & Balint-Kurti, P. J. (2017). A gene encoding maize caffeoyl-CoA O-methyltransferase confers quantitative resistance to multiple pathogens. *Nature Genetics, 49*(9), 1364–1372. https://doi.org/10.1038/ng.3919

Yang, W., Zheng, Y., Zheng, W., & Feng, R. (2005). Molecular genetic mapping of a high-lysine mutant gene (*opaque-16*) and the double recessive effect with *opaque*-2 in maize. *Molecular Breeding, 15*, 257–269. https://doi.org/10.1007/s11032-004-5947-8

Yang, Q., Yin, G., Guo, Y., Zhang, D., Chen, S., & Xu, M. (2010). A major QTL for resistance to Gibberella stalk rot in maize. *Theoretical and Applied Genetics, 121*, 673–687.

Yang, Z., Li, X., Zhang, N., Zhang, Y. N., Jiang, H. W., Gao, J., Kuai, B. K., Ding, Y. L., & Huang, X. Q. (2016). Detection of quantitative trait loci for kernel oil and protein concentration in a B73 and Zheng58 maize cross. *Genetics and Molecular Research, 15*(3), 1–10.

Yuan, Y., Cairns, J. E., Babu, R., Gowda, M., Makumbi, D., Magorokosho, C., et al. (2019). Genome-wide association mapping and genomic prediction analyses reveal the genetic architecture of grain yield and flowering time under drought and heat stress conditions in maize. *Frontiers in Plant Science, 9*, 1919. https://doi.org/10.3389/fpls.2018.01919

Zaidi, P. H., Maniselvan, P., Srivastava, A., Yadav, P., & Singh, R. P. (2010). Genetic analysis of water-logging tolerance in tropical maize (*Zea mays* L.). *Maydica, 55*, 17–26.

Zaidi, P. H., Maniselvan, P., Yadav, P., Singh, A. K., Dureja, P., Singh, N. N., & Srinivasan, G. (2007). Stress-adaptive changes in tropical maize (*Zea mays* L.) under excessive soil moisture stress. *Maydica, 52*, 159–173.

Zaidi, P. H., Rafique, S., Rai, P. K., Singh, N. N., & Srinivasan, G. (2004). Tolerance to excess moisture in maize (*Zea mays* L.): Susceptible crop stages and identification of tolerant genotypes. *Field Crops Research, 90*, 189–202.

Zaidi, P. H., Rashid, Z., Vinayan, M. T., Almeida, G. D., Phagna, R. K., & Babu, R. (2015). QTL mapping of agronomic waterlogging tolerance using recombinant inbred lines derived from tropical maize (*Zea mays* L.) germplasm. *PLoS One, 10*(4), e0124350.

Zhang, W., Yang, W., Wang, M., Wang, W., Zeng, G., Chen, Z., et al. (2013). Increasing lysine content of waxy maize through introgression of *opaque2* and *opaque16* genes using molecular assisted and biochemical development. *PLoS One, 8*, 1–10. https://doi.org/10.1371/journal.pone.0056227

Zhang, W. L., Yang, W. P., Chen, Z. W., Wang, M. C., Yang, L. Q., & Cai, Y. L. (2010). Molecular marker-assisted selection for o2 introgression lines with *o16* gene in corn. *Acta Agronomica Sinica, 36*, 1302–1309. https://doi.org/10.1016/S1875-2780(09)60067-5

Zhang, X., Perez-Rodriguez, P., Semagn, K., Beyene, Y., Babu, R., Lopez-Cruz, M. A., et al. (2015). Genomic prediction in biparental tropical maize populations in water-stressed and well-watered environments using low density and GBS SNPs. *Heredity, 114*, 291–299. https://doi.org/10.1038/hdy.2014.99

Zhang, Y., Xu, L., Fan, X., Tan, J., Chen, W., & Xu, M. (2012). QTL mapping of resistance to gray leaf spot in maize. *Theoretical and Applied Genetics, 125*, 1797–1808.

Zhang, X., Yang, Q., Rucker, E., Thomason, W., & Balint-Kurti, P. (2017). Fine mapping of a quantitative resistance gene for gray leaf spot of maize (Zea mays L.) derived from teosinte (Z. mays ssp. parviglumis). *Theoretical and Applied Genetics, 130*, 1285–1295.

Zhao, X., Tan, G., Xing, Y., Wei, L., Chao, Q., Zuo, W., Lübberstedt, T., & Xu, M. (2012). Marker-assisted introgression of qHSR1 to improve maize resistance to head smut. *Molecular Breeding, 30*(2), 1077–1088. https://doi.org/10.1007/s11032-011-9694-3

Zhu, J. J., Wang, X., Sun, C. X., Zhu, X. M., Li, M., Zhang, G. D., Tian, Y. C., & Wang, Z. L. (2011). Mapping of QTL associated with drought tolerance in a semi-automobile rain shelter in maize (*Zea mays* L.). *Agricultural Sciences in China, 10*(7), 987–996.

Zunjare, R. U., Hossain, F., Muthusamy, M., Baveja, A., Chauhan, H. S., Bhat, J. S., et al. (2018). Development of biofortified maize hybrids through marker-assisted stacking of β-carotene hydroxylase, lycopene-ϵ-cyclase and opaque2 genes. *Frontiers in Plant Science, 9*, 178. https://doi.org/10.3389/fpls.2018.00178

Zunjare, R., Hossain, F., Muthusamy, V., Choudhary, M., Kumar, P., Sekhar, J. C., Guleria, S. K., Singh, N. K., Thirunavukkarasu, N., & Gupta, H. S. (2015). Popping quality attributes of popcorn hybrids in relation to weevil (Sitophilus oryzae) infestation. *Indian Journal of Genetics and Plant Breeding, 75*(04), 510–513.

Zuo, W., Chao, Q., Zhang, N., Ye, J., Tan, G., Li, B., Xing, Y., Zhang, B., Liu, H., Fengler, K. A., Zhao, J., Zhao, X., Chen, Y., Lai, J., Yan, J., & Xu, M. (2015). A maize wall-associated kinase confers quantitative resistance to head smut. *Nature Genetics, 47*(2), 151–157. https://doi.org/10.1038/ng.3170

# Conventional and Molecular Breeding for Sunflower Nutrition Quality Improvement

**Aleksandra Radanović, Sandra Cvejić, Milan Jocković, Boško Dedić, Siniša Jocić, and Dragana Miladinović**

**Abstract** Sunflower is the fourth most important oil plant worldwide and the second most important oil crop in Europe, along with rapeseed. Due to its high content of mono- and polyunsaturated fatty acids as well as vitamin E, sunflower oil is the preferred type of oil in human nutrition in Europe. That is why, as opposed to other different vegetable oils, circa 90% of the total sunflower oil produced is used for food, while 10% is exploited for biodiesel production and other industrial purposes. In human nutrition, sunflower oil is used for cooking, frying, and preparing salads, while in industry, it is used in the frying process and for margarine production. Maintaining secure and sustainable sunflower production is, therefore, of utmost importance. Sunflower breeding led to a significant increase in oil content and change in oil composition, thus increasing both oil quantity and quality. Nowadays, different biotechnological techniques are used to accelerate the creation of superior sunflower genotypes that will be productive in climate changing environments and still be of high quality. The conventional and molecular methods used in sunflower breeding for improved nutrition quality will be addressed in this chapter.

**Keywords** *Helianthus annuus* L. · Oil · Breeding · Marker-assisted selection · Genomic selection · New techniques

## 1 Introduction

Now recognized as one of the most important sources of edible oil for human consumption, sunflower (*Helianthus annuus* L.) has served different purposes for centuries. Native Americans were the first to grow sunflower, with the intention of using it as food, for healing, and for religious purposes. After being introduced to

A. Radanović · S. Cvejić · M. Jocković · B. Dedić · S. Jocić · D. Miladinović (✉)
Institute of Field and Vegetable Crops, National Institute of the Republic of Serbia, Novi Sad, Serbia
e-mail: dragana.miladinovic@ifvcns.ns.ac.rs



A. Raina et al. (eds.), *Advanced Crop Improvement, Volume 2*,
https://doi.org/10.1007/978-3-031-26669-0_13

Europe by Spanish explorers in the sixteenth century, it was grown as an ornamental flower for more than two centuries. Sunflower use as an industrial crop dates from the eighteenth century and the registration of a patent for oil extraction by Arthur Bunyan in England (Vanozzi, 2006). Probably the most important moment in the evolution of the sunflower as a food and oil crop took place in Russia at the end of seventeenth century, when Russian Tsar Peter Alexeyevich (Peter the Great) brought sunflower seeds to Russia. The Russian Tsar was delighted by the beauty of the flower after seeing it in the Netherlands, where he was on his studies. Soon after sunflower introduction in Russia, academician Severegin suggested that seeds be used for oil extraction. In 1829, Russian farmer Daniil Semenovich Bokarev from the village Alekseevka invented the way to extract oil from seeds and initiated the use of sunflower as an oil crop (Pustovoit, 1990). Also, regulations of the Russian Orthodox Church, which prohibited the consumption of numerous oily foods, encouraged the use of sunflower oil because it was not on the prohibited list.

However, it required substantial effort and time to create the modern sunflower. In recent history, one of the initial "sunflower breeders" were Russian farmers who selected individual plants taking into the account some important phenotypic traits such as stay-green property and head and seed size. Many local varieties were created using the "mass selection" method, among which were the famous Fuksinka, Chernyanka, Puzanok, and Zelenka (Pustovoit, 1967). Expansion of sunflower production facilitated the establishment of oil factories and the development of a number of local varieties, which Russian immigrants brought to the United States, Canada, and Argentina, with Russian Mammoth and Giant of Russia being the most famous. Sunflower breeding began in the early twentieth century, when Russian scientists made significant efforts to improve this oil crop.

An important period in sunflower breeding is the first half of the twentieth century, when Pustovoit succeeded to increase oil content from 33% up to 43% by applying modified recurrent selection called seed reserve (Rauf, 2019). The method is based on individual selection of plants, where one part of the seeds of each plant is used for sowing and the other part is kept as a reserve. By the end of 1960s open, pollinated varieties had reached an oil content higher than 50%. Well-known high-yielding open-pollinated varieties, such as Peredovik, VNIIMK 6540, VNIIMK 8883, Armavirkskii 3497, and others, were adopted worldwide and had high influence on the popularization of sunflower as an industrial crop (Jocić et al., 2015; Gavrilova & Anisimova, 2017).

Unrau and White were the first to grow sunflower hybrids in 1944. The idea was further developed by Putt (Vear, 2016). A favorable characteristic of open-pollinated varieties was self-sterility. Because sunflower is cross-pollinated and exhibits heterosis, it was the best way to obtain the highest yields. This resulted in an increase in seed yield of up to 60% and the development of the first hybrid sunflower varieties, such as Advance. These "hybrids" were not uniform, especially not in flowering time, as they were created by crossing heterogeneous genotypes, and their self-sterility was incomplete. Revolutionary changes in sunflower hybrid production were facilitated by the discovery of cytoplasmic male sterility (CMS) in the

interspecific hybrid *H. annuus* × *H. petiolaris* by French scientist Leclercq (1969) and the subsequent discovery of fertility restorer genes (Kinman, 1970). Comparing to open-pollinated varieties, sunflower hybrids have higher yields, higher oil content, uniformity, and improved disease resistance (Škorić et al., 2012). Sunflower single-cross hybrids have been predominant in production for the past 40 years, and sunflower breeders have achieved significant results in increasing seed yield compared to the previously grown open-pollinated varieties.

A step forward in breeding methods was the use of molecular markers, which had been widely used to accelerate the breeding process and make it more efficient. Compared to conventional breeding, where plants were chosen based on their phenotype, marker-assisted selection (MAS) helps breeders select plants with desirable genes based on genetic linkage between the marker and the targeted gene. Since the first sunflower molecular map was made available in 1995, many gene-controlling traits of interest have been mapped. They represent an invaluable tool for overcoming the numerous obstacles encountered by breeders. Over the years, different molecular markers (from restriction fragment length polymorphism (RFLP) to the more specific such as single nucleotide polymorphism (SNP)) have been used for mapping desirable genes. Combining different genes in one genotype (pyramiding) can be very complicated as genes can mask one another; the SNP molecular marker technique can therefore be successfully used for overcoming that issue, as previously described by Qi et al. (2017). Molecular markers are a commonly applied tool in recurrent selection, when it is necessary to incorporate genes for disease and broomrape resistance, change oil quality (high-oleic), and herbicide tolerance, as well as for the recent development and validation of markers for the purpose of fertility restoration gene detection (Imerovski et al., 2014; Louarn et al., 2016; Dimitrijević et al., 2017; Horn et al., 2019). Differences between genotypes in alleles present at quantitative trait loci (QTL) for various traits are particularly important for breeders, considering that the majority of breeding traits are determined by minor genes. The effectiveness of the comparison between QTL positions depends on the proximity of the marker. However, the development of high-density maps will enable more efficient identification of genes for relevant traits. So far, QTL analysis in sunflower using molecular techniques has enabled the identification of gene positions associated with domestication as well as resistance to diseases and many important traits (Burke et al., 2005; Wills & Burke, 2007; Baack et al., 2008; Mandel et al., 2013; Zubrzycki et al., 2017; Imerovski et al., 2019).

Today, sunflower is a significant source of edible oil in both the world and European markets, along with palm trees, soybeans, and rapeseed. The main sunflower seed producers are Ukraine, Russia, the European Union, and Argentina, accounting for 76% of global sunflower production (Martínez-Force et al., 2015). Almost all sunflower oil production is used for food, while only a small part is used for industrial purposes such as biodiesel production (Fig. 1). Due to its high content of mono- and polyunsaturated fatty acids, as well as vitamin E, sunflower oil is the preferred type of oil in human nutrition in Europe. Refined sunflower oil is light-yellow in color and mild in flavor, so it is most commonly used for cooking, frying,

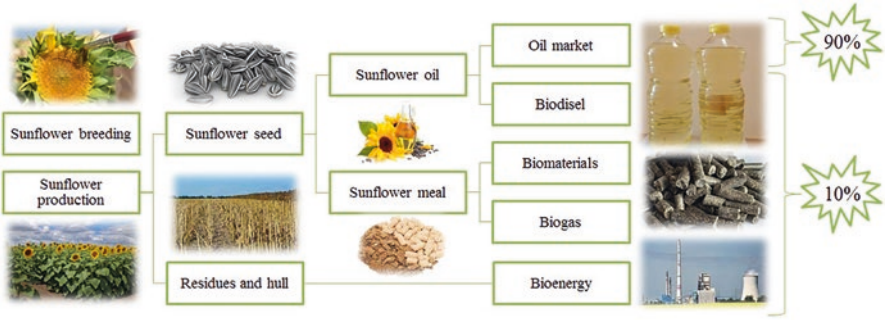



**Fig. 1** Sunflower oil use and its contribution to human nutrition compared to other uses

and preparing salads. It also has a low melting point, making it suitable for preparing sauces and emulsions that need to be refrigerated (Salas et al., 2015). In industry, sunflower oil is used in the frying process and margarine production. During frying, the composition of the oil is altered, so finding the most stable oil type is very important in food preparation. Different types of sunflower oil can be used for frying; although regular oil is widely used, sunflower oil with an altered fatty acid composition proved to be even more suitable for this purpose. Dobarganes et al. (1993) reported that oil stability during frying is strongly dependent on oleic acid content (OAC); sunflower oils with OAC higher than 80% were the most stable. Moreover, Dubinsky and Garces Mancheño (2011) compared the oxidative stability of various oils (including: palm and cotton oils, high-oleic sunflower and canola oil, mid-oleic and regular sunflower oil) and concluded that high-oleic-high-stearic oil type had the highest values. High-stearic oils are, due to their increased content of saturated fats compared to regular sunflower oil, in a semisolid state at room temperature. Thus, this type of oil can be directly used for margarine production, as it helps avoid the use of harmful chemical processes such as transesterification and hydrogenation (Kritchevsky et al., 1995; Fernández-Moya et al., 2002). However, the stability and shelf life of oil also depend on the tocopherol content, which are antioxidants. For example, oils enriched with γ-tocopherol have higher stability and better performance in frying (Lampi & Kamal-Eldin, 1998; García-Moreno et al., 2006).

Besides, confectionery sunflower is gaining in importance as a healthy diet in human nutrition (Hladni & Miladinović, 2019). An integrative approach, combining breeding, -omic technologies, and bioinformatic tools, can be used to improve the sunflower crop to meet future market demands and increasingly pronounced climate change (Miladinović et al., 2019).

This chapter aims to present a review of traditional and molecular breeding approaches used for the improvement of sunflower oil quality and its adaptation to suit different market demands.

## 2 Specificities in Sunflower Breeding

Modern sunflower breeding programs are oriented toward hybrid development by exploiting heterosis achieved by crossing genetically diverse sunflower inbred lines (Jocić et al., 2015). On the genetic level, heterosis is a result of both intra-allelic (domination and super domination) and inter-allelic (epistasis) interactions and is defined as superiority of F1 progeny over its parents. The nature of bi-gender flowers is the main reason why the use of heterosis in sunflowers is only possible through the existence of suitable sources of male sterility and fertility restoration traits. Sunflower hybrids have to show good agronomic performance in different environments and wide adaptation to productive systems to accomplish the requirements of the market and consumer preferences. The breeding of sunflowers needs to deal with the following specific issues:

- *Narrow Genetic Variability of Cultivated Sunflower* Genetic variability of breeding material is essential to successful breeding. Given the narrow genetic base of sunflower, development of new genetic variability is one of the most important aims. Starting material for the development of new sunflower genotypes is usually open-pollinated varieties (Russian, Ukrainian, Argentinian, etc.), land races, public lines, synthetic populations, wild species of the *Helianthus* genus, interspecies hybrids, and other sources (Kaya et al., 2012). Wild *Helianthus* spp. are a significant source of genetic diversity for further improving the cultivated sunflower, including essencial traits such as agronomic and seed-oil characteristics, protein content, fatty acid composition, disease and pest resistance, drought tolerance, cytoplasmic male sterility, and fertility-restoration (Seiler & Rieseberg, 1997; Sukno et al., 1999).
- *Difficulties of Interspecies Hybridization in the Process of Variability Enhancement* Interspecific hybridization is usually applied to transfer genes from wild to cultivated sunflowers, for disease resistance, insect resistance, and resistance to abiotic and biotic stresses. The wild sunflower is frequently used as a male parent (Seiler et al., 2017). Conventional crossing methods have sufficed to produce interspecific hybrids between cultivated sunflower and some wild species of annual diploids. However, most wild diploid species remain in the group of expendable germplasm due to the abortion of embryos at an early developmental stage (Seiler & Rieseberg, 1997).
- *Self-Incompatibility, Self-Fertility, and Inbreeding Depression of Cultivated Inbred Lines* Sunflower is a highly cross-pollinated plant that can tolerate a certain level of self-pollination (Škorić, 2012). The degree of floral self-compatibility and self-fertility depends on genetic factors, environment, and the morphology of floral structure (Miller & Fick, 1997). Self-pollinated plants are uniform in terms of genotype and phenotype, but the occurrence of inbreeding depression causes reductions in productivity, survival, and reproductive ability of genotypes. According to Schuster (1980), after 25 generations of self-pollination, yield decreased by 40% compared to the initial population, while plant height, head

diameter, and 1000-seed weight decreased by an average of around 20%. The significance of inbreeding is in the creation of inbred lines and desirable characteristics that can be used as components in hybrid development. The initial results of crossing two inbred lines of sunflower showed significant heterosis in plant height, head diameter, size, and seed yield.

- *Sunflower Diseases are a Limiting Production Factor and Cause Severe Yield Losses* Over 30 different pathogens can infest sunflowers. Breeding for resistance to these diseases is considered the most effective way to control disease. Sources of resistance or tolerance to most diseases can be found in wild species of the genus *Helianthus*.
- *Long Duration of Breeding* Sunflower breeding is a time-consuming activity that is influenced by numerous factors. Duration can range from a few years (4–6 years), in the case of male line development, to over 15 years when creating CMS lines (Poehlman & Sleper, 1995; Acquaah, 2015). The development of a sunflower hybrid involves: (a) the selection of plants from various germplasm pools for initial breeding crosses; (b) selfing of the selected plants from the breeding crosses for several generations to produce a series of inbred lines (6–8 generations); (c) introduction of CMS into the female line (A-line) using a series of back-crosses (6 generations); (d) crossing the selected female lines with male lines to produce hybrid progeny ($F_1$); (e) the evaluation of hybrid combinations. The selection cycle, from crossing to hybrid release, therefore requires approximately 12–15 years. Time reduction can be achieved by using off-season nurseries at lower latitudes or in the opposite hemisphere and using greenhouses where two more growing seasons can be obtained in order to accelerate the breeding process.
- *Specific Hybrid Sunflower Seed Production is Accomplished by Using Cytoplasmic-Genetic Male Sterility and Maintenance of Male-Sterile (A-) Line* The system encompasses a female parent line (two isogenic lines, A-sterile line and B-fertile line), and a male parent line (restorer or R-line of Rf-line). For hybrid seed production, the female A-line is crossed with the male R-line. The A-line, does not produce pollen since it has CMS and is therefore referred to as "female," R-line restorer plants are self-fertile and produce seeds; they are referred to as "male" as their purpose in hybrid production is to provide pollen to the female lines in the field. The female line cannot be self-pollinated because it is male-sterile. To maintain parental seed stocks of the female line, the A-line is crossed with a B-line, also known as the "maintainer" line.

The objectives of breeding modern sunflower hybrids are primarily focused on seed and oil yield, combined with tolerance to abiotic and biotic stress, with an emphasis on disease resistance and a high adaptability requirement. Moreover, high demand is needed to improve quality traits, such as oil quality traits, which are essential to consumer preferences.

## 3 Main Directions in Sunflower Breeding Related to Nutrition Quality

Basic directions in sunflower breeding programs concerning oil nutrition can be divided into three subcategories: breeding classical oil hybrids, hybrids with altered oil quality, and confectionery sunflower.

### *3.1 Classical Oil Type*

The main direction in sunflower breeding is the development of hybrids with high seed and oil yield. To achieve these goals, sunflower breeders are challenged by limiting factors such as diseases, insects, broomrape, and drought. In order to achieve high and stable yields, sunflower hybrids should be adaptable to different environments, resistant to lodging, and attractive to pollinators. Also, reduced plant height and shorter cycle length (earliness) are considered advantages by many sunflower growers today, as shorter plants are more tolerant to lodging and shorter vegetation enables growing sunflower as a second crop or in environments with a limited number of sunny days.

The productivity of sunflower as an industrial crop is reflected in its oil yield. The main reason for growing sunflowers is the production of sunflower vegetable oil. The development of lines and hybrids with improved oil yield is therefore the most important goal of sunflower breeders. The oil content depends on both the genetic basis and environmental factors such as average daily temperatures and moisture levels during the grain filling period. In an effort to maximize its economic viability as an oily plant species, sunflower breeders have significantly increased the oil content. It is a highly heritable character, and parental lines express a highly significant positive correlation with their hybrids. The highest oil content is 60–65% (Vear, 2010), which is almost double the content that open-pollinated varieties had at the beginning of sunflower breeding. Modified recurrent selection, called "seed reserve" that Pustovoit used for improving sunflower oil content in the first half of the twentieth century is still considered an effective method in breeding for high oil content. Although the genetic variability of sunflower provides the possibility for the development of high-yielding hybrids, progress in breeding for oil content is difficult and slow due to the negative correlation between very high oil content and high seed yield (Škorić, 2012; Jocić et al., 2015).

Defining a breeding goal is of utmost importance for successful hybrid development. Knowledge about the starting material, its selection values in terms of possessing desirable genes, and the genetic basis of important traits (number of genes and mode of inheritance) is of great importance for the development of productive hybrids. Seed yield is characterized by low heritability due to quantitative behavior and high environmental impact, so both additive and nonadditive, or dominant, components of genetic variance have significance in the inheritance process. With

that in mind, sunflower hybrids are tested in multilocation trials in order to evaluate their productivity.

### *3.2 Altered Oil Quality*

Apart from high oil content, great attention is given in sunflower breeding to the alteration in oil quality, such as development of high-oleic genotypes. The constituents of sunflower seed (achene) are oil (44% in average), proteins (18%), cellulose (15%), water (9%), carbohydrates, and minerals (14%) (Andrianasolo et al., 2016). Regular sunflower oil mainly consists of polyunsaturated linoleic fatty acids (18:2) around 70%, and monosaturated oleic acid (18:1) around 20%, while saturated palmitic and stearic fatty acids are found in lower percentages. Moreover, tocopherols (vitamin E) and phytosterols found in sunflower oil have numerous positive effects on human health (Bramley et al., 2000; Patel & Thompson, 2006; Gotar et al., 2008). Besides vitamin E, sunflower oil contains vitamins A, D, and K, in addition to phosphatides, stearin, phenolics, carotenoids, and other compounds. The main parameter on which the fatty acid composition of sunflower oil depends is the final use; it can be used in salads, cooking, for margarine production, biodiesel production, lubricants, and for different purposes in the cosmetic industry. Because of its beneficial effect, sunflower oil is considered to be of premium quality for human consumption. There are two main types of sunflower oil intended for human consumption: classic (linoleic) and high-oleic (>80% oleic acid). The high-oleic type was developed by Soldatov, who used chemical mutagens to induce mutations on variety VNIMK 8931, and created variety Pervenets (Soldatov, 1976). Variety Pervenets with OAC above 80% and other mutant lines with oleic acid above 60% were used as a source for the creation of mid (>60%) and high-oleic (>80%) types of sunflower oil worldwide (Jocić et al., 2015; Rauf, 2019). Besides high-oleic sunflower oil, induced mutations and mutant lines can also be used to develop new types of sunflower oil, such as those with different concentrations of palmitic and stearic fatty acids, among others (Fernández-Martínez et al., 2007; Velasco et al., 2008). As the content of a specific fatty acid can be affected by altering the content of another fatty acid, breeders need to have knowledge of fatty acid synthetic pathways and common steps. Moreover, it is important to get acquainted with the mode of inheritance of an oil quality trait that will be improved by breeding.

In addition to the composition of fatty acids, the quality of sunflower oil also depends on the content of tocopherols and phytosterols. The total content of tocopherols (vitamin E) in standard sunflower oil is approximately 700 mg/kg, and can reach up to 1000 mg/kg. There are four derivatives of tocopherol: α-tocopherol, β-tocopherol, γ-tocopherol, and δ-tocopherol (Demurin et al., 1996). Regular sunflower oil contains mostly α-tocopherol (>90%), but it is possible to obtain different quantities of tocopherols and thus different quality of sunflower oil with combinations of *tph* genes. Škorić (2008) reported that $tph_1$ produces 50% α and 50% β-tocopherol, and $tph_2$ produces 0–5% α and 95–100% γ-tocopherol, while their combination ($tph_1tph_2$) produces 8–40% α, 0–25% β, 25–84% γ, and 8–50% of

δ-tocopherol. Combining genes for high-oleic (*Ol*) acid and genes for different tocopherol (*tph*) content, it is possible to obtain sunflower oil of different quality (Škorić et al., 2008; Škorić, 2012). So far, different mutants have been developed with alterations in saturated fatty acid levels, as well as differences in oleic acid and tocopherol levels than in any other oilseed crop (Fernández-Martínez et al., 2009).

### *3.3 Confectionery Sunflower*

Although sunflower is mostly used to obtain edible oil, non-oil, or confectionery, it has found its place in production in many countries worldwide. Unlike in oil type sunflower, the seeds of confectionery sunflower are usually large, black with white stripes, or colorful with a thick hull loosely attached to the kernel (Jocić et al., 2015). The hull is easily separated from the kernel, which is used in the baking industry as an addition to salads and other foods. Regarding its use as a snack food, one of the main characteristics related to market requirements is achene color and seed size. Black achene color is mainly popular in Russia and some Balkan countries, consumers in Turkey prefer white achene with gray stripes, while in the United States, Spain, and China achene should be gray colored with stripes. One of the main goals regarding confectionery sunflower is high protein content, as it is of interest for human and animal consumption. Partially or completely dehulled achenes are used as a meal for feeding swine and poultry, while whole achenes are used for small animals and birds (Seiler & Jan, 2010). Protein content is determined by multiple genes and is also negatively correlated with oil content. Besides high protein content, other important traits are also amino acid content (lysine), 1000 seed weight, kernel content and low oil content and oil stability, uniformity in seed size and color and easy dehulling (Jocić et al., 2015; Kaya, 2016). In order to develop tasty confectionery sunflower with high quality, higher oleic acid and tocopherol content are also needed, as are different protein qualities like albumin, globulin, and gluten (Kaya, 2016). In addition to the specific goals of confectionery sunflower breeding, its general breeding goals are similar to those of breeding classic oil types: high seed yield, adaptability and stability, high level of tolerance and resistance to main diseases; resistance to parasitic weed broomrape, drought tolerance, and tolerance to herbicides. Due to its positive effects on human nutrition, the production of confectionery sunflower has an increasing trend (Hladni et al., 2017).

## 4 Genetic Resources for Oil Quality Improvement

The sunflower is one of the most adaptable field crops, characterized by a deep root system and moderate water consumption. Taking into account the global trend of climate change, as well as the increasing needs of the growing population for edible oil, there is a constant need for the development of hybrids that will meet current

global demands. Considering that the genetic basis of cultivated sunflower is relatively narrow, the existing genetic resources are an indispensable source of genetic diversity that can be used for the exploitation of valuable genes (Anđelković et al., 2020).

## *4.1* Helianthus *Genus – Crop Wild Relatives (CWR)*

Native to North America, *Helianthus* genus, with 37 perennial and 14 annual species, is an important source of valuable genes for many desirable traits. Greater diversity of wild relatives enables better adaptation to the changing environmental conditions ensuring maintenance of the economic viability of cultivated sunflower. The largest and most important collection of wild sunflower species is located in the USDA-ARS National Plant Germplasm System (NPGS), which is maintained at the North Central Regional Plant Introduction Station (NCRPIS) in Ames, Iowa, USA (Kaya, 2016; Seiler & Jan, 2010). For more than 30 years, accessions from this collection were distributed all around the world as the basis for the development of wild species gene banks and research programs in Argentina, France, Italy, Spain, Germany, Bulgaria, Romania, the Czech Republic, Hungary, Russia, Serbia, India, China, and Mexico (Seiler & Jan, 2010). Thanks to that, important bigger and smaller collections of wild sunflower species are now maintained in other countries like Serbia (Institute of Field and Vegetable Crops, Novi Sad), Bulgaria (Dobroudja Agricultural Institute, General Toshevo), France (National Institute for Agricultural Research, Montpellier), Argentina (Instituto Nacional de Tecnología Agropecuaria, Pergamino), Spain (Institudo de Agricultura Sostenible, Cordoba), Ukraine (Institute of Oilseed Crops, Zaporozhie), and Russia (Vavilov All-Russian Institute of Plant Genetic Resources, Saint Peterburg and Institute of Sunflower, Veidelevka). The usefulness of wild relatives depends to a large extent on the success of crosses with cultivated sunflower and the possibility to transfer genes of interest. Success rates in obtaining interspecies hybrids have been increased by exploiting in vitro techniques. Probably the greatest importance of using wild species is deriving PET1 CMS cytoplasm and fertility restoration genes (*Rf*), which significantly increased the economic viability of growing sunflowers through hybrid cultivation. Moreover, wild sunflower species contain considerable variability regarding oil content and quality. High concentrations of linoleic fatty acid suitable for the production of soft margarine are found in *H. porteri*, *H. debilis* subsp. *Tardiflorus,* and *H. exilis* (Seiler, 2007). Different concentrations of oleic fatty acid have also been found in some annual and perennial wild species, while lower concentrations of saturated palmitic and stearic fatty acids have been documented in *H. annuus* and *H. giganteus* (Seiler, 1998, 2007). Regarding the tocopherol content, Demurin et al. (1996) found little variation in the trait, with α-tocopherol as the predominant form of the ten wild sunflower species maintained in the collection of the Institute of Field and Vegetable Crops.

## 4.2 Local Populations, Open Pollinated Varieties, and Public Lines

Local populations are a valuable source of genetic variability as they are well adapted to specific pedoclimatic conditions and possess tolerance for and resistance to certain constraints. By selecting plants based on their phenotype, Russian farmers were able to develop quite some number of different cultivars, including ones with improved traits like sunflower moth and broomrape resistence (Seiler & Jan, 2010). During the first half of the twentieth century, open-pollinated varieties with improved oil content such as the well-known Kruglik A-41, were introduced into sunflower production. Applying the method of individual selection, popular open-pollinated varieties were developed at the Saratov experimental station, such as Saratovsky 169, 206, 1915, 420, and others (Kaya, 2016). The great importance of open-pollinated sunflower varieties can be attributed to the extensive work of V.S. Pustovoit, who managed to combine resistance to broomrape race B and high productivity with high oil content (>50%), resulting in Peredovik as one of the most famous varieties. The level of oil content achieved in Peredovik by Pustovoit has more or less remained in sunflower hybrids today.

## 4.3 Mutations

Ever since 1930s, plant mutation breeding, based on random genetic variation induction, has successfully been applied in creating new variability (Raina et al., 2016, 2017; Khursheed et al., 2018a, b, c; Laskar et al., 2018a, b). Mutagens have the potential to induce inheritable changes in the genome of plants and thus increase the frequency of obtaining desired individuals (Raina et al., 2020a, b; Goyal et al., 2019a, b). Different types of mutagens have successfully been applied in induced mutation breeding (Laskar et al., 2015, 2019; Khursheed et al., 2019). In general, changes that have been exploited in breeding are the obtained dominant traits, while recessive traits were mainly lost in the process of selection (Wani et al., 2017, 2021; Goyal et al., 2021a, b). Using the Targeting Induced Local Lesion in Genome (TILLING) method, it is possible to detect single polymorphisms in plants and thus create and discover new traits (Sabetta et al., 2011). This method was exploited by Kumar et al. (2013), who developed an ethyl methane sulfonate (EMS) mutant population and identified 26 induced mutations while investigating sunflower seed oil quality.

Induced mutations in sunflower provided many important traits by changing plant characteristics and productivity (Miller & Vick, 1999; Kalaydzhyan et al., 2007; Cvejić et al., 2009, 2011). Alteration in fatty acid content is the most common trait to be changed in sunflower by induced mutation (Cvejić et al., 2014). The first high-oleic sunflower variety, Pervenets, was obtained by exposing seeds of the VNIIMK 8931 variety to a solution of dimethyl sulfate (DMS) and selecting for an

elevated content of oleic acid over 840 g/kg (Soldatov, 1976). Worldwide, Pervenets is used as a high-oleic trait donor in breeding programs. There are publications about other sources of high-oleic mutants with 800 g/kg of oleic acid (Ivanov & Ivanov, 1992) and with 900 g/kg of oleic acid (Andrich et al., 1992). Recently, new genotype with ultra-high-oleic content was developed in which oleic acid content is not affected by temperature during grain filling compared to high-oleic Pervenets and traditional genotypes (León et al., 2013; Alberio et al., 2016). The enhanced oxidative stability of oil containing a higher content of saturated fatty acids makes it more desirable for use. Physical and chemical mutagens were used to develop mutants with different levels of palmitic and stearic acid concentrations (Ivanov et al., 1988; Osorio et al., 1995).

Apart from fatty acid content, significant accomplishments were made in altering tocopherol (vitamin E) content. A spontaneuos mutation raised β-tocopherol content by 50%, approximately, in the self-pollinated variety VNIIMK in Krasnodar in 1983 (Demurin, 1986). This source was used for the development of line LG15 with elevated β-tocopherol content. Soon after, another sunflower line containing 5% α- and 95% γ-tocopherol, LG17, was created (Popov & Demurin, 1987). Chemical mutagen EMS was used to develop the inbred line IAST-1 with more than 90% of γ-tocopherol (Velasco et al., 2004).

## *4.4 Genetic Stocks of Public Breeding Sector*

Genetic stocks developed and maintained in various research institutes in the diverse pedoclimatic conditions are also a valuable source of genetic diversity. One of the greatest sunflower collections is developed at the Institute of Field and Vegetable Crops in Novi Sad, with over 7000 inbred sunflower lines (Atlagić & Terzić, 2014). A part of the collection has been characterized in terms of oil quality, including both fatty acid and tocopherol content (Cvejić et al., 2016; Dimitrijević et al., 2016) and used in crop improvement. Another notable collection of the cultivated sunflower is maintained at the USDA-ARS NPGS in Ames (Marek, 2016). This collection contains an association mapping population consisting of 288 accessions and over 300 pre-breeding sunflower lines that contain introgressions from 11 wild species. Accessions of the NPGS collection are publicly available for research and educational purposes, contrary to some other proprietary collections. A momentous collection is also kept at the Vavilov All-Russian Institute of Plant Genetic Resources from Saint Petersburg, Russia, which significantly contributed to sunflower oil quality improvement, as described above (Gavrilova et al., 2014). The French National Institute for Agricultural Research in Montpellier (INRA) assembled a significant collection of 400 landraces, open-pollinated varieties, and breeding pools (Mangin et al., 2017a). Important research institutes, especially in the Balkan region, are Trakya University from Edirne in Turkey, the Dobroudja Agricultural Institute from General Toshevo, Bulgaria, and the National Agricultural Research and Development

Institute from Fundulea, Romania (NARDI Fundulea), while in South America is the Active Germplasm Bank of the National Institute of Agricultural Technology, (INTA) Manfredi (AGB-IM), Argentina. These Institutes gave considerable contribution and broadened sunflower genetics with multitude of numerous important traits. The collections differ in the level of oil content and composition. Demurin and Borisenko (2011) found great variation in OAC in sunflower inbred lines from VNIIMK, VIR, USDA, and INRA. Moreover, Velasco et al. (2010) found a great variability in vitamin E content (between 119 and 491 mg/kg) in sunflower collection comprising 952 genotypes, of which 631 were different sunflower accessions from the USDA and 321 were sunflower lines and cultivars in the scope of the research program of the Institute for Sustainable Agriculture at Cordoba, Spain.

## 5 Main Objectives in Sunflower Breeding for Nutritional Quality

### *5.1 Yield and Yield Related Traits*

The main objective of developing oilseed sunflower hybrids is to obtain maximum oil yield per unit area. This can be achieved by creating productive $F_1$ hybrids that are high yielding (genetic potential over 5 t/ha), stable, and tolerant to abiotic and biotic stresses. Seed yield is a quantitative trait significantly affected by the environment (Kaya et al., 2012). For improving sunflower productivity, selection of the high yielding plant has been used most often, but genetic gains from such selection were often hampered by the complex nature of the trait and significant G × E interaction effects. By enhancing combining ability and selecting genotypes adapted to particular conditions, e.g., enduring stems, high self-fertility, defined head inclination to improve resistance to stress such as sunburn and bird damage, significant improvements were made concerning seed yield (Fernandez et al., 2009). Particularly, in areas with limited pollinator populations, sowing sunflower genotypes with a high degree of self-fertility is imperative for achieving a high yield (Jan & Seiler, 2007). Moreover, breeders consider the seed number per head an important trait when selecting inbred lines. During the selection process, it is important to choose combinations with a high heterotic effect for a high number of seeds per head (more than 1500). Other relevant traits, significant for sunflower breeding are: the number of plants per hectare (55–60 thousand), hectoliter test weight (50 kg per hl), thousand seed weight (over 80 g), low shell content (20–24%), and high oil content in seed (over 50%). The targeted traits are essential to improve yield and satisfy producers, processors, and market demands. In addition to the above, when creating an ideal model plant, breeders pay special attention to adapting the architecture of the plants to specific environmental conditions. Those traits would include plant height, head shape, size, and position on the stem, leaf size, and number, as well as their duration and position. All of the traits are associated with high yield

and well-developed vegetative plant growth (Jan & Seiler, 2007). Another trait associated with seed yield is the earliness of the hybrids, with an earlier flowering period and physiological maturity duration shorter than 107 days (Kaya et al., 2009).

### 5.1.1 Molecular Tools

Numerous types of molecular markers have been associated with yield and yield-related QTLs. Mestries et al. (1998) examined QTLs associated with seed weight across three generations ($F_2$–$F_4$) of a cross GH × PAC2 by using isoenzyme loci and RFLP markers and detected associations only in $F_3$ generation. Two QTLs were associated with the trait, one major on linkage group (LG) A and an additional one on LG C, explaining 28.5% of the phenotypic variation in total. The authors observed the greatest seed weights in unbranched families. A few years later, Mokrani et al. (2002) identified two QTLs for seed yield per plant on chromosome 9 and one QTL for 1000-seed weight on chromosome 16, explaining 50.7% and 5.4% of the phenotypic variation, respectively, by use of Amplified Fragment Length Polymorphism (AFLP) and Simple Sequence Repeat (SSR) markers. Two QTLs detected on chromosome 9 were common for seed yield per plant and oil percentage. Rachid Al-Chaarani et al. (2004) also used AFLP and SSR markers for mapping four QTLs for seed weight per plant on chromosomes 4, 6, 9, and 21 and three QTLs associated with 1000-seed weight on chromosomes 4, 6, and 9. The QTLs detected explained 43% and 53% of the phenotypic variation, respectively. Chromosome 9 contained a major QTL for 1000-seed weight, explaining 37% of the phenotypic variation.

Tang et al. (2006) exploited SSR markers for a comprehensive study of QTLs associated with different seed traits: seed length, width, and depth, 100-seed weight, 10-kernel weight, 10-pericarp weight, and kernel to pericarp weight ratio. The authors associated between four and six QTLs per examined seed trait, with three quarters of the mapped QTLs clustered on four chromosomes (5, 10, 16, and 17). The authors reported that detected QTLs for tested seed traits on chromosomes 10 and 17 were also tightly linked to apical branching and phytomelanin pigment. Similar findings concerning the association of seed traits with branching were formerly reported by Mestries et al. (1998). Yue et al. (2009) exploited Target Region Amplification Polymorphism (TRAP) and SSR markers and identified 51 QTLs associated with 10 seed morphological traits (including 100-kernel weight, 100-seed weight, kernel-to-seed weight ratio, seed width, length, etc.) in $F_2$ and $F_3$ generations obtained by crossing oilseed and confectionery sunflower. Out of 51 QTLs, 32 were identified in both generations examined. Individually, the identified QTLs explained between 5.1% and 29.3% of the phenotypic variation. Concerning the 100-seed weight trait, four and three QTLs were mapped in the $F_2$ and $F_3$ generations, respectively. Common QTLs for both generations were mapped on chromosomes 1, 10, and 13. Vanitha et al. (2014) associated between two and five SSR markers to seed yield, hull percentage, and 100-seed weight, as well as kernel and hull weight.

As heterosis is an important trait in hybrid breeding, so far there has been no correlation found between SSR-based genetic distances and heterosis in sunflower (Gvozdenović et al., 2009; Kaur et al., 2019).

## *5.2 Oil Content and Quality Traits*

Oil content is a quantitative trait; thus, the expression of the trait is dependent on cultivation methods, environmental conditions, and the genotype. The most common mode of inheritance of oil content in $F_1$ is dominance of the better parent (Pustavojt, 1966; Fick, 1975). However, there were cases of dominance of the parent with lower oil content (Schuster, 1964; Fernández-Martínez et al., 1979), heterosis (Morozov, 1947; Schuster, 1964; Voljf & Dumačeva, 1973) and negative heterosis (Gill & Punia, 1996). The existence of a strong additive effect in the inheritance of oil content in sunflower was reported by numerous authors (Fick, 1975; Škorić, 1976; Rao & Singh, 1977; Rojas & Fernández-Martínez, 1998), while a stronger impact of the dominant component of the genetic variance was reported by Schuster (1964), Kovacik and Skaloud (1972), Petrov (1992), etc. Obtaining hybrids with high oil content is performed by crossing chosen cultivated sunflower genotypes within the existing genetic variability. The breeders choose parental lines that contain a greater number of minor genes that are involved in increasing oil content and combine them to obtain $F_1$ progeny with higher oil content.

Qualitative traits, like tocopherol and fatty acid content, are opposite to oil content and qualitatively inherited, i.e., governed by one or few genes. The phenotypic expression of such traits is thus to a lesser extent affected by environmental conditions compared to quantitative traits (Velasco et al., 2002).

Novel variation within germplasm is necessary for creating hybrids with a modified fatty acid content of oil. In the absence of naturally existing variation, an alternative strategy generates new diversity. These strategies would include the examination of existing germplasm as well as performing interspecific crossing or using somatic hybridization, induced mutagenesis, or gene transfer (Velasco et al., 2002). As was shown previously, induced mutations are commonly exploited for increasing genetic variability in sunflower and have enabled the creation of numerous mutants with altered fatty acid content (Soldatov, 1976; Fernandez-Martinez & Dominguez-Gimenez, 1988; Ivanov et al., 1988; Ivanov & Ivanov, 1992; Andrich et al., 1992; Osorio et al., 1995; Miller & Fick, 1997; Velasco et al., 2004). However, mutant genotypes that refer to novel oil qualities most often demand the use of additional breeding efforts in order to eliminate undesirable seed quality or agronomical traits before becoming suitable for commercial exploitation. Therefore, several selection cycles are recommended before the breakthrough of high-yielding and high-quality hybrid development (Velasco et al., 2002).

Primarily, the advantage of sunflower seeds is for oil usage. However, meal residues after oil extraction are a valuable product due to their high protein content, as with other oilseeds (González-Pérez, 2015). Depending on the process used for oil

extraction, sunflower meal contains nearly 40% protein when mechanical extraction of hulled seeds is exploited, around 50% if an organic solvent is utilized (Robertson et al., 1972), and 53–66% if defatted meal is prepared from dehulled seeds (Bau et al., 1983). Sunflower proteins have been appraised as a suitable food ingredient but are not frequently used in human consumption. Instead, they enrich the food with sunflower flour, especially dairy and meat products, baby formulas, pasta, and bakery products (González-Pérez & Vereijken, 2007). There is a huge and growing demand for the use of plant proteins worldwide. Plant proteins as functional ingredients in food production are an economical and viable alternative to animal proteins. Oil crops are a supreme source of protein production. So far, proteins obtained from soybean grain have been mainly exploited for this purpose. However, other crops such as peas, chickpeas, husks, sunflowers, rapeseed, etc. could be appropriate for protein use. Sunflower seeds are notably attractive due to their widespread production and availability in areas where soybeans are not or rarely grown. Moreover, sunflower is an acclaimed crop to both farmers and processors because of its extensive oil extraction use. González-Pérez and Vereijken (2007) reported that sunflower seeds contain small amounts of anti-nutritional factors (such as cyanogens, protease inhibitors, and glycosylates), while their amino acid compositions (except lysine) are consistent with the FAO pattern. However, the development of sunflower seeds as a source of dietary protein is generally prevented by two factors: the presence of phenolic compounds, chlorogenic acid (CGA- dark coloration of sunflower protein products), and protein denaturation during oil processing and extraction. Therefore, sunflower breeding can be focused on increased protein functionality and the development of genotypes with low chlorogenic acid. Such sunflowers will broaden the range of livestock feed, aquaculture, and human food production applications.

### 5.2.1 Molecular Tools

As breeding for oil quantity is a major breeding trait, it is no surprise that the initiation of molecular marker application in sunflower studies initiated an immediate examination of the oil quantity-marker connection. Nowadays, great efforts have been put into developing markers for high OAC, a highly demanded trait.

#### Oil Content

Molecular studies on associating markers with oil traits were initiated with the construction of one of the first sunflower linkage maps, when Leon et al. (1995) mapped six OTLs that explained 57% of the genetic variability of seed oil percentage by use of RFLP markers, with additive gene action as a predominant mode of inheritance (Table 1). As quantitative traits are both genetically and environmentally dependent, the same mapping population was assessed at four locations in the USA and Argentina (Leon et al., 2003). In total, eight QTLs were detected on seven

**Table 1** QTLs and major genes associated with oil traits detected with different mapping populations and marker systems

| Trait | Trait/gene abbreviation | Cross | Population | Linkage groups/ chromosomes | Number of QTLs | Total variance explained | Markers linked to trait | Distance (cM) | References |
|---|---|---|---|---|---|---|---|---|---|
| Oil quantity | | | | | | | | | |
| Seed oil percentage | *sop* | ZENB8 × HA89 | $F_2$: $F_3$ | B, C, I, N | 6 | 57.0 | | | León et al. (1995) |
| Seed oil percentage | *sop* | ZENB8 × HA89 | $F_2$: $F_3$ | B, C, G, I, L, M, N | 8 | 88.0 | | | León et al. (2003) |
| Oil content | / | GH × PAC2 | $F_2$ | A, Q | 2 | 19.4 | | | Mestries et al. (1998) |
| Oil content | / | GH × PAC2 | $F_3$ | A, Q, C | 3 | 53.8 | | | Mestries et al. (1998) |
| Oil content | / | GH × PAC2 | $F_4$ | A, Q | 2 | 26.1 | | | Mestries et al. (1998) |
| Oil percentage in seed | *pog* | L1 × L2 | $F_2$ | 9, 11, 12, 13 | 7 | 90.4 | | | Mokrani et al. (2002) |
| Oil content | *oil* | XRQ × PSC8 | $F_2$ | 2, 3, 5, 7, 12 | 5 | 68.1 | | | Bert et al. (2003) |
| Oil content | *oil* | XRQ × PSC8 | $F_2$ | 2, 4, 5, 7, 12 | 5 | 70.1 | | | Bert et al. (2003) |
| Oil percentage in seed | *pog* | PAC2 × RHA266 | RIL | 8, 11, 13, 21 | 4 | 39.0 | | | Rachid Al-Chaarani et al. (2004) |
| Seed oil concentration | *soc* | RHA280 × RHA801 | RIL | 1, 4, 9, 10, 16, 17 | 6 | 55.7 | | | Tang et al. (2006) |
| Oil content (%) | *OC* | COSF 7A 9 HO 5–13 | $F_2$ | 7 and 8 | 2 | /* | | | Premnath et al. (2016) |

(continued)

**Table 1** (continued)

| Trait | Trait/gene abbreviation | Cross | Population | Linkage groups/ chromosomes | Number of QTLs | Total variance explained | Markers linked to trait | Distance (cM) | References |
|---|---|---|---|---|---|---|---|---|---|
| Oleic acid content | | | | | | | | | |
| Oleic acid content | *Ol1* | HAOL-9 × CAS-3 | $F_2$ | / | | | AC10-765<br>F15-690 | 7.2<br>7.0 | Dehmer and Friedt (1998) |
| Oleic acid content | *C18: 1* | CAS-3 × HAOL-9 | $F_2$ | 1, 8, 14 | 3 | 58.4 | | | Pérez-Vich et al. (2002) |
| Oleic acid content | *Ol* | RHA280 × RHA801 | RIL | 14 | | | F13/R5<br>F14/R13<br>ORS391<br>ORS832<br>ORS1180<br>CRT20<br>CRT25<br>ORS333<br>ZVG64 | 0.0<br>0.0<br>0.0<br>0.0<br>0.0<br>2.6<br>2.6<br>2.6<br>3.2 | Schuppert et al. (2006) |
| Oleic acid content | *Ol* | BD40713 × BE78079 | $F_2$ | | | | Fsp-b_R1 | 0.0 | Lacombe et al. (2009) |
| Oleic acid content | *OAC* | COSF 7A 9 HO 5–13 | $F_2$ | 8, 9, 14 | 3 | /* | | | Premnath et al. (2016) |
| Oleic acid content | *OAC* | L-1-OL-1 × 86-1 | $F_2$ | 6, 9 | 3 | /* | | | Zhou et al. (2018) |
| Stearic acid content | | | | | | | | | |
| Stearic acid content | *C18: 0* | HA89 × CAS-3 | $F_2$ | 1, 7 | 2 | 79.3 | | | Pérez-Vich et al. (2002) |
| Stearic acid content | *C18: 0* | HAOL-9 × CAS-3 | $F_2$ | 1, 3, 8, 14 | 4 | 84.4 | | | Pérez-Vich et al. (2002) |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Stearic acid content | *C18: 0* | HA-89 × CAS-20 | $F_2$ | 3, 11, 13 | 3 | 43.6 | | | Pérez-Vich et al. (2004) |
| Stearic acid content | *C18: 0* | P-21 × CAS-3 | $F_2$: $F_3$ | | | | ORS243<br>ORS1161 | 0.5<br>3.9 | Pérez-Vich et al. (2006) |
| Tocopherol content | | | | | | | | | |
| Beta-tocopherol content | *Tph1* | CAS-12 × T589 | $F_2$: $F_3$ | 1 | | | ORS1093<br>ORS222<br>ORS598 | 0.0<br>0.0<br>0.0 | Vera-Ruiz et al. (2006) |
| Gamma-tocopherol content | *Tph2* | CAS-12 × IAST-540 | $F_2$ | 8 | | | ORS599<br>ORS312 | 1.9<br>3.6 | García-Moreno et al. (2006) |
| Total tocopherol content | *TTC* | PAC2 × RHA266 | RIL | 1, 8, 10, 14 | 5 | 45.0 | | | Haddadi et al. (2012) |

/* The value of total variance explained with all makers was not provided by the authors

chromosomes, explaining 88% of the genetic variability for seed oil concentration, with the majority of QTLs having an additive effect and some being dominant or overdominant. All QTLs associated with high oil content originated from the parental line with higher seed oil content (HA 89). Mestries et al. (1998) identified between two and three QTLs that are associated with oil content in each generation: $F_2$, $F_3$, and $F_4$ of a cross GH × PAC2, accounting for 19.4%, 53.8%, and 26.1% of phenotypic variability, respectively. The identified QTLs originated from both parental lines.

Mokrani et al. (2002) mapped six QTLs on chromosomes 9, 11–13 using AFLPs and SSRs, thereby explaining 90.4% of the phenotypic variance for oil percentage. The QTL *pog-13-1* mapped on chromosome 13, with an epistatic effect, was characterized as the most important one, accounting for 47% of phenotypic variance. Henceforth, Bert et al. (2003) mapped five QTLs for oil content in both $F_2$ and $F_3$ populations, obtained by crossing XRQ × PSC8, which in total explained 68.1% and 70.1% of the phenotypic variation, respectively. Contrary to these studies, Rachid Al-Chaarani et al. (2004) identified four QTLs with a moderate phenotypic effect, explaining in total 39% of the phenotypic variance ($R^2$ ranging between 8 and 13% per QTL) in RILs obtained from a cross of PAC2 × RHA266. All identified alleles with a positive effect on the percentage of seed oil originated from RHA266.

Tang et al. (2006) exploited SSR and insertion-deletion (INDEL) markers for the identification of QTLs associated with seed oil concentration in RILs obtained from a cross of RHA280 × RHA801, in which RHA801 is an oilseed parental line. In total, six QTLs were detected on chromosomes 1, 4.9, 10, 16, and 17, explaining 55.7% of the phenotypic variation. Alleles with the positive effect of the increased oil trait originated from RHA801.

Premnath et al. (2016) identified two QTLs associated with oil content on chromosomes 7 and 8, explaining 12.8% and 14.9% of the phenotypic variance, respectively, in the $F_2$ population obtained by crossing COSF 7A 9 (high oil content) and HO 5–13 (moderate oil content).

### Increased Oleic Acid Content

As mentioned previously, the complexity of the increased high-oleic trait lies in the nature of the trait, as OAC can vary in different genetic backgrounds of the recipient line as well as different environmental conditions. A lot of molecular studies were conducted on examining high-oleic genotypes of sunflower, in which the high OAC trait originates from cultivar Pervenets (Soldatov, 1976). Increased OAC is a consequence of gene silencing caused by a partial duplication of the *FAD2-1* (oleoyl-phosphatidyl choline desaturase) gene (Lacombe et al., 2002; Schuppert et al., 2006). This duplication is termed the "*Ol* mutation/locus" (Dimitrijevic et al., 2017) or sometimes "$Ol_1$," as the different inheritance patterns have been reported. Dehmer and Friedt (1998) examined several $F_2$ combinations. However, only HA89 × HA-OL9 displayed a 3:1 segregation ratio for high OAC. Two Random Amplified Polymorphic DNA (RAPD) Polymerase Chain Reaction (PCR)

fragments, AC10-765 and F15-690, were mapped in the HA89 × HA-OL 9 $F_2$ population, 7.2 cM and 7.0 cM from the *Ol*$_1$ locus, respectively. Pérez-Vich et al. (2002) crossed HAOL-9 with the high-stearic acid mutant CAS-3 and mapped three QTLs on chromosomes 1, 8, and 14 using RFLP and AFLP markers. Of the three QTLs, the one on chromosome 14 was marked as the most important, explaining 56.5% of the phenotypic variation. Significant epistatic interaction was observed between QTL on chromosome 14 originating from HAOL-9 and QTL on chromosome 8 originating from CAS-3. The authors reported that an oleoyl-PC desaturase locus (OLD7) co-segregated with the *Ol* gene on chromosome 14. Similar findings, reporting that the *Ol* gene was located on chromosome 14, were obtained by Schuppert et al. (2006), who developed diagnostic dominant INDEL markers for detection of the tandem *FAD2-1* repeats and identified different SNPs, SSRs, and INDELs in downstream sequences of *FAD1-2* and the 3'UTR of *FAD2-1*. As the authors developed the dominant markers, they suggested combining the dominant INDEL marker with flanking codominant markers for the identification of potential heterozygous plants (Table 1). Furthermore, Lacombe et al. (2009) used a codominant SSR marker for amplification of the intron of the oleate desaturase allele, closely linked to the mutation, as well as mutation-specific dominant markers. As the background of the recipient of the *Ol* mutation is important in creating high-oleic sunflower genotypes, identification of the appropriate markers is also crucial. Several studies dealt with the validation of different types of markers across diverse genetic backgrounds (Nagarathna et al., 2011; Bilgen, 2016; Dimitrijević et al., 2016, 2017). However, further development of markers and their validation is still required.

Premnath et al. (2016) recently examined OAC and identified three QTLs on chromosomes 8, 9, and 14. The QTLs have previously been reported, and the authors singled out the potential use of markers ORS 762 and HO_Fsp_b (designed by Schuppert et al. (2006)) linked to QTLs on chromosomes 8 and 14, respectively, in MAS. Furthermore, Zhou et al. (2018) exploited specific-locus amplified fragment sequencing (SLAF-seq) for mapping three QTLs, two on chromosome 6 and one on chromosome 9, associated with high OAC in the cross L-1-OL-1 × 86-1. The authors created a map of 2221.86 cM, while the average genetic distance between SLAFs was 0.36 cM.

### Increased Stearic Acid Content

Pérez-Vich et al. (1999) identified two partially recessive genes, *es*$_1$ and *es*$_2$, controlling high SAC. Out of the two, *es*$_1$ has the greater effect on stearic acid content (SAC) compared to *es*$_2$, and it co-segregates to a stearoyl-ACP desaturase locus (*SAD17A*) on chromosome 1, explaining approximately 80% of the phenotypic variation (Pérez-Vich et al., 2002). In addition to this QTL, the authors mapped several minor QTLs in two $F_2$ mapping populations (Table 1). Later, Pérez-Vich et al. (2004) identified three QTLs affecting SAC on chromosomes 3, 11, and 13 in a cross of HA89 × CAS-20. The sunflower line CAS-20 (*Es*$_1$ *Es*$_1$*es*$_2$*es*$_2$) was

developed from the previously mentioned CAS-3 ($es_1es_1es_2es_2$) mutant line. Compared to CAS-3 with 25% stearic acid, CAS-20 exhibited decreased SAC (8.6% in its seed oil). However, the SAC is still increased when compared to regular sunflower oil which has less than 6% (Pérez-Vich et al., 2004, 2006). All of the reported QTLs for increased SAC originated from CAS-20 and were attributed to segregating for the recessive gene $es_2$. Contrary to CAS-3, in the CAS-14 mutant line, SAC was found to be controlled by the $es_3$ gene located on the distal part of chromosome 8 and strongly affected by temperature during seed maturation (Pérez-Vich et al., 2006). Also, the expression of the trait is not uniform along the seed. CAS-14 had a SAC more than 35%, significantly higher than in regular sunflower oil. The flanking, linked SSR markers to the $es_3$ were discovered using bulk-segregant analysis: ORS243 and ORS1161, located 0.5, and 3.9 cM from the gene, respectively. Moreover, during the analysis of the CAS-3 × CAS-14 $F_2$:$F_3$ population, two QTLs were identified on chromosomes 1 and 8, representing $Es_1$ and $Es_3$ loci from CAS-3 and CAS-14, respectively. The authors reported a significant epistatic interaction between the two detected QTLs.

## Tocopherols

Tocopherol content was not the subject of many molecular studies, despite its contribution to increasing the nutritional and technological properties of sunflower oil. As there are four naturally occurring tocopherols (α-, β-, γ-. and δ-tocopherol), designing oils with appropriate tocopherol content can be very beneficial for consumers. As the most prevalent type of tocopherol in regular sunflower oil, α-tocopherol has some advantages, as it has great vitamin E value but low in vitro activity (Vera-Ruiz et al., 2006). Contrary to α-tocopherol, β-tocopherol has a better balance of in vivo and in vitro antioxidant properties, making it desirable for a particular oil use. Vera-Ruiz et al. (2006) thus mapped the $Tph_1$ gene responsible for increased β-tocopherol content (more than 30%) in sunflower line T589. The $Tph_1$ gene co-segregated with three SSR markers, ORS222, ORS598 and ORS1093 and was located on the upper end of chromosome 1. The same year, García-Moreno et al. (2006) mapped the $Thp_2$ gene from a high γ-tocopherol source. Four lines with high γ-tocopherol content were developed, and it was determined that this trait is controlled by recessive alleles at the $Tph_2$ locus. This locus was located on chromosome 8, flanked by ORS312 and ORS599, 3.6 cM proximal and 1.9 cM distal, respectively.

Genetic control of tocopherol content was analyzed on a molecular level by Haddadi et al. (2012), who mapped five QTLs on chromosomes 1, 8, 10, and 14, explaining in total 45% of the phenotypic variation. Using a candidate gene approach, the authors identified four candidate genes co-localizing with mapped QTLs: VTE4 (tocopherol methyl-transferase), HPPD (p-hydroxyphenylpyruvate dioxygenase), GST (glutathione S-transferase), and Droug1.

## *5.3 Abiotic Stress Tolerance and Nutrition Quality Traits*

Environmental constraints, including abiotic stress factors such as drought, salinity, and low and high temperatures, severely limit crop productivity. Improvement of traits conferring tolerance to these stresses is a complex issue that could be accomplished using traditional and modern breeding methods (Sreenivasulu et al., 2007). Abiotic stress tolerance has been less studied in sunflower than biotic stress tolerance since it has polygenic inheritance and may be conditioned by multiple interacting mechanisms (Miklas et al., 2006). These and other factors make abiotic stress tolerance especially difficult to study, both physiologically and genetically. According to Škorić (2009), drought is the main abiotic constraint in sunflower production, as it affects 26% of arable land. Drought stress can cause decreases in fertility, yield, and product quality. Moreover, drought stress occurring at vegetative and reproductive stages can affect sunflower oil quantity and quality as well as protein quantity (Nel et al., 2002; Rauf, 2008; Ali et al., 2009; Hussain et al., 2015). Second in line according to their importance are mineral toxicities/deficiencies, while frost stands as the third most important abiotic stress.

Unfavorable conditions, especially drought, can influence the composition of sunflower seeds. A major seed growth-limiting factor that also affects oil and protein content is water deficiency during all growth and developmental stages. Both have medium-high heritability and depend on stress conditions. In sunflower, water stress leads to a significant decrease in oil content (Muriel & Downes, 1974; Nel et al., 2002) and an increase in protein content at maturity (Ebrahimi et al., 2009).

Even though oil quality traits are governed by one or a few genes, the traits are still affected by the environment. The content of fatty acids, especially the oleic/linoleic acid ratio, can therefore vary among the same type of sunflower, depending on the cultivation area and climatic factors (precipitation, temperature, sunlight, relative humidity, etc.). Temperature during seed formation is an important factor affecting oil quality traits (Canvin, 1965).

Higher temperatures during seed maturation produced oil high in oleic and low in linoleic acid compared to production during low-temperature periods (Kovacik et al., 1998). Implementation of drought stress from the flowering stage to physiological maturity (in comparison to the control group) has also been reported to increase oleic acid and reduce linoleic acid (Flagella et al., 2000). High-temperature regimes can influence the ratio of oleic and linoleic acids in sunflower (Trémoliéres et al., 1982). Moreover, oleic acid content increases when night temperatures are high, regardless of the daily minimum temperatures (Izquierdo et al., 2002). Besides temperature, other factors such as total solar radiation can lead to a significant decrease in linoleic acid content, while the impact of day length remains unclear (Seiler, 1983). As mentioned above, even though tocopherol composition shows genetic and phenotypic variability (Demurin et al., 1996), there is no information in the current literature on whether drought stress can influence changes in tocopherol content (Ali et al., 2009).

Numerous strategies are utilized in breeding for drought stress in crop species through the modification and introduction of plant traits related to drought tolerance and associated with high yield (Rauf, 2008). Generally, sunflower shows more drought tolerance than other field crops due to its specific plant architecture, better adaptation, and ability to grow in different agro-ecological conditions. Sunflower breeders made significant efforts in changing morphological traits and developmental phenophases, e.g., shorter genotypes with lower leaf mass, deeper root system, earliness, etc., which ensured sunflower as a well-adapted crop to existing climate changes. Moreover, a good strategy for avoiding drought stress could lie in arranging sowing depending on the onset of the dry period in summer seasons (Miladinović et al., 2019). Success in breeding for abiotic stress tolerance has been accomplished by using the stay-green effect, which delays leaf senescence during the grain-filling phase. Exploitation of this effect can lead to achieving a higher yield potential through increments in the biomass production or yield stability under conditions of water shortage, high plant population density, or late sowings (Sala et al., 2012). According to the same authors, more target traits in sunflower can confer yield advantages in stress-prone environments, such as tolerance to stem-logging, reduced height, and herbicide-resistant hybrids.

Sunflower and other wild relatives represent a great source of tolerance for drought stress. They are adapted to different types of habitats (grasslands, deserts, swamps, mountains, forests, roads, and fields) and possess a significant variability in terms of resistance or tolerance to most abiotic and biotic stresses (Seiler et al., 2017). As an example, *H. deserticola* and *H. anomalus* present a potentially good source of drought tolerance traits as they are native to drought-prone environments (Seiler & Marek, 2016).

### 5.3.1 Molecular Tools

As drought tolerance is a quantitative trait independent from yield properties, the use of molecular markers for the introduction of tolerant QTLs in high-yielding sunflower genotypes would lead to further crop improvement and ensure stable sunflower oil yields (Chiementi et al., 2002).

Some of the relatively recent works addressed the mining of QTLs associated with oil quantity and quality parameters under different water regimes. Ebrahimi et al. (2008) identified 62 QTLs associated with seed quality traits in water-stressed conditions, and 56 QTLs in well-watered conditions in greenhouses or field trials. The authors examined oil content, palmitic acid content (PAC), SAC, OAC, and linoleic acid content (LAC) in RILs obtained from PAC2 × RHA266. Of the detected QTLs, 12 were common under both water-stressed and well-watered conditions. Also, the authors identified overlapping QTL regions associated with several examined traits and/or water regimes. Overlapping QTLs for oil content were found in regions on chromosomes 2, 10, 12, and 16, of which the region on chromosome 16 was identified as the most important. In total, it contained three QTLs associated with water-stressed conditions and two QTLs associated with well-watered

conditions. Some of the QTLs also overlapped with QTLs for OAC and LAC, indicating a common genetic base for the traits. The authors found a negative correlation between SAC and OAC and oil content, and a positive correlation between PAC and LAC and oil content. Moreover, an important overlap was discovered on chromosome 8 among QTLs for OAC, SAC, and LAC, which were also associated with the SSR marker SSU217. A year later, Ebrahimi et al. (2009) used the same mapping population to examine QTLs associated with seed protein content and other seed properties, such as kernel and hull weight, and seed density in greenhouse or field conditions under water stress compared to the non-stressed conditions. Similar to the previous experiment, a great deal of variation was found on the phenotypic level among the analyzed plants, while nonspecific and specific QTLs were identified. QTLs associated with protein content were found on the majority of chromosomes, while chromosome 14 was reported to be the most important as it contained five QTLs associated with the trait in all examined conditions. The authors also detected four regions on chromosomes 1, 8, 10, and 16, in which QTLs associated with protein content were overlaid with QTLs for oil content reported by Tang et al. (2006) and Ebrahimi et al. (2008). Ebrahimi et al. (2009) suggested that the regions may contain genes that are involved in the synthesis of proteins that have an indirect effect on oil content or they may contain genes that control substrate partitioning between protein and oil synthesis. Haddadi et al. (2010) examined QTLs associated with not only protein and oil content but also fatty acid content in the RILs (PAC2 × RHA266) subjected to well-, partial-irrigated system, and late-sowing conditions. Similar to the previous study, common regions for several traits were found. For example, chromosomes 10 and 15 contained common regions for percentage of seed oil and SAC; also, seven QTLs associated with PAC, OAC, SAC, and LAC were detected on chromosome 14. Identification of QTLs and QTL-associated markers can lead to increased efficiency of MAS for creating sunflower genotypes tolerant to water-stressed conditions and providing a sustainable oil source for consumption or other specific purposes.

Recently, Gezeljeh et al. (2018) associated two SSRs and three retrotransposon markers to the oil content trait in natural and water-limited conditions. The authors performed an association study on 100 inbred lines from Europe, the USA, and Iran. Overlapping QTLs in both conditions may be very useful in MAS for creating sunflower genotypes adapted to different environmental conditions and ensuring potentially stable oil content.

According to Debeake et al. (2017), in future cropping systems, sunflower could be recognized as an oil-protein and environmentally friendly crop suited for low-input production. As a climate-smart model crop, adapted to the conditions in which irrigation water is available to a lesser extent, sunflower can be used as a (moderately) drought-tolerant crop and grown in conditions with no systematic irrigation. Sunflower adaptations could be developed through breeding, crop management, and shifting of the growing areas. Consequently, in the future, sunflower can be the oil crop of preference thanks to its ability to grow in diverse agro-ecological conditions as well as its moderate drought tolerance, especially bearing in mind the global environmental changes.

### 5.3.2 Genomic Selection for Sunflower Improvement

Genomic selection (GS), as a potentially powerful tool in breeding, is introduced modestly in sunflower breeding. Genomic selection is applied for the prediction of performance through the use of a model. In other words, it is used for selecting individuals based on their genomic breeding values (Meuwissen et al., 2001). Contrary to MAS, where only a few markers linked to a trait of interest are used, a larger number of markers are exploited in GS, covering both major and minor genes throughout the genome. Moreover, the development, availability, and, most importantly, reduction in the price of using high-throughput genotyping platforms have made them more attractive in recent years (Dimitrijević & Horn, 2018). Genomic selection was used for predicting sunflower hybrid performance (associated with phenotyping data of flowering time and leaf senescence), oil content in hybrid sunflower, as well as *Sclerotinia* resistance (Livaja et al., 2016; Bonnafous et al., 2016; Mangin et al., 2017b). Livaja et al. (2016) assessed the performance of the genome-based best linear unbiased prediction (GBLUP) model for the prediction of resistance to *Sclerotinia* mid-stalk rot in a biparental population that was genotyped with the 25 K SNP array. The authors obtained highly predictive abilities for the stem lesion length resistance trait, while lower predictive abilities were obtained for the speed of fungal growth and leaf lesion length, which expressed lower heritability. For the prediction of seed oil content, Mangin et al. (2017b) compared the prediction accuracy of classical general combining aptitude (GCA) to that of whole genome sequencing. An incomplete factorial design was used for this purpose, in which 36 CMS lines were crossed with 36 restorer lines. The characterization of 452 hybrids concerning oil content (additive trait with high heritability) was performed in a multi-environment trial. Genomic selection and GCA were equally accurate in predicting hybrid performance if all parental lines were well characterized. However, GS proved to be more accurate compared to GCA in cases where at least one parental line was uncharacterized. Initial experimental trials for GS showed the potential and limitations of using GS in sunflower breeding (Dimitrijevic & Horn, 2018).

## 6 Future Breeding Challenges

As mentioned in this chapter, sunflower is one of the main oil sources worldwide, while in some regions it is the main source. Maintaining and increasing its yield is therefore important for securing sustainable oil production. Growing sunflower has several advantages, including its wide distribution in different agroecological conditions and moderate drought tolerance. Considering the current and expected climate changes, as well as the increasing human population, sunflower breeders have a demanding task: designing sunflower genotypes able to keep the current and increase future productivity under the changing and unpredictable environment. What is notable is that each growing season is different from the other; we are

witnessing fast environmental changes, such as rain showers followed by drought periods or extensive drought periods accompanied by high temperatures. In order to obtain a sustainable sunflower yield for ensuring oil security, breeders need to develop sunflower genotypes, that will be high oil yielding and resistant to abiotic and biotic stress.

This chapter outlines the major conventional and molecular breeding efforts made so far, with the aim of improving sunflowers. However, how will the breeders face future challenges?

They will need to utilize all available tools and develop new low-cost, high-throughput tools for sunflower breeding. These tools will most likely be involved in aided high-throughput phenotyping and remote sensing, molecular tools, as well as developing reliable prediction models and methods/software for dealing with and integrating large amounts of data.

High-throughput phenotyping is considered the bottleneck of breeding advancement (Araus et al., 2018). Precise phenotyping is the basis for classical breeding and the development of molecular markers (GWAS and GS). High-throughput phenotyping platforms can be very expensive, which is why they are mainly utilized in developed countries and the private sector. Different phenotyping networks are established in order to enable the sharing of such platforms, both on the national and international level, such as the EPPN (European Plant Phenotyping Network), the NAPPN (North American Plant Phenotyping Network), and the IPPN (International Plant Phenotyping Network). So far, only a few platforms have been established and tested for sunflower phenotyping. "Heliaphen" is an outdoor high-throughput phenotyping platform designed for analysis of drought scenarios as well as monitoring sunflowers throughout its lifecycle (Gosseau et al., 2018). Phenotyping platforms can also be very useful in phenotyping traits invisible to the naked eye, such as the root system. GROWSCREEN-Rhizo was thus successfully used for root phenotyping of cultivated and wild sunflower genotypes (Dimitrijević et al., 2018). Moreover, 3D reconstruction can be very useful in sunflower phenotyping (Nguyen et al., 2016; Gélard et al., 2017). Nguyen et al. (2016) reported that their custom stereovision system outperformed the structure-from-motion approach in 3D reconstruction of small details and leaf edges of sunflower plants. Furthermore, appropriate software and approaches are required for providing as accurate an evaluation of the plant as possible. Zorić et al. (2020) used a new Flower Color Image Analysis (FloCIA) software for digital image segmentation of sunflower ray florets and automatic color class classification.

Remote sensing techniques and crop models have been utilized for crop yield estimation. Obtaining accurate and timely estimation of crop yields prior to harvest allows proper crop yield management decision-making at a regional scale, which is imperative for national food policy and security assessment (Jin et al., 2018). In sunflower, Litvischenko et al. (2017) used microwave radiation for remote sensing of seed moisture content diagnosis for sunflower seed ripeness in field conditions, providing farmers with information about the optimal time for harvest. Furthermore, remote sensing in sunflower was used for yield and leaf area index estimations (Tunca et al., 2018; Zeng et al., 2018), biomass estimation (Claverie et al., 2012),

nitrogen status measurement (Agüera et al., 2011), evaluation of vegetation stage (Herbei & Sala, 2015), estimation of chlorophyll and water status in leaves (Neto et al., 2017), estimation of crop height and leaf area index (Fieuzal & Baup, 2016), retrieval of bio-physical parameters (Routh et al., 2019), and weed mapping in the early season that can help herbicide savings in sunflower crop production (López-Granados et al., 2016). As it can be seen, remote sensing can help breeders and farmers in sunflower crop production in numerous ways. However, appropriate sensing methods combined with suitable data management methods are imperative for obtaining the most precise results possible.

Besides phenotyping, genotyping is currently performed on larger scales. The use of high-throughput genotyping platforms helps generate a lot of data and analyze quantitative traits in more detail. Genome wide association study (GWAS), besides the already mentioned GS, has been used in examining *Sclerotinia* head rot resistance by Fusari et al. (2012), flowering time (Cadic et al., 2013; Bonnafous et al., 2018), and branching (Nambeesan et al., 2015). At the moment, GWAS is still unavailable to many breeders due to the high cost of the analysis (Dimitrijevic & Horn, 2018). With the advancement of technology, the development of powerful and accurate prediction models has become imperative. As sunflower breeding is based on hybridization, prediction models should include dominant and epistatic effects in addition to the already included additive effect. The addition of dominance to the model and testing several models have been performed in sunflower (Bonnafous et al., 2016, 2018).

Public availability of the sunflower genome sequence has also opened doors for expanding molecular research in sunflower (Badouin et al., 2017). The researchers provided the sunflower community with not only the sequence but also insight into oil metabolism and flowering, as well as asterid evolution. Sequence data is at its beginning because the examination of quantitative traits requires analysis of gene expression at the genome level. Examining the transcriptome, proteome, metabolome, and epigenome should give the "whole" molecular picture of sunflower behavior in certain environmental conditions, as well as detailed mechanisms involved in its response to stress, which could lead to identifying "weak spots" and exploiting different breeding methods for sunflower improvement. These could include the conventional, but also genome editing techniques. But how far are we from applying all the mentioned techniques to sunflower breeding?

Concerning the use of modern "omics" techniques in sunflower breeding, initial studies were conducted on disease resistance (Guo et al., 2017; Yang et al., 2017), as well as abiotic stress resistance to drought (Liang et al., 2017; Ghaffari et al., 2017; Sarazin et al., 2017; Moschen et al., 2017), and leaf senescence, which strongly affects sunflower yield (Moschen et al., 2016a, b). Contrary to "omics" techniques, genome editing techniques have not been used to change any properties in sunflower. Chantry-Darmon et al. (2018) reported improvement and adaptation of the first steps of the CATCH method (Cas9-Assisted Targeting of Chromosomal Segments as described by Jiang et al., 2015) for cutting a specific locus in *Medicago truncatula* and use of this approach on a more complex and larger genome region in sunflower. Major obstacles to the successful application of modern genome editing

in sunflower include difficulties that arise during plant regeneration as well as the low number of obtained transgenic regenerants per assay (Miladinović et al., 2021). Therefore, further improvement needs to be made in the transformation protocols.

Appropriate evaluation of the performance of the newly-developed sunflower genotypes in particular environmental conditions can lead to better exploitation of the genotypes and obtaining higher yields. Casadebaig et al. (2011) developed a model, SUNFLO, for assisting the improvement of genotyping assessment in sunflower crops. The obtained results suggested that SUNFLO seems to be sufficiently robust for the estimation of the influence of breeding traits on yield or for exploring new management practices. However, the model does not take into account biotic stress or biomass allocation, and SUNFLO was further improved. According to Mangin et al. (2017c), the model simulates major plant and soil processes, namely, nitrogen content and soil water, root growth, plant nitrogen uptake and transpiration, leaf expansion and senescence, and biomass accumulation, as a function of dominant environmental constraints (temperature, radiation, water, and nitrogen deficit). SUNFLO was further used by Picheny et al. (2017) for designing sunflower ideotypes for certain environments.

All the mentioned techniques imply gathering plenty of data, which needs to be appropriately stored and analyzed. Moreover, the accessibility of data can be beneficial not only for researchers but also for farmers. In research, it would allow deposition of the new research data and avoid multiplication of results, while helping sunflower producers make proper sowing choices and optimizing crop management. As suggested by Kamilaris et al. (2017), if developed and made more accessible, farmers would benefit from several key points, such as tools for yield and demand prediction, guidance provided to farmers based on the responsiveness of their crops to fertilizers, herbicides and pesticides for more appropriate crop management, etc.

Numerous breeding aspects can be improved thanks to technological advancement. However, will all these techniques be enough to initiate a new "green evolution" or are more needed? Technology advancements are very quick, thereby making the prices of previously developed technology affordable to poor countries, which frequently have abundant genetic resources that are, in most cases, insufficiently examined. Utilization of these tools may be promising in mining for traits and helping create new sunflower ideotypes of tomorrow, thereby allowing secure food sources for future generations. It may also enable expansion of the sunflower cultivation area beyond the current 25 million ha and production of more than the current 36 Mt/year. Moreover, one of the advantages of growing sunflower is their low growing demands, while sunflower oil has always been the oil of preference for domestic consumption. At present, not only regular sunflower oil is in high demand but also specific oils such as highly stable high-oleic or relatively recently developed high-oleic-high-stearic. Future advancements may allow for the creation of sunflower oil with specific fatty acid and tocopherol content for greater health value and stability.

**Acknowledgements** This work was done as a part of the activities of the Center of Excellence for Innovations in Breeding of Climate-Resilient Crops-Climate Crops, Institute of Field and Vegetable Crops, Novi Sad, Serbia. It was supported by the Ministry of Education, Science, and Technological Development of the Republic of Serbia, grant number: 451-03-9/2021-14/200032, as well as by the Science Fund of the Republic of Serbia, through IDEAS project "Creating climate smart sunflower for future challenges" (SMARTSUN), grant number 7732457 and COST Actions CA 16212, CA18111, and CA19125, and IAEA project RER5024.

## References

Acquaah, G. (2015). Conventional plant breeding principles and techniques. In J. Al-Khayri, S. Jain, & D. Johnson (Eds.), *Advances in plant breeding strategies: Breeding, biotechnology and molecular tools* (pp. 115–158). Springer.

Agüera, F., Carvajal, F., & Pérez, M. (2011). Measuring sunflower nitrogen status from an unmanned aerial vehicle-based system and an on the ground device. *The International Archives of the Photogrammetry, Remote Sensing and Spatial Information Sciences, 38*, 33–37.

Alberio, C., Izquierdo, N. G., Galella, T., Zuil, S., Reid, R., Zambelli, A., & Aguirrezábal, L. A. (2016). A new sunflower high oleic mutation confers stable oil grain fatty acid composition across environments. *The European Journal of Agronomy, 73*, 25–33.

Ali, Q., Ashraf, M., & Anwar, F. (2009). Physico-chemical attributes of seed oil from drought stressed sunflower (*Helianthus annuus* L.) plants. *Grasas y Aceites, 60*(5), 477–483.

Anđelković, V., Cvejić, S., Jocić, S., Kondić-Špika, A., Marjanović Jeromela, A., Mikić, S., Prodanović, S., Radanović, A., Savić Ivanov, M., Trkulja, D., & Miladinović, D. (2020). Use of plant genetic resources in crop improvement – Example of Serbia. *Genetic Resources and Crop Evolution, 67*, 1935.

Andrianasolo, F. N., Debaeke, P., Champolivier, L., & Maury, P. (2016). Analysis and modelling of the factors controlling seed oil concentration in sunflower: A review. *OCL, 23*(2), D206.

Andrich, G., Balzini, S., Zinnai, A., Fiorentini, R., Baroncelli, S., & Pugliesi, C. (1992). The oleic/linoleic ratio in achenes coming from sunflower lines treated with hard X-rays. In *Proceedings of the 13th international sunflower conference*. Pisa, Italy, 7–11 September 1992. ISA, Paris, Frnace, pp. 1544–1549.

Araus, J. L., Kefauver, S. C., Zaman-Allah, M., Olsen, M. S., & Cairns, J. E. (2018). Translating high-throughput phenotyping into genetic gain. *Trends in Plant Science, 23*(5), 451–466.

Atlagić, J., & Terzič, S. (2014). Sunflower genetic resources – Interspecific hybridization and cytogenetics in prebreeding. In J. I. Arribas (Ed.), *Sunflowers: Growth and development, environmental influences and pests/diseases* (pp. 95–130). Nova Science Publishers.

Baack, E. J., Sapir, Y., Chapman, M. A., Burke, J. M., & Rieseberg, L. H. (2008). Selection on domestication traits and quantitative trait loci in crop–wild sunflower hybrids. *Molecular Ecology, 17*(2), 666–677.

Badouin, H., Gouzy, J., Grassa, C. J., Murat, F., Staton, S. E., Cottret, L., Lelandais-Brière, C., Owens, G. L., Carrère, S., Mayjonade, B., & Legrand, L. (2017). The sunflower genome provides insights into oil metabolism, flowering and Asterid evolution. *Nature, 546*(7656), 148.

Bau, H. M., Mohtadi-Nia, D. J., Mejean, L., & Debry, G. (1983). Preparation of colorless sunflower protein products: Effect of processing on physicochemical and nutritional properties. *Journal of the American Oil Chemists' Society, 60*(6), 1141–1148.

Bert, P. F., Jouan, I., De Labrouhe, D. T., Serre, F., Philippon, J., Nicolas, P., & Vear, F. (2003). Comparative genetic analysis of quantitative traits in sunflower (*Helianthus annuus* L.). 2. Characterisation of QTL involved in developmental and agronomic traits. *Theoretical and Applied Genetics, 107*(1), 181–189.

Bilgen, B. B. (2016). Characterization of sunflower inbred lines with high oleic acid content by DNA markers. In *Proceedings of the 19th international sunflower conference*. Edirne, Turkey, 29 May–3 June 2016. ISA, Paris, France, pp. 662–668.

Bonnafous, F., Langdale, N., Sunrise Consortium, Mangin, B. (2016). Inclusion of dominance effect in genomic selection model to improve predictive ability for sunflower hybrid performance. In *Proceedings of the 19th international sunflower conference*. Edirne, Turkey, 29 May–3 June 2016. ISA, Paris, France, pp. 285.

Bonnafous, F., Fievet, G., Blanchet, N., Boniface, M. C., Carrère, S., Gouzy, J., Legrand, L., Marage, G., Bret-Mestries, E., Munos, S., & Pouilly, N. (2018). Comparison of GWAS models to identify non-additive genetic control of flowering time in sunflower hybrids. *Theoretical and Applied Genetics, 131*(2), 319–332.

Bramley, P. M., Elmafda, I., Kafatos, A., Kelly, F. J., Manios, Y., Roxborough, H. E., Schuch, W., Sheehy, P. J. A., & Wagner, K. H. (2000). Vitamin E. *Journal of the Science of Food and Agriculture, 80*(7), 913–938.

Burke, J. M., Knapp, S. J., & Rieseberg, L. H. (2005). Genetic consequences of selection during the evolution of cultivated sunflower. *Genetics, 171*(4), 1933–1940.

Cadic, E., Coque, M., Vear, F., Grezes-Besset, B., Pauquet, J., Piquemal, J., Lippi, Y., Blanchard, P., Romestant, M., Pouilly, N., & Rengel, D. (2013). Combined linkage and association mapping of flowering time in sunflower (*Helianthus annuus* L.). *Theoretical and Applied Genetics, 126*(5), 1337–1356.

Canvin, D. T. (1965). The effect of temperature on the oil content and fatty acid composition of the oils from several oil seed crops. *Canadian Journal of Botany, 43*(1), 63–69.

Casadebaig, P., Guilioni, L., Lecoeur, J., Christophe, A., Champolivier, L., & Debaeke, P. (2011). SUNFLO, a model to simulate genotype-specific performance of the sunflower crop in contrasting environments. *Agricultural and Forest Meteorology, 151*(2), 163–178.

Chantry-Darmon, C., Satgé, C., Milon, N., Bellec, A., Marande, W., Cauet, S., Callot, C., Duriez, R., Munos, S., Bancaud, A., & Berges, H. (2018). Development of a CRISPR/Cas9 large DNA fragment targeting technique for plant genomes. In *Prooceedings of plant & animal genome conference XXVI*. San Diego, USA, 13–17 January 2018. Posters P0003.

Chimenti, C. A., Pearson, J., & Hall, A. J. (2002). Osmotic adjustment and yield maintenance under drought in sunflower. *Field Crops Research, 75*(2–3), 235–246.

Claverie, M., Demarez, V., Duchemin, B., Hagolle, O., Ducrot, D., Marais-Sicre, C., Dejoux, J. F., Huc, M., Keravec, P., Béziat, P., & Fieuzal, R. (2012). Maize and sunflower biomass estimation in Southwest France using high spatial and temporal resolution remote sensing data. *Remote Sensing of Environment, 124*, 844–857.

Cvejić, S., Prodanović, S., & Jocić, S. (2009). Enhancement of genetic variability for the seed oil composition by induced mutations in sunflower collection. In *Proceedings of the 19th EUCARPIA conference*. Ljubljana, Slovenia, 26–29 May 2009. EUCARPIA, pp. 75.

Cvejić, S., Jocić, S., Prodanović, S., Terzić, S., Miladinović, D., & Balalić, I. (2011). Creating new genetic variability in sunflower using induced mutations. *Helia, 34*(55), 47–54.

Cvejić, S., Miladinović, D., & Jocić, S. (2014). Mutation breeding for changed oil quality in sunflower. In N. B. Tomlekova, M. I. Kozgar, & M. R. Wani (Eds.), *Mutagenesis: Exploring genetic diversity of crops* (pp. 77–96). Wageningen Academic Publishers.

Cvejić, S., Jocić, S., Jocković, M., Jeromela, A. M., Miladinović, D., Imerovski, I., & Dimitrijević, A. (2016). Variability of seed oil quality in sunflower germplasm. In *Prooceedings of III international congress, "Food technology, quality and safety"*. Novi Sad, Serbia, 25–27 October, pp. 509–513.

Debaeke, P., Casadebaig, P., Flenet, F., & Langlade, N. (2017). Sunflower crop and climate change: Vulnerability, adaptation, and mitigation potential from case-studies in Europe. *OCL, 24*(1), D102.

Dehmer, K. J., & Friedt, W. (1998). Development of molecular markers for high oleic acid content in sunflower (*Helianthus annuus* L.). *Industrial Crops and Products, 7*(2–3), 311–315.

Demurin, Y. (1986). Phenotypic variability and correlation between tocopherol content and some biochemical characters in sunflower seeds. *Science Technology Bulletin VNIIMK Krasnodar, 93*, 21–24.

Demurin, Y., & Borisenko, O. (2011). Genetic collection of oleic acid content in sunflower seed oil. *Helia, 34*(55), 69–74.

Demurin, Y., Skoric, D., & Karlovic, D. (1996). Genetic variability of tocopherol composition in sunflower seeds as a basis of breeding for improved oil quality. *Plant Breeding, 115*(1), 33–36.

Dimitrijević, A., & Horn, R. (2018). Sunflower hybrid breeding: From markers to genomic selection. *Frontiers in Plant Science, 8*, 2238.

Dimitrijević, A., Imerovski, I., Miladinović, D., Jocković, M., Cvejić, S., Jocić, S., Zeremski, T., & Sakač, Z. (2016). Screening of the presence of *Ol* gene in NS sunflower collection. In *Proceedings of the 19th international sunflower conference*. Edirne, Turkey, 29 May–3 June 2016. ISA, Paris, France, pp. 661–667.

Dimitrijević, A., Imerovski, I., Miladinović, D., Cvejić, S., Jocić, S., Zeremski, T., & Sakač, Z. (2017). Oleic acid variation and marker-assisted detection of Pervenets mutation in high- and low-oleic sunflower cross. *Crop Breeding and Applied Biotechnology, 17*(3), 235–241.

Dimitrijević, A., Galinski, A., Miladinović, D., Cvejić, S., Jocić, S., Terzić, S., Nagel, K., & Fiorani, F. (2018). Root phenotyping of NS sunflower. *Botanica Serbica, 42*(Suppl 1), 164.

Dobarganes, M. C., Marquez-Ruiz, G., & Perez-Camino, M. C. (1993). Thermal stability and frying performance of genetically modified sunflower seed (*Helianthus annuus* L.) oils. *Journal of Agricultural and Food Chemistry, 41*(4), 678–681.

Dubinsky, E., & Garcés Mancheño, R. (2011). High-stearic/high-oleic sunflower oil: A versatile fat for food applications. *International News on Fats, Oils and Related Materials, 22*(6), 369–372.

Ebrahimi, A., Maury, P., Berger, M., Poormohammad Kiani, S., Nabipour, A., Shariati, F., Grieu, P., & Sarrafi, A. (2008). QTL mapping of seed-quality traits in sunflower recombinant inbred lines under different water regimes. *Genome, 51*(8), 599–615.

Ebrahimi, A., Maury, P., Berger, M., Calmon, A., Grieu, P., & Sarrafi, A. (2009). QTL mapping of protein content and seed characteristics under water-stress conditions in sunflower. *Genome, 52*(5), 419–430.

Fernández-Martínez, J. M., & Dominguez-Gimenez, J. (1988). Development of sunflower parental lines using EMS treatments. In *Proceedings of the 12th international sunflower conference*. Novi Sad, Yugoslavia, 25–29 July 1988. ISA, Paris, France, pp. 415–418.

Fernández-Martínez, J., Portero, F., & Ortíz, J. (1979). Genética del contenido en aceite de la semilla de girasol, *Helianthus annuus* L. Ann Comun INIA. *Serie Produccion Vegetal, 10*, 93–100.

Fernández-Martínez, J. M., Pérez-Vich, B., Velasco, L., & Domínguez, J. (2007). Breeding for specialty oil types in sunflower. *Helia, 30*(46), 75–84.

Fernández-Martínez, J. M., Pérez-Vich, B., & Velasco, L. (2009). Sunflower. In J. Vollmann & I. Rajcan (Eds.), *Oil crops* (pp. 155–232). Springer.

Fernández-Moya, V., Martínez-Force, E, & Garcés, R. (2002). Temperature effect on a high stearic acid sunflower mutant. *Phytochemistry, 59*(1), 33–37.

Fick, G. N. (1975). Heritability of oil content in sunflowers 1. *Crop Science, 15*(1), 77–78.

Fieuzal, R., & Baup, F. (2016). Estimation of leaf area index and crop height of sunflowers using multi-temporal optical and SAR satellite data. *International Journal of Remote Sensing, 37*(12), 2780–2809.

Flagella, Z., Rotunno, T, Di Caterina, R., De Simone, G., Ciciretti, L., & De Caro, A. (2000). Effect of supplementary irrigation on seed yield and oil quality of sunflower (*Helianthus annus* L.) grown in a sub-arid environment. In *Proceedings of the 15th international sunflower conference*. Toulouse, France, 25–29 July 2000. ISA, Paris, France, pp. 139–144.

Fusari, C. M., Di Rienzo, J. A., Troglia, C., Nishinakamasu, V., Moreno, M. V., Maringolo, C., Quiroz, F., Álvarez, D., Escande, A., Hopp, E., & Heinz, R. (2012). Association mapping in sunflower for sclerotinia head rot resistance. *BMC Plant Biology, 12*(1), 93.

García-Moreno, M. J., Vera-Ruiz, E. M., Fernández-Martínez, J. M., Velasco, L., & Pérez-Vich, B. (2006). Genetic and molecular analysis of high gamma-tocopherol content in sunflower. *Crop Science, 46*(5), 2015–2021.

Gavrilova, V. A., & Anisimova, I. N. (2017). Genealogy of the sunflower lines created on the basis of Russian varieties. *Helia, 40*(67), 133–146.

Gavrilova, V. A., Rozhkova, V. T., & Anisimova, I. N. (2014). Sunflower genetic collection at the Vavilov institute of plant industry. *Helia, 37*(60), 1–16.

Gélard, W., Devy, M., Herbulot, A., & Burger, P. (2017). Model-based segmentation of 3D point clouds for phenotyping sunflower plants. In *Prooceedings of 12th international joint conference on computer vision, imaging and computer graphics theory and applications (VISAPP 2017)*. Porto, Portugal, 27 February–01 March 2017, pp. 459–467.

Gezeljeh Ali, S., Darvishzadeh, R., Ebrahimi, A., & Bihamta, M. R. (2018). Identification of SSR and retrotransposon-based molecular markers linked to morphological characters in oily sunflower (*Helianthus annuus* L.) under natural and water-limited states. *Journal of Genetics, 97*(1), 189–203.

Ghaffari, M., Toorchi, M., Valizadeh, M., & Shakiba, M. (2017). Proteomic prospects for tolerance of sunflower (*Helianthus annuus*) to drought stress during the flowering stage. *Crop & Pasture Science, 68*(5), 457–465.

Gill, H. S., & Punia, M. S. (1996). Expression of heterosis in single-, double-and three-way cross hybrids of sunflower (*Helianthus annuus* L.). *Helia, 19*, 111–118.

González-Pérez, S. (2015). Sunflower proteins. In E. Martínez-Force, N. Turgut Dunford, & J. J. Salas (Eds.), *Sunflower: Chemistry, production, processing, and utilization* (pp. 331–393). AOCS Press.

González-Pérez, S., & Vereijken, J. M. (2007). Sunflower proteins: Overview of their physicochemical, structural and functional properties. *Journal of the Science of Food and Agriculture, 87*(12), 2173–2191.

Gosseau, F., Blanchet, N., Varès, D., Burger, P., Campergue, D., Colombet, C., Gody, L., Liévin, J. F., Mangin, B., Tison, G., & Vincourt, P. (2018). Heliaphen, an outdoor high-throughput phenotyping platform for genetic studies and crop modeling. *Frontiers in Plant Science, 9*, 1908.

Gotar, A. A., Berger, M., Labalette, F., Centis, S., Dayde, J., & Calmon, A. (2008). Estimation of breeding potential for tocopherols and phytosterols in sunflower. In *Proceedings of the 17th international sunflower conference*. Cordoba, Spain, 08–12 June 2008. ISA, Paris, France, pp. 555–559.

Goyal, S., Wani, M. R., Laskar, R. A., Raina, A., & Khan, S. (2019a). Assessment on cytotoxic and mutagenic potency of gamma rays and EMS in *Vigna mungo* L. Hepper. *Biotecnología Vegetal, 19*, 193–204.

Goyal, S., Wani, M. R., Laskar, R. A., Raina, A., Amin, R., & Khan, S. (2019b). Induction of morphological mutations and mutant phenotyping in black gram [*Vigna mungo* (L.) Hepper] using gamma rays and EMS. *Vegetos, 32*(4), 464–472.

Goyal, S., Wani, M. R., Raina, A., Laskar, R. A., & Khan, S. (2021a). Phenotypic diversity in mutagenized population of urdbean (*Vigna mungo* (L.) Hepper). *Heliyon, 7*(5), e06356.

Goyal, S., Wani, M. R., Raina, A., Laskar, R. A., & Khan, S. (2021b). Quantitative assessments on induced high yielding mutant lines in urdbean [*Vigna mungo* (L.) hepper]. *Legume Science, 4*(3), e125.

Guo, S., Zuo, Y., Zhang, Y., Wu, C., Su, W., Jin, W., Yu, H., An, Y., & Li, Q. (2017). Large-scale transcriptome comparison of sunflower genes responsive to *Verticillium dahliae*. *BMC Genomics, 18*(1), 42.

Gvozdenović, S., Saftić-Panković, D., Jocić, S., & Radić, V. (2009). Correlation between heterosis and genetic distance based on ssr markers in sunflower (*Helianthus annuus* L.). *Journal of Agricultural Sciences (Belgrade), 54*(1), 1–10.

Haddadi, P., Yazdi-Samadi, B., Langlade, N. B., Naghavi, M. R., Berger, M., Kalantari, A., Calmon, A., Maury, P., Vincourt, P., & Sarrafi, A. (2010). Genetic control of protein, oil and fatty acids content under partial drought stress and late sowing conditions in sunflower (*Helianthus annuus*). *The African Journal of Biotechnology, 9*(40), 6768–6782.

Haddadi, P., Ebrahimi, A., Langlade, N. B., Yazdi-Samadi, B., Berger, M., Calmon, A., Naghavi, M. R., Vincourt, P., & Sarrafi, A. (2012). Genetic dissection of tocopherol and phytosterol in

recombinant inbred lines of sunflower through quantitative trait locus analysis and the candidate gene approach. *Molecular Breeding, 29*(3), 717–729.

Herbei, M. V., & Sala, F. (2015). Use landsat image to evaluate vegetation stage in sunflower crops. *AgroLife Scientific Journal, 4*(1), 79–86.

Hladni, N., & Miladinović, D. (2019). Confectionery sunflower breeding and supply chain in Eastern Europe. *OCL, 26*, 29.

Hladni, N., Terzić, S., Mutavdžić, B., & Zorić, M. (2017). Classification of confectionary sunflower genotypes based on morphological characters. *The Journal of Agricultural Science, 155*, 1594–1609.

Horn, R., Radanović, A., Fuhrmann, L., Sprycha, Y., Hamrit, S., Jocković, M., Miladinović, D., & Jansen, C. (2019). Development and validation of markers for the fertility restorer gene $Rf_1$ in sunflower. *International Journal of Molecular Sciences, 20*(6), 1260.

Hussain, S., Saleem, M. F., Iqbal, J., Ibrahim, M., Ahmad, M., Nadeem, S. M., Ali, A., & Atta, S. (2015). Abscisic acid mediated biochemical changes in sunflower (*Helianthus annuus* L.) grown under drought and well-watered field conditions. *The Journal of Animal and Plant Sciences, 25*, 406–416.

Imerovski, I., Dimitrijević, A., Miladinović, D., Jocić, S., Dedić, B., Cvejić, S., & Šurlan-Momirović, G. (2014). Identification and validation of breeder-friendly DNA markers for $Pl_{arg}$ gene in sunflower. *Molecular Breeding, 34*(3), 779–788.

Imerovski, I., Dedić, B., Cvejić, S., Miladinović, D., Jocić, S., Owens, G. L., Kočiš Tubić, N., & Rieseberg, L. H. (2019). BSA-seq mapping reveals major QTL for broomrape resistance in four sunflower lines. *Molecular Breeding, 39*, 41.

Ivanov, P., & Ivanov, I. (1992). Biochemical characteristics of several sunflower mutants. In *30th Anniversary of Institute 'Dobrudja'*. Sofia, Bulgaria, pp. 98–102.

Ivanov, P., Petakov, V., Nikolova, V., & Petchev, E. (1988). Sunflower breeding for high palmitic acid content in the oil. In *Proceedings of 12th international sunflower conference*. Novi Sad, Yugoslavia, 25–29 July 1988. ISA, Paris, France, pp. 463–465.

Izquierdo, N., Aguirrezábal, L., Andrade, F., & Pereyra, V. (2002). Night temperature affects fatty acid composition in sunflower oil depending on the hybrid and the phenological stage. *Field Crops Research, 77*(2–3), 115–126.

Jan, C. C., & Seiler, G. J. (2007). Sunflower. In R. J. Singh (Ed.), *Genetic resources, chromosome engineering, and crop improvement* (pp. 103–165). CRC Press.

Jiang, W., Zhao, X., Gabrieli, T., Lou, C., Ebenstein, Y., & Zhu, T. F. (2015). Cas9-assisted targeting of CHromosome segments CATCH enables one-step targeted cloning of large gene clusters. *Nature Communications, 6*, 8101.

Jin, X., Kumar, L., Li, Z., Feng, H., Xu, X., Yang, G., & Wang, J. (2018). A review of data assimilation of remote sensing and crop models. *The European Journal of Agronomy, 92*, 141–152.

Jocić, S., Miladinović, D., & Yalcin, K. (2015). Breeding and genetics of sunflower. In E. Martinez-Force, N. T. Dunford, & J. J. Salas (Eds.), *Sunflower chemistry, production, proccessing and utilization* (pp. 1–25). AOCS Press.

Kalaydzhyan, A. A., Khlevnoy, L. V., Neshchadim, N. N., Golovin, V. P., Vartanyan, V. V., & Burdun, A. M. (2007). *Rossiyskiy solnechnyy tsvetok.-Krasnodar: Sovet* (pp. 1–352). Kuban. (In Russian).

Kamilaris, A., Kartakoullis, A., & Prenafeta-Boldú, F. X. (2017). A review on the practice of big data analysis in agriculture. *Computers and Electronics in Agriculture, 143*, 23–37.

Kaur, K., Dhillon, S. K., Gill, B. S., & Kaur, G. (2019). Association of SSR based genetic distances with heterosis in sunflower. *Journal of Environmental Biology, 40*(5), 1102–1108.

Kaya, Y. (2016). Sunflower. In S. K. Gupta (Ed.), *Breeding oilseed crops for sustainable production: Opportunities and constraints* (pp. 55–88). Academic.

Kaya, Y., Evci, G., Durak, S., Pekcan, V., & Gucer, T. (2009). Yield components affecting seed yield and their relationships in sunflower (*Helianthus annuus* L.). *Pakistan Journal of Botany, 41*(5), 2261–2226.

Kaya, Y., Jocić, S., & Miladinović, D. (2012). Sunflower. In S. K. Gupta (Ed.), *Technological innovations in major world oil crops* (Vol. 1, pp. 85–129). Springer.

Khursheed, S., Raina, A., Amin, R., Wani, M. R., & Khan, S. (2018a). Quantitative analysis of genetic parameters in the mutagenized population of faba bean (*Vicia faba* L.). *The Research on Crops, 19*(2), 276–284.

Khursheed, S., Raina, A., Laskar, R. A., & Khan, S. (2018b). Effect of gamma radiation and EMS on mutation rate: Their effectiveness and efficiency in faba bean (*Vicia faba* L.). *Caryologia, 71*(4), 397–404.

Khursheed, S., Raina, A., & Khan, S. (2018c). Physiological response of two cultivars of faba bean using physical and chemical mutagenesis. *International Journal of Advance Research in Science and Engineering, 7*(4), 897–905.

Khursheed, S., Raina, A., Parveen, K., & Khan, S. (2019). Induced phenotypic diversity in the mutagenized populations of faba bean using physical and chemical mutagenesis. *Journal of the Saudi Society of Agricultural Sciences, 18*(2), 113–119. https://doi.org/10.1016/j.jssas.2017.03.001

Kinman, M. L. (1970). New developments in the USDA and state experiment station sunflower breeding programs. In *Proceedings of the 4th international sunflower conference*. ISA, Paris, France, pp. 181–183.

Kovacik, A., & Skaloud, V. (1972). Combining ability and prediction of heterosis in sunflower (*Helianthus annuus* L.). *Scientia Agric Bohemoslovaca, 4*(4), 263–273.

Kovacik, A. V., Skaloud, V., & Vickova, V. (1998). Variability of sunflower oil yield in Europe as influenced by cultivar oil content. *Helia, 11*, 25–28.

Kritchevsky, D., Tepper, S. A., Kuksis, A., Eghtedary, K., & Klurfeld, D. M. (1995). Influence of triglyceride structure on experimental arteriosclerosis. *The Federation of American Societies for Experimental Biology, 9*(3), A320.

Kumar, A. P., Boualem, A., Bhattacharaya, A., Parikh, S., Desai, N., Zambelli, A., Leon, A., Chatterjee, M., & Bendahmane, A. (2013). SMART – Sunflower mutant population and reverse genetic tool for crop improvement. *BMC Plant Biology, 13*, 38.

Lacombe, S., Leger, S., Kaan, F., Berville, A., & Sas, M. (2002). Genetic, molecular and expression features of the Pervenets mutant leading to high oleic acid content of seed oil in sunflower. *OCL, 9*(1), 17–23.

Lacombe, S., Souyris, I., & Bervillé, A. J. (2009). An insertion of oleate desaturase homologous sequence silences via siRNA the functional gene leading to high oleic acid content in sunflower seed oil. *Molecular Genetics and Genomics, 281*(1), 43–54.

Lampi, A. M., & Kamal-Eldin, A. (1998). Effect of α-and γ-tocopherols on thermal polymerization of purified high-oleic sunflower triacylglycerols. *The Journal of the American Oil Chemists, 75*(12), 1699–1703.

Laskar, R. A., Khan, S., Khursheed, S., Raina, A., & Amin, R. (2015). Quantitative analysis of induced phenotypic diversity in chickpea using physical and chemical mutagenesis. *Journal of Agronomy, 14*, 3–102.

Laskar, R. A., Laskar, A. A., Raina, A., & Amin, R. (2018a). Induced mutation analysis with biochemical and molecular characterization of high yielding lentil mutant lines. *International Journal of Biological Macromolecules, 109*, 167–179.

Laskar, R. A., Wani, M. R., Raina, A., Amin, R., & Khan, S. (2018b). Morphological characterization of gamma rays induced multipodding mutant (mp) in lentil cultivar pant L 406. *International Journal of Radiation Biology, 94*(11), 1049–1053.

Laskar, R. A., Khan, S., Deb, C. R., Tomlekova, N., Wani, M. R., Raina, A., & Amin, R. (2019). Lentil (*Lens culinaris* Medik.) diversity, cytogenetics and breeding. In J. M. Al-Khayri et al. (Eds.), *Advances in plant breeding: Legumes*. Springer. https://doi.org/10.1007/978-3-030-23400-3_9

Leclercq, P. (1969). Une stérilité cytoplasmique chez le tournesol. *Ann Amélior Plant, 19*, 99–106.

León, A. J., Lee, M., Rufener, G. K., Berry, S. T., & Mowers, R. P. (1995). Use of RFLP markers for genetic linkage analysis of oil percentage in sunflower seed. *Crop Science, 35*(2), 558–564.

León, A. J., Andrade, F. H., & Lee, M. (2003). Genetic analysis of seed-oil concentration across generations and environments in sunflower. *Crop Science, 43*(1), 135–140.

León, A. J., Zambelli, A. D., Reid, R. J., Morata, M. M., Kaspar, M., & Advanta International BV. (2013). *Nucleotide sequences mutated by insertion that encode a truncated oleate desaturase protein, proteins, methods and uses*. U.S. Patent Application 13/822,279.

Liang, C., Wang, W., Wang, J., Ma, J., Li, C., Zhou, F., Zhang, S., Yu, Y., Zhang, L., Li, W., & Huang, X. (2017). Identification of differentially expressed genes in sunflower (*Helianthus annuus*) leaves and roots under drought stress by RNA sequencing. *Botanical Studies, 58*(1), 42.

Litvischenko, V. L., Nikiforov, I. Y., & Ershov, I. V. (2017). Remote measurement of sunflower seed moisture content by the use of microwaves. *Journal of the Science of Food and Agriculture, 97*(14), 4880–4882.

Livaja, M., Unterseer, S., Erath, W., Lehermeier, C., Wieseke, R., Plieske, J., Polley, A., Luerßen, H., Wieckhorst, S., Mascher, M., & Hahn, V. (2016). Diversity analysis and genomic prediction of *Sclerotinia* resistance in sunflower using a new 25 K SNP genotyping array. *Theoretical and Applied Genetics, 129*(2), 317–329.

López-Granados, F., Torres-Sánchez, J., Serrano-Pérez, A., de Castro, A. I., Mesas-Carrascosa, F. J., & Pena, J. M. (2016). Early season weed mapping in sunflower using UAV technology: Variability of herbicide treatment maps against weed thresholds. *Precision Agriculture, 17*(2), 183–199.

Louarn, J., Boniface, M. C., Pouilly, N., Velasco, L., Perez-Vich, B., Vincourt, P., & Munos, S. (2016). Sunflower resistance to broomrape (*Orobanche cumana*) is controlled by specific QTLs for different parasitism stages. *Frontiers in Plant Science, 7*, 590.

Mandel, J. R., Nambeesan, S., Bowers, J. E., Marek, L. F., Ebert, D., Rieseberg, L. H., Knapp, S. J., & Burke, J. M. (2013). Association mapping and the genomic consequences of selection in sunflower. *PLoS Genetics, 9*(3), e1003378.

Mangin, B., Pouilly, N., Boniface, M. C., Langlade, N. B., Vincourt, P., Vear, F., & Muños, S. (2017a). Molecular diversity of sunflower populations maintained as genetic resources is affected by multiplication processes and breeding for major traits. *Theoretical and Applied Genetics, 130*(6), 1099–1112.

Mangin, B., Bonnafous, F., Blanchet, N., Boniface, M. C., Bret-Mestries, E., Carrère, S., Cottret, L., Legrand, L., Marage, G., Pegot-Espagnet, P., & Munos, S. (2017b). Genomic prediction of sunflower hybrids oil content. *Frontiers in Plant Science, 8*, 1633.

Mangin, B., Casadebaig, P., Cadic, E., Blanchet, N., Boniface, M. C., Carrère, S., Gouzy, J., Legrand, L., Mayjonade, B., Pouilly, N., & André, T. (2017c). Genetic control of plasticity of oil yield for combined abiotic stresses using a joint approach of crop modelling and genome-wide association. *Plant, Cell & Environment, 40*(10), 2276–2291.

Marek, L. F. (2016). Sunflower genetic resources. In *Proceedings of the 19th international sunflower conference*. Edirne, Turkey, 29 May–3 June 2016. ISA, Paris, France, pp. 31–44.

Martínez-Force, E., Dunford, N. T., & Salas, J. J. (2015). *Sunflower: Chemistry, production, processing, and utilization*. Elsevier.

Mestries, E., Gentzbittel, L., de Labrouhe, D. T., Nicolas, P., & Vear, F. (1998). Analyses of quantitative trait loci associated with resistance to shape *Sclerotinia sclerotiorum* in sunflowers (*Helianthus annuus* L.) using molecular markers. *Molecular Breeding, 4*(3), 215–226.

Meuwissen, T. H. E., Hayes, B. J., & Goddard, M. E. (2001). Prediction of total genetic value using genome-wide dense marker maps. *Genetics, 157*(4), 1819–1829.

Miklas, P. N., Kelly, J. D., Beebe, S. E., & Blair, M. W. (2006). Common bean breeding for resistance against biotic and abiotic stresses: From classical to MAS breeding. *Euphytica, 147*(1–2), 105–131.

Miladinović, D., Hladni, N., Radanović, A., Jocić, S., & Cvejić, S. (2019). Sunflower and climate change: Possibilities of adaptation through breeding and genomic selection. In C. Kole (Ed.), *Genomic designing of climate-smart oilseed crops* (pp. 173–238). Springer.

Miladinović, D., Antunes, D., Yildirim, K., Bakhsh, A., Cvejić, S., Kondić-Špika, A., Marjanovic Jeromela, A., Opsahl-Sorteberg, H. G., Zambounis, A., & Hilioti, Z. (2021). Targeted plant

improvement through genome editing: From laboratory to field. *Plant Cell Reports*. https://doi.org/10.1007/s00299-020-02655-4

Miller, J. F., & Fick, G. N. (1997). The genetics of sunflower. In A. A. Schneiter (Ed.), *Sunflower technology and production* (Agronomy monograph 35) (pp. 395–439). ASA-CSSA-SSSA.

Miller, J. F., & Vick, B. A. (1999). Inheritance of reduced stearic and palmitic acid content in sunflower seed oil. *Crop Science, 39*, 364–367.

Mokrani, L., Gentzbittel, L., Azanza, F., Fitamant, L., Al-Chaarani, G., & Sarrafi, A. (2002). Mapping and analysis of quantitative trait loci for grain oil content and agronomic traits using AFLP and SSR in sunflower (*Helianthus annuus* L.). *Theoretical and Applied Genetics, 106*(1), 149–156.

Morozov, V. K. (1947). *Sunflower breeding in the USSR* (pp. 1–274). Pishchepromizdat. (In Russian).

Moschen, S., Bengoa Luoni, S., Di Rienzo, J. A., Caro, M. D. P., Tohge, T., Watanabe, M., Hollmann, J., González, S., Rivarola, M., García-García, F., & Dopazo, J. (2016a). Integrating transcriptomic and metabolomic analysis to understand natural leaf senescence in sunflower. *Plant Biotechnology Journal, 14*(2), 719–734.

Moschen, S., Higgins, J., Di Rienzo, J. A., Heinz, R. A., Paniego, N., & Fernandez, P. (2016b). Network and biosignature analysis for the integration of transcriptomic and metabolomic data to characterize leaf senescence process in sunflower. *BMC Bioinformatics, 17*(5), 174.

Moschen, S., Di Rienzo, J. A., Higgins, J., Tohge, T., Watanabe, M., González, S., Rivarola, M., García-García, F., Dopazo, J., Hopp, H. E., & Hoefgen, R. (2017). Integration of transcriptomic and metabolic data reveals hub transcription factors involved in drought stress response in sunflower (*Helianthus annuus* L.). *Plant Molecular Biology, 94*(4–5), 549–564.

Muriel, J. L., & Downes, R. W. (1974). Effect of periods of moisture stress during various. phases of growth of sunflowers in the greenhouse. In *Proceedings of 6th international sunflower conference*. Bucharest, Romania, July 22–24 1974. ISA, Paris, France, pp. 127–131.

Nagarathna, T. K., Shadakshari, Y. G., & Ramanappa, T. M. (2011). Molecular analysis of sunflower (*Helianthus annuus* L.) genotypes for high oleic acid using microsatellite markers. *Helia, 34*(55), 63–68.

Nambeesan, S. U., Mandel, J. R., Bowers, J. E., Marek, L. F., Ebert, D., Corbi, J., Rieseberg, L. H., Knapp, S. J., & Burke, J. M. (2015). Association mapping in sunflower (*Helianthus annuus* L.) reveals independent control of apical vs. basal branching. *BMC Plant Biology, 15*(1), 84.

Nel, A. A., Loubser, H. L., & Hammes, P. S. (2002). Development and validation of relationships between sunflower seed quality and easily measurable seed characteristics for grading purposes. *The South African Journal of Plant and Soil, 19*(4), 201–205.

Neto, A. J. S., Lopes, D. C., Pinto, F. A., & Zolnier, S. (2017). Vis/NIR spectroscopy and chemometrics for non-destructive estimation of water and chlorophyll status in sunflower leaves. *Biosystems Engineering, 155*, 124–133.

Nguyen, T. T., Slaughter, D. C., Townsley, B., Carriedo, L., Julin, N. N., & Sinha, N. (2016). Comparison of structure-from-motion and stereo vision techniques for full in-field 3d reconstruction and phenotyping of plants: An investigation in sunflower. In *2016 ASABE annual international meeting* (p. 1). American Society of Agricultural and Biological Engineers.

Osorio, J., Fernández-Martínez, J., Mancha, M., & Garcés, R. (1995). Mutant sunflowers with high concentration of saturated fatty acids in the oil. *Crop Science, 35*(3), 739–742.

Patel, M. D., & Thompson, P. D. (2006). Phytosterols and vascular disease. *Atherosclerosis, 186*, 12–19.

Pérez-Vich, B., Garcés, R., & Fernández-Martínez, J. M. (1999). Genetic control of high stearic acid content in the seed oil of the sunflower mutant CAS-3. *Theoretical and Applied Genetics, 99*(3–4), 663–669.

Pérez-Vich, B., Fernandez-Martinez, J. M., Grondona, M., Knapp, S. J., & Berry, S. T. (2002). Stearoyl-ACP and oleoyl-PC desaturase genes cosegregate with quantitative trait loci underlying high stearic and high oleic acid mutant phenotypes in sunflower. *Theoretical and Applied Genetics, 104*(2–3), 338–349.

Pérez-Vich, B., Knapp, S. J., Leon, A. J., Fernández-Martínez, J. M., & Berry, S. T. (2004). Mapping minor QTL for increased stearic acid content in sunflower seed oil. *Molecular Breeding, 13*(4), 313–322.

Pérez-Vich, B., Leon, A. J., Grondona, M., Velasco, L., & Fernández-Martínez, J. M. (2006). Molecular analysis of the high stearic acid content in sunflower mutant CAS-14. *Theoretical and Applied Genetics, 112*(5), 867–875.

Petrov, P. (1992). Use of heterosis in sunflower in Bulgaria. In *Proceedings of the 13th international sunflower conference*. Pisa, Italy, 7–11 September 1992. ISA, Paris, France, pp. 1216.

Picheny, V., Trépos, R., & Casadebaig, P. (2017). Optimization of black-box models with uncertain climatic inputs – Application to sunflower ideotype design. *PLoS One, 12*(5), e0176815.

Poehlman, J. M., & Sleper, D. A. (1995). Methods in plant breeding. In *Breeding field crops* (pp. 172–174).

Popov, P. S., & Demurin, Y. N. (1987). Mutation variability and inheritance of tocopherol composition in sunflower seeds of VIR collection. *Breeding and Genetics of Technical Crops, 113*, 30–33.

Premnath, A., Narayana, M., Ramakrishnan, C., Kuppusamy, S., & Chockalingam, V. (2016). Mapping quantitative trait loci controlling oil content, oleic acid and linoleic acid content in sunflower (*Helianthus annuus* L.). *Molecular Breeding, 36*(7), 106.

Pustavojt, V. S. (1966). Nasledovanie važnejših hozjajstveno cennih priznakov. In V. S. Pustavojt (Ed.), Izabrannie trudi, (94–96). Kolos, Moskva. in Russian.

Pustovoit, V. S. (1967). *Handbook of selection and seed growing of oil plants*. U.S. Department of Commerce.

Pustovoit, V. S. (1990). *Selected works* (p. 367). Agropromizdat.

Qi, L. L., Talukder, Z. I., Hulke, B. S., & Foley, M. E. (2017). Development and dissection of diagnostic SNP markers for the downy mildew resistance genes $Pl_{Arg}$ and $Pl_8$ and maker-assisted gene pyramiding in sunflower (*Helianthus annuus* L.). *Molecular Genetics and Genomics, 292*(3), 551–563.

Rachid Al-Chaarani, G., Gentzbittel, L., Huang, X. Q., & Sarrafi, A. (2004). Genotypic variation and identification of QTLs for agronomic traits, using AFLP and SSR markers in RILs of sunflower (*Helianthus annuus* L.). *Theoretical and Applied Genetics, 109*(7), 1353–1360.

Raina, A., Laskar, R. A., Khursheed, S., Amin, R., Parveen, K., & Khan, S. (2016). Role of mutation breeding in crop improvement-past, present and future. *Asian Research Journal of Agriculture, 2*, 1–13.

Raina, A., Laskar, R. A., Khursheed, S., Khan, S., Parveen, K., & Amin, R. (2017). Induce physical and chemical mutagenesis for improvement of yield attributing traits and their correlation analysis in chickpea. *International Letters of Natural Sciences, 61*, 14–22.

Raina, A., Laskar, R. A., Tantray, Y. R., Khursheed, S., Wani, M. R., & Khan, S. (2020a). Characterization of induced high yielding cowpea mutant lines using physiological, biochemical and molecular markers. *Scientific Reports, 10*(1), 1–22.

Raina, A., Khan, S., Sahu, P., & Roa, R. (2020b). Increasing rice grain yield under abiotic stresses: Mutagenesis, transgenics and genomics approaches. In C. Aryadeep (Ed.), *Rice research for quality improvement: Genomics and genetic engineering* (pp. 753–777). Springer. https://doi.org/10.1007/978-981-15-4120-9_31

Rao, N. M., & Singh, B. (1977). Inheritance of some quantitative characters in sunflower (*Helianthus annuus* L.). *Pantnagar Journal of Research, 2*(2), 144–146.

Rauf, S. (2008). Breeding sunflower (*Helianthus annuus* L.) for drought tolerance. *Communications in Biometry and Crop Science, 3*(1), 29–44.

Rauf, S. (2019). Breeding strategies for sunflower (*Helianthus annuus* L.) genetic improvement. In J. M. Al Khayri, S. M. Jain, & D. V. Johnson (Eds.), *Advances in plant breeding strategies: Industrial and food crops* (Vol. 6, pp. 637–673). Springer.

Robertson, J. A., Morrison, W. H., Burdick, D., & Shaw, R. (1972). Flavor and chemical evaluation of partially hydrogenated sunflower oil as a potato chip frying oil. *American Potato Journal, 49*(11), 444–450.

Rojas, P., & Fernández-Martínez, J. M. (1998). Combining ability of oil and protein among six sunflower lines. In *Proceedings of the EUCARPIA-international symposium on breeding of protein and oil crops*. Pontevedra, Spain EUCARPIA, pp. 117–118.

Routh, P. K., Sarkar, N. C., Das, P. K., Debnath, D., Bandyopadhyay, S., & Raj, U. (2019). Retrival of bio-physical parameters in sunflower crop (*Helianthus annuus*) using field based hyperspectral remote sensing. In *Proocedings of international archives of the photogrammetry, remote sensing & spatial information sciences*. New Delhi, India, 18–20 February 2019, Vol. XLII-3/W6, pp. 623–629.

Sabetta, W., Alba, V., Blanc, A., & Montemurro, C. (2011). sunTILL: A TILLING resource for gene function analysis in sunflower. *Plant Methods, 7*(1), 20.

Sala, C. A., Bulos, Altieri, E., & Ramos, M. L. (2012). Sunflower: Improving crop productivity and abiotic stress tolerance. In N. Tuteja, S. S. Gill, A. F. Tiburcio, & R. Tuteja (Eds.), *Improving crop resistance to abiotic stress* (pp. 1203–1249). Wiley-VCH Verlag GmbH & KgaA.

Salas, J. J., Bootello, M. A., & Garcés, R. (2015). Food uses of sunflower oils. In E. Martínez-Force, N. T. Dunford, & J. J. Salas (Eds.), *Sunflower: Chemistry, production, processing, and utilization* (pp. 441–464). Elsevier/AOCS Press.

Sarazin, V., Duclercq, J., Guillot, X., Sangwan, B., & Sangwan, R. S. (2017). Water-stressed sunflower transcriptome analysis revealed important molecular markers involved in drought stress response and tolerance. *Environmental and Experimental Botany, 142*, 45–53.

Schuppert, G. F., Tang, S., Slabaugh, M. B., & Knapp, S. J. (2006). The sunflower high-oleic mutant *Ol* carries variable tandem repeats of FAD2-1, a seed-specific oleoyl-phosphatidyl choline desaturase. *Molecular Breeding, 17*(3), 241–256.

Schuster, W. (1964). *Inbreeding and heterosis in sunflower (Helianthus annuus L.)* (p. 135). Wilhem Schmitz.

Schuster, W. (1980). Investigations of the effects of continued inbreeding from the I0 to the I25 on different characters in the sunflower (*Helianthus annuus* L.). *Zeitschrift fur Pflanzenzuchtung, 84*(2), 148–116.

Seiler, G. J. (1983). Effect of genotype, flowering date, and environment on oil content and oil quality of wild sunflower seed 1. *Crop Science, 23*(6), 1063–1068.

Seiler, G. J. (1998). The potential use of wild *Helianthus* species for selection of low saturated fatty acids in sunflower oil. In *Proceedings of the international symposium on breeding of protein and oil crops*. EUCARPIA, Pontevedra, Spain, 1–4 April 1998. EUCARPIA, pp. 109–110.

Seiler, G. J. (2007). The potential of wild sunflower species for industrial uses. *Helia, 30*(46), 175–198.

Seiler, G., & Jan, C. C. (2010). Basic information. In J. Hu, G. Seiler, & C. Kole (Eds.), *Genetics, genomics and breeding of sunflower* (pp. 1–40). Science Publishers/CRC Press.

Seiler, G. J., & Rieseberg, L. H. (1997). Systematics, origin, and germplasm resources of the wild and domesticated sunflower. In *Sunflower technology and production* (pp. 21–65). American Society Agron.

Seiler, G. J., & Marek, L. F. (2016). Collection of Wild Helianthus anomalus and deserticola Sunflower from the Desert Southwest USA. *Helia, 39(65), 139–155.*

Seiler, G. J., Qi, L. L., & Marek, L. F. (2017). Utilization of sunflower crop wild relatives for cultivated sunflower improvement. *Crop Science, 57*(3), 1083–1101.

Škorić, D. (1976). Mode of inheritance of oil content in sunflower seed of F1 generation and components of genetic variability. In *Proceedings of 7th international sunflower conference, Krasnodar, Russia*. ISA, Paris, France, pp. 376–388.

Škorić, D. (2009). Sunflower breeding for resistance to abiotic stresses. *Helia, 32*(50), 1–16.

Škorić, D. (2012). Sunflower breeding. In D. Škorić & Z. Sakač (Eds.), *Sunflower genetics and breeding* (pp. 164–344). Serbian Academy of Sciences (SASA), Branch in Novi Sad.

Škorić, D., Jocić, S., Sakac, Z., & Lecić, N. (2008). Genetic possibilities for altering sunflower oil quality to obtain novel oils. *The Canadian Journal of Physiology and Pharmacology, 86*, 215–221.

Škorić, D., Seiler, G. J., Liu, Z., Jan, C. C., Miller, J. F., & Charlet, L. D. (2012). *Sunflower genetics and breeding* (International monograph) (p. 519). Serbian Academy of Sciences and Arts Branch in Novi Sad.

Soldatov, K. I. (1976). Chemical mutagenesis in sunflower breeding. In *Proceedings of the 7th international sunflower conference*. Krasnodar, USSR, 23 June–3 July 1976. ISA, Paris, France, pp. 352–357.

Sreenivasulu, N., Sopory, S. K., & Kishor, P. K. (2007). Deciphering the regulatory mechanisms of abiotic stress tolerance in plants by genomic approaches. *Gene, 388*(1–2), 1–13.

Sukno, S., Melero-Vara, J. M., & Fernández-Martínez, J. M. (1999). Inheritance of resistance to *Orobanche cernua* Loefl. in six sunflower lines. *Crop Science, 39*(3), 674–678.

Tang, S., Leon, A., Bridges, W. C., & Knapp, S. J. (2006). Quantitative trait loci for genetically correlated seed traits are tightly linked to branching and pericarp pigment loci in sunflower. *Crop Science, 46*(2), 721–734.

Trémoliéres, A., Dubacq, J. P., & Drapier, D. (1982). Unsaturated fatty acids in maturing seeds of sunflower and rape: Regulation by temperature and light intensity. *Phytochemistry, 21*(1), 41–45.

Tunca, E., Köksal, E. S., Çetin, S., Ekiz, N. M., & Balde, H. (2018). Yield and leaf area index estimations for sunflower plants using unmanned aerial vehicle images. *Environmental Monitoring and Assessment, 190*(11), 682.

Vanitha, J., Manivannan, N., & Chandirakala, R. (2014). Qualitative trait loci analysis for seed yield and component traits in sunflower. *African Journal of Biotechnology, 13*(6), 754–761.

Vanozzi, G. P. (2006). The perspectives of use of high oleic sunflower for oleochemistry and energy raws. *Helia, 29*(44), 1–24.

Vear, F. (2010). Classic genetics and breeding. In J. Hu, G. Seiler, & C. Kole (Eds.), *Genetics, genomics and breeding of sunflower* (pp. 51–77). Science Publishers/CRC Press.

Vear, F. (2016). Changes in sunflower breeding over the last fifty years. *OCL, 23*(2), D202.

Velasco, L., Fernández-Martínez, J. M., Garcia-Ruiz, R., & Domínguez, J. (2002). Genetic and environmental variation for tocopherol content and composition in sunflower commercial hybrids. *The Journal of Agricultural Science, 139*(4), 425–429.

Velasco, L., Pérez-Vich, B., & Fernández-Martínez, J. M. (2004). Novel variation for the tocopherol profile in a sunflower created by mutagenesis and recombination. *Plant Breeding, 123*(5), 490–492.

Velasco, L., Pérez-Vich, B., & Fernández-Martínez, J. M. A. (2008). New sunflower mutant with increased levels of palmitic acid in seed oil. *Helia, 31*(1), 55–60.

Velasco, L., Del Moral, L., Pérez-Vich, B., & Fernández-Martínez, J. M. (2010). Selection for contrasting seed tocopherol content in sunflower seeds. *The Journal of Agricultural Science, 148*(4), 393–400.

Vera-Ruiz, E. M., Velasco, L., Leon, A. J., Fernández-Martínez, J. M., & Pérez-Vich, B. (2006). Genetic mapping of the *Tph1* gene controlling beta-tocopherol accumulation in sunflower seeds. *Molecular Breeding, 17*(3), 291–296.

Voljf, V. G., & Dumačeva, P. L. (1973). *Pojavlenije geterozisa u gibridov pervogo pokolenija podsolnečnika* (pp. 40). Geterozis kulturnih rastenij. Rez, Varna.

Wani, M. R., Dar, A. R., Tak, A., Amin, I., Shah, N. H., Rehman, R., Baba, M. Y., Raina, A., Laskar, R., Kozgar, M. I., & Khan, S. (2017). Chemo-induced pod and seed mutants in mungbean (*Vigna radiata* L. Wilczek). *SAARC Journal of Agriculture, 15*(2), 57–67.

Wani, M. R., Laskar, R. A., Raina, A., Khan, S., & Khan, T. U. (2021). Application of chemical mutagenesis for improvement of productivity traits in lentil (*Lens culinaris* Medik). *Annals of Biology, 37*(1), 69–75.

Wills, D. M., & Burke, J. M. (2007). Quantitative trait locus analysis of the early domestication of sunflower. *Genetics, 176*, 2589–2599.

Yang, C., Xu, L., Zhang, N., Islam, F., Song, W., Hu, L., Liu, D., Xie, X., & Zhou, W. (2017). iTRAQ-based proteomics of sunflower cultivars differing in resistance to parasitic weed *Orobanche cumana*. *Proteomics, 17*(13–14), 1700009.

Yue, B., Cai, X., Yuan, W., Vick, B., & Hu, J. (2009). Mapping quantitative trait loci (QTL) controlling seed morphology and disk diameter in sunflower (*Helianthus annuus* L.). *Helia, 32*(50), 17–36.

Zeng, W., Xu, C., Zhao, G., Wu, J., & Huang, J. (2018). Estimation of sunflower seed yield using partial least squares regression and artificial neural network models. *Pedosphere, 28*(5), 764–774.

Zhou, F., Liu, Y., Liang, C., Wang, W., Li, C., Guo, Y., Ma, J., Yu, Y., Fan, L., Yao, Y., & Zhao, D. (2018). Construction of a high-density genetic linkage map and QTL mapping of oleic acid content and three agronomic traits in sunflower (*Helianthus annuus* L.) using specific-locus amplified fragment sequencing (SLAF-seq). *Breeding Science, 68*, 18051.

Zorić, M., Cvejić, S., Mladenović, E., Jocić, S., Babić, Z., Marjanović Jeromela, A., & Miladinović, D. (2020). Digital image analysis using FloCIA software for ornamental sunflower ray floret color evaluation. *Frontiers in Plant Science, 11*, 584822.

Zubrzycki, J. E., Maringolo, C. A., Filippi, C. V., Quiroz, F. J., Nishinakamasu, V., Puebla, A. F., Di Rienzo, J. A., Escande, A., Lia, V. V., Heinz, R. A., Hopp, H. E., Cervigni, G. D. L., & Paniego, N. B. (2017). Main and epistatic QTL analyses for *Sclerotinia* Head Rot resistance in sunflower. *PLoS One, 12*(12), e0189859.

# Mendelian to Genomics and Bioinformatics Approaches in Cytoplasmic Male Sterility and Fertility Restoration in Sorghum Breeding


**Krishnananda P. Ingle, Mangesh P. Moharil, Santosh J. Gahukar, Pravin V. Jadhav, Rameshwar Ghorade, Gholamareza Abdi, Gopal W. Narkhede, and Atul Singh**

**Abstract** *Sorghum bicolor* L. Moench is a fodder crop and annual cereal grain belonging to Poaceae family and is popularly known as 'King of Millet.' Sorghum breeders have been harnessing yield-associated heterosis to facilitate hybrid seed production. In this regard, cytoplasmic-nuclear interaction majorly contributes to cytoplasmic genetic male sterility. The A1 (*milo*) source of cytoplasm in sorghum is used extensively to commercialize hybrid seed production. Nuclear genes, the restorers of fertility (*Rf*), counteract the male-sterile phenotype and hence can restore the pollen fertility in plants and overcome the deleterious interaction in the genome of the mitochondrion. Cytoplasmic male sterility (CMS) *Rf* systems significantly boost the production of hybrid seed, and these *Rf* genes are shown to encode pentatricopeptide repeat (PPR) containing proteins. These proteins mainly target chloroplasts and mitochondria and are involved in RNA processing, site-specific endonucleolytic cleavage, and RNA degradation. The development of candidate genes responsible for fertility restoration is now considered significant in using

K. P. Ingle (✉) · A. Singh
College of Agriculture, Koneru Lakshmaiah University, Guntur, Andhra Pradesh, India
e-mail: krisona369@gmail.com

M. P. Moharil · S. J. Gahukar · P. V. Jadhav
Biotechnology Centre, Department of Agricultural Botany, Post Graduate Institute, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, India

R. Ghorade
Sorghum Research Scientist, Department of Agricultural Botany, Post Graduate Institute, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, India

G. Abdi
Department of Biotechnology, Persian Gulf Research Institute, Persian Gulf University, Bushehr, Iran

G. W. Narkhede
Associate Scientist (Backcross Breeding), Kalash Seeds Pvt Ltd., Jalna, India



A. Raina et al. (eds.), *Advanced Crop Improvement, Volume 2*,
https://doi.org/10.1007/978-3-031-26669-0_14

them functional markers. The present review gives an account of the genetic and molecular perspectives of the CMS and fertility restoration in post-rainy sorghum.

**Keywords** Cytoplasmic male sterility · Fertility restoration · *In silico* analysis · Pentatricopeptide repeat protein · Programmed cell death

## 1 Introduction

After wheat, rice, corn, and barley, *Sorghum bicolor* (L.) Moench is the most widely farmed cereal grain in the world, feeding around 500 million people in Africa (Morris et al., 2013). As food and fodder, it is primarily farmed in semi-arid tropical climates (Food and Nations 2019). Sorghum yields are nearly three times higher in developed countries than in the rest of the world (Upadhyaya et al., 2019). It is a $C_4$ *Panicoideae* crop with a diploid genome (~730 Mb) with ten chromosomes (2n = 20). As a $C_4$ crop, it has excellent tolerance to biotic stresses such as drought and temperature (Xin et al., 2017). Sorghum is often considered a cross-pollinated crop, although it is self-pollinated because of stigma receptivity before the anthesis (Celarier, 1958). It is a resilient and dependable crop that grows in dry climates and hence contributes significantly to raising food production in the semi-arid tropics (Pattanashetti, 2014). To meet the ever-increasing need for food and feed in developing countries, climate-resilient and high-yielding crop types are required (Muleta et al., 2019).

## 2 Sorghum Area and Production

The world sorghum area under cultivation is around 40.53 million ha with a production of 58.28 million mt and 1.44 mt per ha productivity, whereas the United States has productivity of 4.58 mt per ha. As compared to the total foreign productivity to the United States, there is a considerable gap of 3.48 mt per ha of difference that can be observed. The countries like India, Pakistan, Uganda, Niger, and Sudan, have productivity of <1 mt per ha (https://apps.fas.usda.gov/psdonline/circulars/production.pdf). According to comparative research, the area under kharif sorghum was 0.621 million ha, and the area under post-rainy (*rabi*) sorghum was 2.597 million ha, with 0.368 and 0.837 million tonnes produced, respectively. Productivity in a kharif was 594 kg $ha^{-1}$ and in post-rainy 322 kg $ha^{-1}$ (Ingle, 2020). In the Vidarbha region, the area under kharif sorghum was 0.119 million ha, and production was 0.70 million mt with productivity 859 kg $ha^{-1}$, whereas post-rainy Sorghum occupied area of 0.273 million ha, and production was 0.136 million

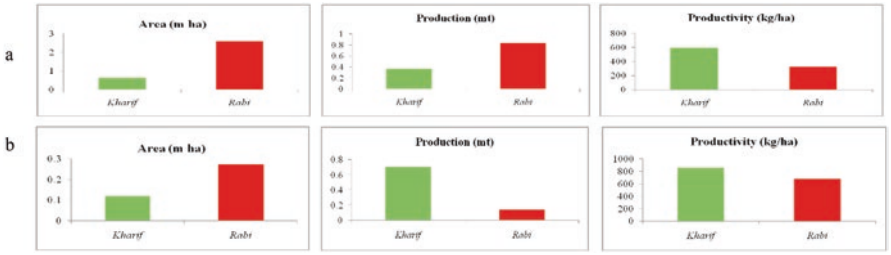



**Fig. 1** Post-rainy (*rabi*) and *kharif* sorghum cultivated area, production, and productivity in (**a**) Vidarbha region and (**b**) Maharashtra region of India source

tonnes with productivity 684 kg ha$^{-1}$ (https://www.districtsofindia.com/maharashtra/alldistricts/agriculture/index.aspx) (Fig. 1).

Male cytoplasmic sterility (CMS) is a maternally inherited condition that comes from the plant's inability to generate pollen. Mitochondrial abnormalities have been identified as the cause of all CMS cases so far. CMS-associated regions contain unique ORFs, which are frequently chimeric in form and transcribed alongside traditional mitochondrial genes. Nuclear genes (called fertility inhibitors (*Rf*)) can suppress the male sterile phenotype, allowing plants with damaging mitochondrial genomes to produce pollen again. CMS *Rf* system will make hybrid seed production easier by removing the need for tedious manual labour and assuring that each seed is the result of cross-pollination (Schnable & Wise, 1998). In sorghum, two reproductive repair genes for cytoplasm A1 have been found, with the operational genes being members of the pentatricopeptide repeat gene (PPR) family (Jordan et al., 2010; Klein et al., 2005). Genes that code for PPR proteins are frequently linked to reproductive recovery. Successive repeats of a degenerate 35 amino acid motif are the main character of PPR proteins. The PPR family is hypothesized to be involved in RNA processing, site-specific endonucleolytic cleavage, and degradation in mitochondria and chloroplasts. The capacity to restore fertility and maintain sterility is segregated in most landraces, demonstrating the necessity to select for this ability. Post-rainy season sorghum is a major import sorghum variety cultivated in dryland crops across India, including Maharashtra, Gujarat, Karnataka, Andhra Pradesh, and Telangana, necessitating the creation of varieties particularly suited for the post-rainy season to boost yields and productivity. However, there is lack of appropriate hybrids with acceptable seed setting and grain quality as many factors responsible for this temperature, photoperiod, and fertility restoration. To develop hybrids with high ability to restore fertility, it is necessary to exploit the candidate genes responsible for fertility restoration that could be used as a functional marker against the parental lines and restorers to choose the best restorer (s) with the best fertility with the other CMS lines.

The capacity to restore fertility and maintain sterility is segregated in most indigenous breeders, showing the necessity to select for this ability. Sorghum is an important dryland crop in Maharashtra, Karnataka, Gujarat, Telangana, and Andhra Pradesh; hence, varieties that are especially suited for the post-rainy season are

needed to boost production and productivity. Many variables responsible for this restoration of photoperiod, temperature, and fertility, however, include a shortage of appropriate hybrids with acceptable seed and grain quality. Therefore, it is necessary to exploit the candidate genes responsible for the restoration of fertility that could be used as a functional marker against the parental and restorer lines to choose the good restorers that have the best fertility with the other CMS lines to develop hybrids with high ability to restore fertility. To speed up the creation of sorghum varieties with high genetic yield potential, modern breeding concepts and technology are urgently needed (Hao et al., 2021). Therefore, developing the hybrids for the post-rainy season is required to increase the yield and performance by incorporating high-yielding combinations using marker-assisted selection. This chapter describes the exploration of genes in the sorghum restorers and in landraces responsible for the fertility restoration to develop high-yielding post-rainy season hybrids and depicts the model responsible for the CMS.

## 3 Constraints in Post-rainy Sorghum Hybrid Breeding

Despite proving its importance, post-rainy sorghum has not attained prime importance to date. Despite the large areas under cultivation, the continuing marginal production of post-rainy sorghum indicates a low impact of crop improvement efforts on productivity (Reddy et al., 2012). Several constraints need to be addressed to make post-rainy sorghum more remunerative for the breeders, including the following:

(a) Growing in a large area of medium to shallow soils where drought occurrence is much faster than in deep soils.
(b) Charcoal rot and shoot fly susceptibility infestation is a major constraint for higher productivity in post-rainy season.
(c) Low temperature (Mukri et al., 2010) is critical to success of post-rainy season hybrids which significantly affects the pollen fertility and seed setting percentage.
(d) Lack of appropriate hybrids with improper fertility restoration leads significant reduction in the yield and, therefore, a need of selection of landraces having post-rainy adaptations (Ganapathy et al., 2012).
(e) Fertility restoration assessment, especially under low temperatures, normally observed in post-rainy season (Praveen et al., 2015).

Among all the constraints, the most significant one is fertility restoration. It is necessary to derive a system that will facilitate the transfer of restorer genes to different nuclear backgrounds and exploit the candidate genes responsible for the fertility restoration, which can be utilized as functional markers against the parental lines and restorer lines to select the best restorer(s) with the best fertility with the

other CMS lines. Identifying these genes aids in the development of hybrids with high fertility restoration capabilities as well as the knowledge of the molecular process of fertility restoration.

Cytoplasmic male sterility (CMS) is predominantly used for the exploitation of the hybrid vigour owing to its heritable nature to transfer the desired gametophyte to induce sterility or fertility, which remains a cost-effective system to promote efficient hybrid production as compared to the Mendelian fashion and restoration of fertility and its maintenance (Budar & Pelletier, 2001). The phenomenon of male sterility was firstly reported in India (Ayyangar & Ponnaiya, 1937) and in America (Stephens, 1937). Cytoplasmic male sterility refers to the production of non-functional pollen grains, anther dehiscence, and non-viable male gametes (Mayr, 1986). Male sterility provides an essential breeding tool to harness heterosis in hybrid crops and offers valuable insight to study the cytoplasmic-nuclear genomic interactions (Kaul, 1988; Schnable & Wise, 1998; Hanson & Bentolila, 2004). Fertility restoration is often governed by nuclear-encoded genes known as fertility restoration genes (*Rf*) (Kofuji et al., 2012). These *Rf* genes encode a protein called pentatricopeptide repeat (PPR). These restorers PPR proteins rectify the nuclear-mitochondrial interaction and restore fertility by reducing the accumulation of their cognate CMS-associated mitochondrial proteins (Barkan & Small, 2014). Mitochondrial-nuclear interaction can also encode fertility restorer genes (*Rf*), which mostly counteract the effect of sterilizing factors of the mitochondria (Touzet & Budar, 2004). Hence, it is essential to investigate male sterility and identify genes responsible for fertility restoration and gain insight into molecular mechanisms for fertility restoration in post-rainy sorghum to facilitate crop improvement.

## 4 Cytoplasm Diversity in Sorghum

After maize, sorghum is the second cereal crop where the CMS approach has been effectively used to mass-produce F1 hybrids to enhance the productivity (Praveen et al., 2015). CMS in sorghum was first discovered through the introduction of the '*kafir*' nuclear genome into '*milo*,' having an incompatible cytoplasmic background (Reddy & Stenhouse, 1994; Reddy et al., 2008). Sorghum F1 hybrids are superior by 50–60% in their grain yield than the traditional landraces. The discovery of A1 (*milo*) CMS in sorghum (Stephens & Holland, 1954) has revolutionized sorghum production worldwide and subsequently exploited for mass production of hybrid seeds Reddy (et al., 2007). Almost all commercially released *post-rainy* sorghum hybrids are based on the A1 (*milo*) CMS system. The utilization of CMS in a breeding programme made it feasible to incorporate the specific character into hybrid parents (Garcia et al., 2019). Hybrid seeds enhance crop production as they harness hybrid vigour (Singh et al., 2015). In addition to source $A_1$, several other cytoplasmic sources have been found such as $A_2$ (Schertz & Ritchey, 1978), $A_4$ (Worstell


**Fig. 2** A fertile panicle and CMS systems in sorghum: *Milo* ($A_1$) and *non-milo* ($A_2$, $A_3$ & $A_4$). (Source: Reddy et al. 2008)

et al., 1984), $A_3$ (Quinby, 1981), Indian $A_4$ ($A_4$M, $A_4$VZM, $A_4$G) (Rao et al., 1984), KS cytoplasm's (Ross & Hackerott, 1972), and $A_5$, $A_6$, 9E (Webster & Singh, 1964). The A1 (*milo*) CMS system is predominantly used in the development of the commercial hybrids in India, China, America, and Australia. Prominently, $A_1$, $A_2$, $A_3$, and $A_4$ cytoplasm are used extensively (Fig. 2). The majority of breeding lines act as restorers on A1 (*milo*) cytoplasm, making restoration relatively easy. Commercial sorghum hybrids all over the world employ A1 cytoplasm (Reddy & Stenhouse, 1994).

The A1 (*milo*) CMS method has been utilized to successfully commercialize sorghum hybrids and develop male parent lines (R-lines or restorers that carry the dominant genes that restore male fertility in hybrid crops). Restoration of fertility in A1 (*milo*) cytoplasm is challenging, according to traditional genetics research, with modifier genes and primary genes accounting for most of the genotypic diversity (Maunder & Pickett, 1959; Erichsen & Ross, 1963; Miller & Pickett, 1964). Non-milo cytoplasm repair is difficult, and research in this area is restricted. It was reported that restoration on A1 (*milo*) cytoplasm is easy because most of the breeding lines act as restorers, whereas $A_2$, $A_3$, and $A_4$ cytoplasm appear difficult for fertility restoration (Kante et al., 2018). Apart from high restoration frequency on A1 (*milo*) cytoplasm, the hybrids developed on this cytoplasm showed resistance to grain mould infection (Reddy et al., 2011). Some restorers are available for $A_2$ and other cytoplasm but are not acceptable agronomically because of stability (Jilani et al., 2000). Therefore, there is a need to upgrade the restorers for diverse cytoplasmic male sterile lines and identify the fertility restoration genes in the sorghum restorers and hybrids through marker-aided selection/marker-assisted breeding approaches.

## 5 Cytoplasmic Male Sterility: *Rf* (Fertility Restoration) System

Cytoplasmic male sterility is inherited maternally, enabling plants to produce non-functional pollen (Schnable & Wise, 1998). CMS provides insight into nuclear-mitochondrial interaction because aberrant mitochondrial genome organization causes dysfunction in pollen development (Chase, 2007; Hanson & Bentolila, 2004). Mostly sporophytic male sterility primarily affects tapeta and meiocytes and leads to abortion of pollen (Guo & Liu, 2012), whereas gametophytic male sterility mainly affects the microspores or pollen grains development (Chen & Liu, 2014). Thus, male sterility in CMS is expressed under the presence of dominant allele present in S-cytoplasm, whereas the recessive allele of restorer is located in the nuclear genome, which is conditioned by the nuclear and mitochondrial interaction but neither the cytoplasmic factor nor the genetic factors alone can regulate sterility (Mishra & Kumari, 2018). Nuclear restorer (*Rf* or *Fr*) genes suppress male-sterile phenotype and mitochondrial abnormalities by diverse mechanisms (Schnable & Wise, 1998). To create reliable male-fertility-restoring lines for 9E type CMS, the progeny of test crosses of fertile revertant lines in 9E cytoplasm, which were obtained because of reversions in sterile $F_1$ hybrids induced by conditions of high-water availability, with CMS lines in this cytoplasm were grown in selective backgrounds (Elkonin & Sarsenova, 2020). The possible mechanisms responsible for fertility restoration through nuclear genes that occur in CMS/*Rf* systems are postulated in this review.

## 6 Molecular Basis: The Mitochondrial Route

Restorers of fertility (*Rf*) are nuclear genes that suppress the male-sterile phenotype in plants with a deleterious mitochondrial genome and restore pollen production (Chase, 2007). CMS Rf systems make hybrid seed production much easier and eliminate the need for hand emasculation (Schnable & Wise, 1998). CMS/*Rf* systems play a significant role in elucidating mitochondrial and nuclear genetic interactions and cooperative functions in plants (Fujii & Toriyama, 2008). Mitochondrial defects are responsible for cytoplasmic male sterility. The regions whose expression is associated with CMS are often chimeric in structure and contain unusual open reading frames and are frequently co-transcribed with conventional mitochondrial genes (Chen & Liu, 2014). Many CMS genes arise from mitochondrial genomic rearrangements. It has been shown that ten mitochondrial genes are actively involved in the formation of CMS genes through the mitochondrial electron transfer chain (mtETC) pathway. Among these mitochondrial genes, *atp8*, *atp6*, and *cox1* are frequently involved in the CMS genes origination. In sorghum, mitochondrial gene *orf107* encodes a protein with a segment of *atp9* on the $A_3$ cytoplasm at the N

terminus, and the remaining portion is similar to ORF79 (Tang et al., 1996). The mitochondrial gene *coxI* has been reported for the cytoplasm 9E in a CMS line of sorghum.

Respiratory chain enzyme complexes (NADH dehydrogenase, cytochrome oxidase, and cytochrome reductase) play a pivotal role in the electron transfer system in plants and play an important role in linking electron transfer to proton transport outside the mitochondria and generating proton stimulus, membrane that induces ATP synthesis by the $H^+$-ATPase enzyme (Luo et al., 2013; Kim et al., 2007). The overproduction of reactive oxygen species (ROS) and release of cytochrome c from mitochondria to cytosol have been reported to cause programmed cell death (PCD) or plant apoptosis (Greenberg & Yao, 2004; Liu et al., 1996; Yao et al., 2002), the coding region of *coxI* gene in an extension of 303 nucleotides (Bailey-Serres et al., 1986). COXI is an assembly factor for *cytochrome c oxidase*, which plays a crucial role in mitochondrial respiration. In rice, COXII has another role in the degradation of hydrogen peroxide, thereby reducing the free radicals and overcoming programmed cell death (Luo et al., 2013). Sorghum hybrid breeding consists of a three-line system, male-sterile (CMS), male fertile (maintainer), and restorer line (restorer). In the male fertile line (maintainer), the fertile cytoplasm is responsible for its self-fertility due to *Rf* genes' absence. In male sterile lines, CMS mitochondrial genomic rearrangement results in the accumulation of CMS proteins preferentially in another tapetum, thereby inhibiting COXI function through physical interaction. This deleterious interaction inhibits the function of COXI in hydrogen peroxide metabolism; thus, there is an early release of *cytochrome c* and triggers the ROS (reactive oxygen species), which forcibly leads to premature tapetal death at the meiotic stage. These abnormal molecular events significantly show the pollen abortion and tapetal degeneration responsible for male sterility. As a result of a negative interaction between modifier genes (mitochondrial genes) and conserved genes (essential nuclear genes), cytoplasmic-nuclear incompatibility occurs (Luo et al., 2013; Van et al., 2008).

The nucleus-encoded *Rf*1, *Rf*2, *Rf*5, and *Rf*6 genes (these *RF* genes are targeted to mitochondria and interact with cytoplasmic male sterile genes with a view to restore fertility by suppressing the CMS genes expressions) of encodes PPR proteins. This protein binds to CMS proteins and rectifies the nuclear-mitochondrial defects through RNA editing, splicing, transcriptionally, or post-transcriptionally or through translationally or post-translationally to reduce CMS protein accumulation and CMS-RNA level and, thus, restore fertility (Chen & Liu, 2014). It was reported that the CMS line synthesized a unique nucleotide chain (Pring et al., 1982) a variant of COXI mitochondrial gene leading to the replacement of 38 KDa polypeptide in the male fertile line sorghum (Dixon et al., 1982). This mitochondrial genomic rearrangement leads to the synthesis of the variant enzyme, which results in forming a novel-mitochondrial open reading frame (ORF) (Bailey-Serres et al., 1986). Thus, it has been suggested that mitochondrial biogenesis or function impaired by the presence of variant enzyme could interfere with pollen fertility, and this pollen fertility restore by the PPR protein through rectification of mitochondrial-nuclear genomic rearrangement. Therefore, cytoplasm male sterility is due to the chimeric

association of ORFs, and fertility restoration is often associated with nuclear-encoded *Rf* (fertility restoration) genes encoding PPR proteins (Hanson & Bentolila, 2004).

## 7 Molecular Basis: The Nuclear Route

Selection for full sterility in female parents and complete fertility recovery in CMS hybrids by male parents reveal that breeding genes and reproductive repair genes in hybrid breeding programmes are significantly characterized by classical genetics (Jordan et al., 2011). Thus, a negative interaction between modifier genes (mitochondrial genes) and conserved genes (essential nuclear genes) causes cytoplasmic-nuclear incompatibility (Luo et al., 2013; Van et al., 2008). Restoration frequency is higher in the $A_1$ cytoplasm (65%) compared to $A_2$ (56%) female parents. Fertility restoration by dominant nuclear gene(s) is essential to mass-produce $F_1$ hybrid seed as it is produced on a male sterile seed parent (Praveen et al., 2015). Different numbers of nuclear genes responsible for fertility recovery have been cloned in different crops.

The majority of these genes encode and target pentatricopeptide replication proteins (PPRs), which work by specifically reducing the expression of sterility-causing mitochondrial transcripts (Chen & Liu, 2014; Hu et al., 2012; Klein et al., 2005; Koizuka et al., 2003; Wang et al., 2006; Dahan & Mireau, 2013).

Till date, five reproductive repair (*Rf*) genes encoding the pentatricopeptide repeat protein (PPR) have been mapped, and potential candidate genes have been identified as part of the PPR gene family (Klein et al., 2001; Jordan et al., 2010; Praveen et al., 2015) listed in Table 1.

PPR proteins (characterized by a 35-amino-acid motif in tandem arrays of 2-27 repeats per peptide) bind RNA proteins that target the mitochondria or chloroplast and restore fertility in the CMS line through post-transcriptional, transcriptional, translational, post-translational, and metabolic modification (Chen & Liu, 2014; Schmitz-Linneweber & Small, 2008).

PPR genes are involved in fertility restoration in $Rf_1$ sorghum (Klein et al., 2001, 2005), $Rf_2$ (Jordan et al., 2010), $Rf_5$ (Jordan et al., 2011), and $Rf_6$ (Praveen et al., 2015). $Rf_1$ can significantly reduce the level of the sterilizing factor (Liu et al., 2001), whereas $Rf_2$ has been recognized for putative aldehyde dehydrogenase, an enzyme involved in metabolic functions and reactions. $Rf_5$ and $Rf_6$ are the mitochondrial precursor that rectifies the mitochondrial, nuclear interaction and encodes for PPR protein family that restore fertility in CMS line, which yields high-yielding hybrids. *Rf* proteins are transcribed in the mitochondria after being encoded in the nucleus (Melonek et al., 2021).

**Table 1** Candidate gene targeted fertility restoration traits in sorghum encoding PPR protein

| SN | Gene | LG | Transcript name | Forward sequence | Reverse sequence | Size (bp) |
|---|---|---|---|---|---|---|
| 1 | *RF1* | 8 | Sobic.008G037700 | AGATGGTTGAGGGAATGCGG | AAGCTTTGGCAATAGCCCCT | 624 |
| 2 | *RF1* | 8 | Sb08g020970 | GTTGTGGCTTGCAACGTGAT | AGCCCTATTTGTGCCCCTTC | 567 |
| 3 | *RF2* | 2 | Sobic.002G057000 | TCATGCCAGACATAGTCGCC | CTTTCACGGTTACACCTCGT | 1100 |
| 4 | *RF2* | 2 | Sb02g004530 | ACCAGGTCTTGTTGAACTCGTC | CACGTGCAGGAGCTAAACCT | 734 |
| 5 | *RF3* | 3 | Sb03g010260.1 | GCTGGTCACACTGAAAAGGC | TTGTTGGATCAAGCAGGCCA | 793 |
| 6 | *RF5* | 5 | Sb05g002606 | AGCTTTGCACAGGCCATCAA | TCGCTTCATTGATCCGGAGG | 835 |
| 7 | *RF5* | 5 | Sb05g002620 | CAGGCGTAACTCCCTTCCTC | TGCAAGAATGGAAAGTGCAATG | 805 |
| 8 | *RF6* | 4 | Sobic.004G004100 | CTCGCCAATCTCTTCGGGAC | CAAACCTGGCGTTGTTGACC | 224 |
| 9 | *RF6* | 4 | Sobic.004G143100 | CTTCAACGAGGAGGGCAGAC | GCGGTGTAGGTGACTACGTT | 460 |

Source: Ingle et al. (2019)
*RF* fertility restoration genes, *LG* location in genome, *bp* base pairs

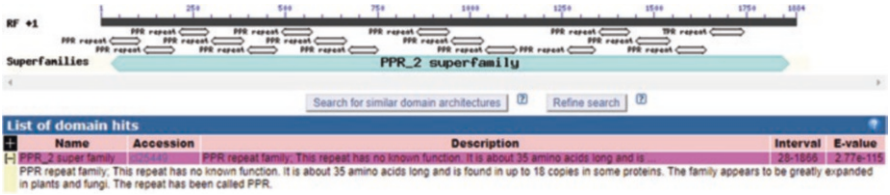



**Fig. 3** *Coding sequences* sequence of gene Sb08g020970 targeted protein pentatricopeptide repeat-containing protein (putative mitochondrial protein). (Source: https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi)

## 8 In Silico Analysis of Candidate Genes Encoding PPR Protein

Our in silico analysis of sorghum gene Sb08g020970 (Table 1) revealed the targeted protein PPR family. The *cds* sequence of gene Sb08g020970 was searched in conserved domain databases (Fig. 3).

The predicted alignment indicated a highly conserved amino acid sequence of PPR protein (Fig. 4), whereas expression analysis using phytozome of the respective gene revealed that gene encoding PPR protein showed high expression during anthesis/initiation of the flowering period (Fig. 5). Similarly, all the listed genes (Table 1) showed that these encode for PPR protein.

## 9 Pentatricopeptide Repeat Protein (PPR) Family

The restoration in the CMS phenotype is done via nuclear-derived RNA-binding proteins that encode for pentatricopeptide repeat protein (Barkan & Small, 2014). PPR proteins are made up of tandem arrays of a degenerate 35 amino acid motif that binds to RNA in a sequence-specific way (Schmitz-Linneweber & Small, 2008). A typical PPR protein targets the mitochondria, chloroplasts, and other organelle transcripts, thereby altering the expression of RNA sequence or translation and restoring fertility. The primary function of PPR is the restoration of pollen fertility (Akagi et al., 2004; Brown et al., 2003; Desloire et al., 2003; Hu et al., 2012; Kazama & Toriyama, 2003; Koizuka et al., 2003; Klein et al., 2005), suggesting that PPR has two prominent families, P and PLS (Fig. 6). P subfamily consisted only P motif, whereas PLS is termed as plant combinatorial modular proteins (Aubourg et al., 2000). P motif which is made up of 35 amino acids, L motif (35–36 amino acids) and S motif 31 amino acids) plays an important role in RNA editing (Rivals et al., 2006). Most P class motif consists of one or more tracts of PPR and an additional conserved domain named small MutS-related (SMR) domain (Fig. 6).

This domain works for RNA or DNA endonuclease activity in other proteins, but in the case of PPR protein, they have an unknown function (Fukui & Kuramitsu,

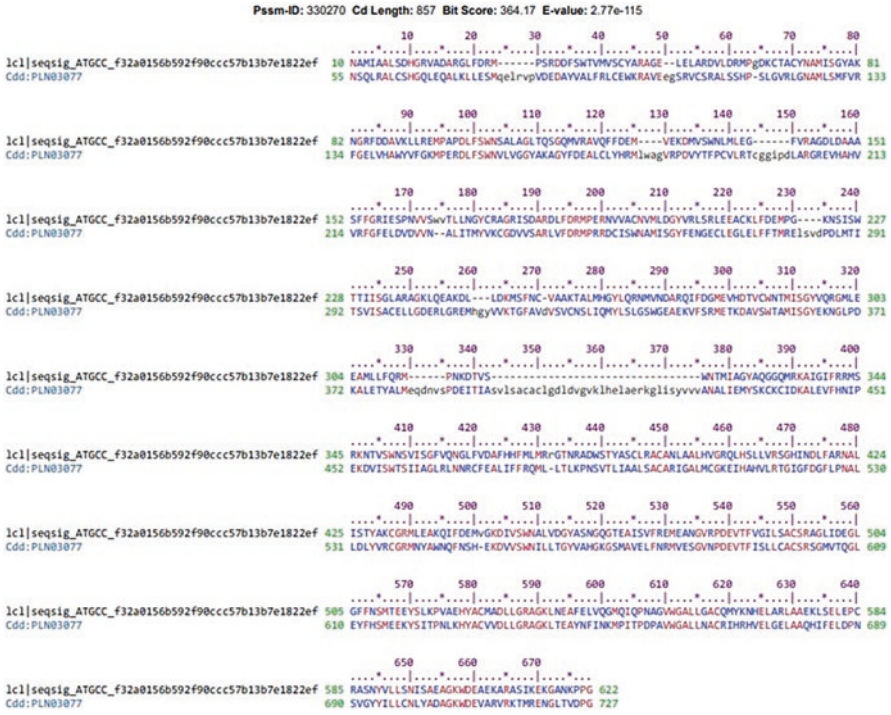



**Fig. 4** Predicted sequence alignment of gene Sb08g020970 encoding protein PPR, colour indicates amino acid sequence conservation across the PPR protein: highly conserved (blue), semi-conserved (black), and poorly conserved (red). An asterisk (*) indicates places where there is just one fully conserved residue

**PSSM ID: The location-specific scoring matrix (PSSM) was used for protein BLAST searches in which amino acid substitution scores were given independently for each position in a multiple sequence alignment. A positive score means that amino acid replacement is more common. Bit-score S' is a normalized score given in bits that helps you to estimate how much search space you should be looking for in order to find a decent or better score at random**
**E value: The Expectation value is dependent on the database size and the length of the query sequence. The lower the E value, the better the hits; dots are used for semi-conserved substitutions (similar residues); dash indicates gap. (Source: https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi)**

2011). PLS comprises three motifs, P, L, and S, at C terminal domains, denoted by E or DYW (Fig. 6). PLS domain explicitly involved in RNA editing in plant (Okuda et al., 2007, 2009). DYW is the conserved domain and has the active-site signature of known cytidine deaminases (Salone et al., 2007). PPR interacting proteins that could severely affect the PPR protein effects (Wang et al., 2021).

Pentatricopeptide repeat-containing protein has two subfamilies, P and PLS. P family comprises of one or more P motif, whereas PLS family consisted of P, L, and S combinatorial modular protein in plants. The P class family sometimes contains additional motif named PPR-small MutS-related (SMR) protein, whereas PLS


**Fig. 5** Expression of the gene Sb08g020970-targeted protein PPR (putative mitochondrial protein) during anthesis/floral initiation is high. Fragments Per Kb (Kilobase) of transcript per M (Million) mapped reads is abbreviated as FPKM. The amount of cDNA fragments generated by a transcript determines its relative expression in RNA-Seq. (Source: https://phytozome-next.jgi.doe.gov/)

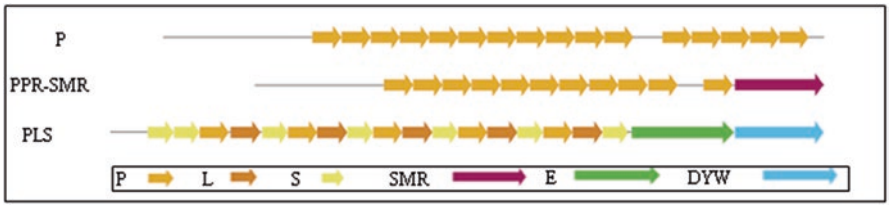



**Fig. 6** Structure of pentatricopeptide repeat-containing protein. (Source: Modified from Barkan & Small, 2014)

family consisted of E domain, and, in many cases, it may also contain DYW domain having conserved signature of nucleotide deaminases. Therefore, a need to identify the candidate genes is associated with fertility restoration under the influence of this conserved family. Up to now, six fertility restoration genes, namely, $Rf_1$, $Rf_2$, $Rf_3$, $Rf_5$, and $Rf_6$, have been identified in sorghum, most of which showed good restoration for A1 cytoplasm and encoded for PPR gene family members (Klein et al., 2005; Jordan et al., 2010; Praveen et al., 2015). Several identified putative genes encoding PPR protein in other crops and sorghum associated with fertility restoration are mentioned in Table 2.

## 10 Molecular Models for Mitochondrial-Nuclear Gene Interaction and Fertility Restoration

Fertility restoration occurs through the expression of dominant genes resulting in fertile plants. However, it is mandatory for successful exploitation of male sterility and therefore needs to understand the underneath of the molecular mechanism that restores fertility. Studies in CMS systems have shown the phenomena through four models, which are described below.

**Table 2** Several identified characterized genes encoding PPR proteins (*Rf-PPR*) associated with fertility restoration

| PPR gene | Plant | Target RNA | Possible function | Loss of function | References |
|---|---|---|---|---|---|
| CRR2 | Arabidopsis | Chloroplast | Controlling ndhD expression by transcript processing | Reduced chloroplast NA (P) DH dehydrogenase activity | Hashimoto et al. (2003) |
| GUN1 | Arabidopsis | Chloroplast | Chloroplast development | Does not repress photosynthesis-related nuclear genes | Koussevitzky et al. (2007) |
| PPR2 | Maize | Chloroplast | Required for chloroplast translation machinery | Chloroplast lack ribosomes, albino seedlings | Williams and Barkan (2003) |
| PPR4 | Maize | Chloroplast | Regulate splicing and chloroplast ribosome biogenesis | Chloroplast lack ribosomes, albino seedlings | Hattori et al. (2007) |
| *Rf*1a, PPR-791, PPR8-1 | Rice | Mitochondria | Reduces level of aberrant CMS-associated proteins, RNA editing | Male sterility | Wang et al. (2006) |
| *Rfo* | Radish | Mitochondria | Decrease accumulation of CMS-associated protein O*RF* 138 but do not decrease transcript level | Male sterility, accumulation of O*RF* 138 protein product | Brown et al. (2003) |
| *Rf*-1 | Sorghum | Mitochondria | RNA editing? | Male sterility | Klein et al. (2005) |
| *Rf*-2 | Sorghum | Mitochondria | Unknown | Male sterility | Jordan et al. (2010) |
| *Rf*-3 | Sorghum | Mitochondria | Unknown | Male sterility | Jordan et al. (2010) |
| *Rf*-4 | Sorghum | Mitochondria | Unknown | Male sterility | Jordan et al. (2011) |
| *Rf*-5 | Sorghum | Mitochondria | Unknown | Male sterility | Jordan et al. (2011) |
| *Rf-6* | Sorghum | Mitochondria | Unknown | Male sterility | Praveen et al. (2015) |

## 11 Cytotoxicity Model

In the cytotoxicity model, mitochondrial genomic rearrangement gives rise to 10–35 kDa CMS protein, a transmembrane protein with a hydrophobic region that directly kills the cells. This CMS protein hampers mitochondrial function leading to male abortion (Levings, 1993) (Fig. 7). Cytotoxic peptide and its C terminus encode by *orf*79, which is necessary for cytotoxicity (Wang et al., 2006).

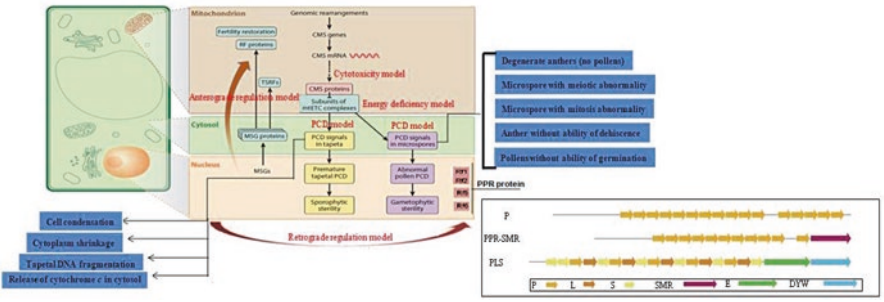



**Fig. 7** Molecular mechanism/model for fertility restoration. (Source: This figure was constructed by K. P. Ingle and modified from Chen & Liu, 2014). (**a**) Schematic representation of nuclear-mitochondrial interaction interpreted by different molecular models, (**b**) PCD signals in microspores, (**c**) PCD signals in tapetum, (**d**) PPR protein structure and *Rf*-PPR interaction restore fertility via retrograde and anterograde regulation model. The mitochondrial sorting genes (MSGs) codes for tissue-specific regulatory factors (TSRfs) and encode for *Rf* protein (fertility restoration protein). Mitochondrial genomic rearrangement developed cytotoxic CMS protein, which directly kills the cells (cytotoxicity model) or interacts with the mitochondrial electron transfer chain complexes (mtETC). This detrimental interaction fails to meet the cellular energy (ATP) for the development of male organ, leading to male abortion (energy deficiency model); this complex releases *cytochrome c* into cytosol, triggers the overproduction of ROS, and leads to apoptosis (programmed cell death) and nuclear genes (*Rf*1, *Rf*2, *Rf*5, and *Rf*6) encode pentatricopeptide repeat-containing protein (PPR), and this *Rf*-PPR through endonucleolytic cleavage, RNA editing and processing, and at post-transcriptional and translational level rectifies the detrimental effect of CMS protein and thus restore fertility

## 12 Energy Deficiency Model

CMS proteins lead the mitochondrial dysfunction, and therefore, the cells become unable to meet the substantial demand of energy required for the development of male reproductive organs. The mitochondrion is the cell's powerhouse and plays a vital role in producing ATP via mtETC of the respiratory complexes. Compared to other organs, the development of the sporophytic and gametophytic cells requires more cellular energy. In such a situation, cells may produce more energy (ATP) either through enhancing the metabolic activity of mitochondria or via increasing the number of mitochondria. The mitochondrion fails to meet the demand of cellular energy to develop male gametophyte, leading to male abortion or pollen sterility (Lee & Warmke, 1979; Chen & Liu, 2014) (Fig. 7).

## 13 Programmed Cell Death Model (PCD)

Programmed cell death is a cellular process that involves plasma membrane blistering (zygosity), cell density, cytoplasmic contraction, nuclear DNA fragmentation, pollen sterilization, male gamete abortion, and release of cytochrome

c into the cytosol. Plant PCD has been shown to play an important role in growth processes such as limb growth, ageing, xylem and aerosol formation, seed germination, root tip elongation, and disease resistance (Reape & McCabe, 2010). The reactive oxygen species (ROS) is triggered due to the release of cytochrome c from mitochondria to cytosol (Yao et al., 2002). This overproduction of ROS primarily affects the development of the normal tapetum; the tapetum gets degenerated and leads progression of PCD. The early or delayed tapetum development is resulting male sterility (Ji et al., 2013; Kawanabe et al., 2006) (Fig. 7). RBPs (RNA-binding proteins) regulate messenger RNA (mRNA) at each stage of its life cycle (Kramer et al., 2018; Muller-McNicoll et al., 2019).

## 14 Retrograde Regulation Model

In the retrograde regulation model, CMS genes (mitochondrial or chloroplast genes) regulate the expression of nuclear genes, whereas nuclear genes, so-called *Rf* genes, primarily hamper the function of chloroplast (plastid) genes in the case of anterograde regulation (Chen & Liu, 2014). The nuclear genes called *Rf* genes encode PPR protein, and *Rf*-PPR proteins targeted to mitochondria and interact with CMS genes where they suppress the expression of CMS gens or through the elimination of detrimental effects of CMS proteins via a post-transcriptional mechanism, such as RNA cleavage and editing, degradation of target mRNA, and restore fertility (Fig. 7). The nucleus-encoded *Rf*1, *Rf*2, *Rf*5, and *Rf*6 genes code for PPR protein, and, thus, these *Rf*-PPR were shown to target mitochondria and responsible for the considerable reduction in the accumulation of CMS-associated mtRNA/proteins. These PPR proteins specifically bind to mitochondrial transit peptide (mtRNA) and thus prevent from interacting with other proteins or RNA. These PPR proteins play a vital role in RNA stabilization and eventually define the position of processed RNA terminus by blocking exoribonucleases (Ingle et al., 2019).

## 15 Marker-Assisted Selections for Fertility Restoration Trait

In plants, cytoplasmic male sterility (CMS) is because of nuclear-mitochondrial interaction, whereas genic male sterility (GMS) is because of nuclear genes alone. Both the cytoplasmic male sterile and genetic male sterile systems significantly facilitate the hybrid seed production and allow the breeders to develop high-yielding hybrids with high heterotic values. Understanding the genetic relationships between parental lines is critical in heterosis breeding, and DNA markers such as simple sequence repeat (SSR), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), and random amplified polymorphic DNA (RAPD) are useful in identifying genomic regions with desired traits. Because of its co-dominant and multi-allelic nature, an SSR marker could be useful in the

future for molecular mapping of the gene(s)/QTL(s) responsible for fertility restoration using quantitative genomics approaches (Rakshit et al., 2012) and thus contributed significantly to the commercial success of CGMS-hybrids. There is a strong correlation between molecular markers and *rf* loci, which allows us to identify genetically different lines for heterotic crossings in sorghum hybrid breeding (Rakshit et al., 2012). A, B, and R-lines are involved in a three-line traditional breeding method for hybrid creation. A male sterile line (A-line). Cognate maintenance line (B-line) and restorer line (R-line). By crossing A-line with its maintainer B-line, the sterile line is maintained. The B-line is carried by selfing or crossing with another B-line, while the $F_1$ hybrid seed is produced by crossing an A-line with a specific R-line. As a result, maintaining the genetic purity of these lines is critical for commercial hybrid seed production success. Conventionally, the genetic purity is maintained by conducting a grow-out test (GOT) based on the evaluation of phenotypic characters or defined morphological descriptors but is subjective as environmental effects influence GOT. The DNA markers were then advocated for the genetic purity testing and the assessment of different hybrid seed lots (Fig. 8) and allow the identification of real hybrids and study of the variation of hybrids and lines/cultivars in sorghum (Madhusudhana et al., 2015).

Several molecular markers that are tightly linked to fertility restoration traits are enlisted in Table 3. Similarly, the linkage analysis indicated that $Rf_1$ locus is flanked by Xtxp250 and Xtxp18 and defined a position at a genetic distance of 10.8 cM and 12 cM, respectively, and associated with fertility restoration and could be incorporated in the hybrid breeding programme as candidate markers in the screening of putative restorer lines having fertility restoration genes (Klein et al., 2001). It was reported that pentatricopeptide repeat-containing protein (PPR) gene (Sobic.002G057000) tightly linked to Xtxp616 and Xtxp304 loci and found similar to $Rf_1$ loci in Rice (Praveen et al., 2015). It was suggested in sorghum that primers TS050 and TS304T have close association with fertility restorer genes. They stated that these primers helped to identify different lines for improving the breeding programme (Kushalappa et al., 2015). Recently, the SSR marker MS-SB02-37912, which encodes the PPR protein, has been widely used in marker-assisted breeding for $Rf_2$ fertility restoration in sorghum breeding (Madugula et al., 2018). The availability of these genic SSR markers (Table 3) allows for effective germplasm line (*milo*) CMS system screening as well as marker-assisted *Rf* gene pyramiding into diverse restorer parents to generate high-yielding hybrids with high fertility restoration.

## 16 Conclusions and Prospects

SSR markers may be widely used in the future for molecular mapping of the gene(s)/QTL(s) responsible for fertility recovery traits. SSR markers that are tightly bound to Rf loci can be used to routinely screen parental lines to select the best restorers that have the best fertility against sterile lines to show *post-rainy* sorghum

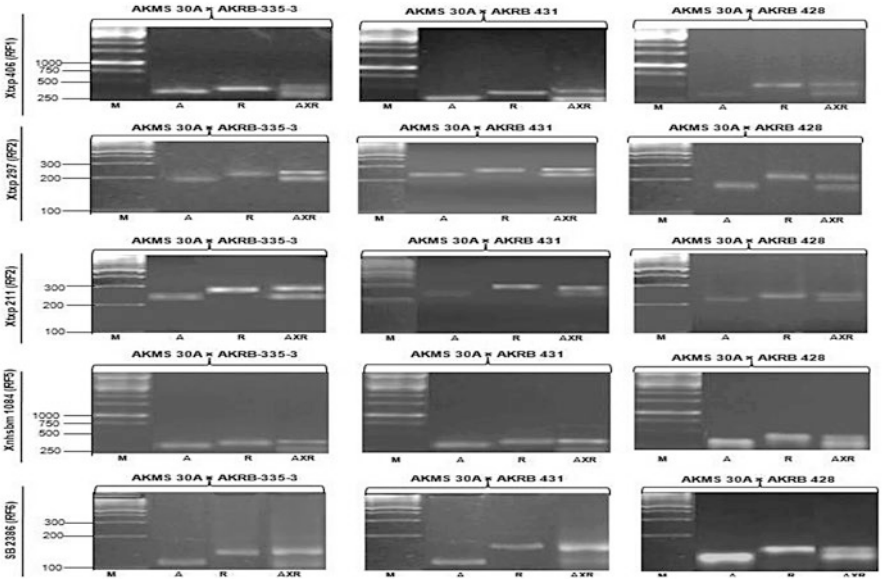



**Fig. 8** Molecular hybridity profiling utilizing Rf-linked SSR markers AxR (AKMS 30A × AKRB 335-3, AKMS 30A × AKRB 431, and AKMS 30A × AKRB 428), A (AKMS 30A), and R (AKMS 30A) (AKRB 335-3, AKRB 431, and AKRB 428). On the side of each gel picture, the name of each SSR marker is provided. M marker (100 bp) and 1 kb DNA ladder on extreme left. (Source: Ingle et al., 2019)

hybrids with high heterotic values. Knowing the position of the *Rf* genes and their defined position in the genome enables more effective strategies for the growth of male and female parents by allowing breeders to identify the best alleles based on marker selection. Marker-assisted selection can prove helpful to the breeders in screening different restoration factors in restorers and provide documentation for breeding/selection of high restoration ability restorers having the good combining ability with CMS lines to develop high-yielding *post-rainy* sorghum hybrids. The identified fertility restoration (*Rf*) genes will assist in the establishment of functional indicators linked with fertility restoration features, allowing for more effective hybrid breeding for commercial hybrid seed production. *Rf* will make it easier to design functional markers for restoring fertility in the future, allowing CMS to be used more effectively as a pollination control tool in hybrid breeding. The high-throughput technology will be useful in identifying haplotypes that target fertility/sterility features, which can then be used in marker-assisted breeding to generate high fertility and high-yielding hybrids during the *rabi* season.

**Declarations Conflict of Interest** The authors certify that they have no financial or personal conflicts of interest.

**Table 3** Details of *Rf* loci linked SSR markers associated with fertility restoration trait

| Marker | Gene | Forward sequence | Reverse sequence | Tm (F) | Tm (R) | Size (bp) | References |
|---|---|---|---|---|---|---|---|
| Xtxp18 | *Rf*1 | ACTGTCTAGAACAAGCTGCG | TTGCTCTAGCTAGGCATTTC | 57.3 | 55.3 | 231 | Kong et al. (2000) |
| Xtxp250 | *Rf*1 | GCACATCCTCTAAAACTACTTAGT | GAACAGGACGATGTGATAGAT | 57.6 | 55.9 | 283 | Bhattramakki et al. (2000) |
| Xtxp406 | *Rf*1 | GGCCTGAATCTCAGTGTTAAG | AGTTGCCTGCTTCGACACTT | 57.9 | 57.3 | 287 | Klein et al. (2005) and Klein et al. (2001) |
| Xtxp297 | *Rf*2 | GACCCATATGTGGTTTAGTCGCAAAG | GCACAATCTTCGCCTAAATCAACAAT | 63.2 | 60.1 | 220 | Taramino et al. (1997) |
| Xtxp211 | *Rf*2 | TCAACGGCCAATGATTTCTAAC | AGGTTGCGAATAAAAGGTAATGTG | 56.5 | 57.6 | 221 | Bhattramakki et al. (2000) |
| Xtxp50 | *Rf*2 | TGATGTTGTTACCCTTCTGG | AGCCTATGTATGTGTTCGTCC | 55.3 | 57.9 | 310 | Bhattramakki et al. (2000) |
| Xtxp616 | *Rf*2 | GCATTTCTTTCCTGCAATGAC | GCAGACAAGATCTCACCCAAG | 55.9 | 59.8 | 280 | Jordan et al. (2010) |
| Xtxp304 | *Rf*2 | ACATAAAAGCCCCTCTTC | CTTTCACACCCTTTATTCA | 51.4 | 50.2 | 206 | Jordan et al. (2010) |
| Xtxp31 | *Rf*3 | TGCGAGGCTGCCCTACTAG | TGGACGTACCTATTGGTGC | 61.0 | 56.7 | 222 | Kong et al. (2000) |
| Xtxp38 | *Rf*3 | ACAAACCGCGACGAAGTAAC | ACAAGGCAAAGCACAAAGC | 57.3 | 54.5 | 437 | Kong et al. (2000) |
| Xtxp34 | *Rf*3 | TGGTTCGTATCCTTCTCTACAG | CATATACCTCCTCGTCGCTC | 58.4 | 59.4 | 360 | Kong et al. (2000) |
| Xnhsbm1083 | *Rf*5 | TGACTGGTCAACAACGAGGA | CTCTCCCGTGCATGTACTCA | 57.3 | 59.4 | 219 | Jordan et al. (2011) |
| Xnhsbm1084 | *Rf*5 | CATTTCACATTCAAGGTCATGG | ACATTTATGGGTGCGTGCTT | 56.5 | 55.3 | 280 | Jordan et al. (2011) |
| Xnhsbm1085 | *Rf*5 | CGTGAATGAATGAACGAACG | GAGAGCAGAGGGGTAACTGC | 55.3 | 61.4 | 248 | Jordan et al. (2011) |
| SB2386 | *Rf*6 | GGCGGTAGGTGTAAAAAGGAAGGA | GCATGCCCTACGACTCTTGTGTCT | 62.7 | 64.4 | 169 | Praveen et al. (2015) |
| Xnhsbm1195 | *Rf*6 | CTAAAGGAACTCGGCGATTG | GTCGTGTCCTTCGGCATTAT | 57.3 | 57.3 | 255 | Praveen et al. (2015) |
| Xnhsbm1197 | *Rf*6 | CTGCAGAGGTCCTAGTGACAAA | GAACGACTTATAATTTGAGCCAGA | 60.3 | 57.6 | 262 | Praveen et al. (2015) |

*Rf* fertility restoration genes, *Tm* melting temperature, *bp* base pairs

## References


Akagi, H., Nakamura, A., Yokozeki-Misono, Y., Inagaki, A., Takahashi, H., Mori, K., & Fujimura, T. (2004). Positional cloning of the rice *Rf*$_1$ gene, a restorer of BT-type cytoplasmic male sterility that encodes a mitochondria-targeting PPR protein. *Theoretical and Applied Genetics, 108*(8), 1449–1457.

Aubourg, S., Boudet, N., Kreis, M., & Lecharny, A. (2000). In Arabidopsis thaliana, 1% of the genome codes for a novel protein family unique to plants. *Plant Molecular Biology, 42*(4), 603–613.

Ayyangar, G. N. R., & Ponnaiya, B. W. X. (1937). The occurrence and inheritance of ear heads with empty anther sacs in sorghum. *Current Science, 5*, 309.

Bailey-Serres, J., Hanson, D. K., Fox, T. D., & Leaver, C. J. (1986). Mitochondrial genome rearrangement leads to extension and relocation of the cytochrome c oxidase subunit I gene in sorghum. *Cell, 47*(4), 567–576.

Barkan, A., & Small, I. (2014). Pentatricopeptide repeat proteins in plants. *Annual Review of Plant Biology, 65*, 415–442.

Bhattramakki, D., Dong, J., Chhabra, A. K., & Hart, G. E. (2000). An integrated SSR and RFLP linkage map of *Sorghum bicolor* (L.) Moench. *Genome, 43*, 988–1002.

Brown, G. G., Formanová, N., Jin, H., Wargachuk, R., Dendy, C., Patil, P., Laforest, M., Zhang, J., Cheung, W. Y., & Landry, B. S. (2003). The radish Rfo restorer gene of Ogura cytoplasmic male sterility encodes protein with multiple pentatricopeptide repeats. *The Plant Journal, 35*(2), 262–272.

Budar, F., & Pelletier, G. (2001). Male sterility in plants: Occurrence, determinism, significance and use. *Comptes Rendus de l'Academie des Sciences. Serie III, Sciences de la Vie, 324*(6), 543–550.

Celarier, R. P. (1958). Cytotaxonomy of the Andropogoneae III. Subtribe Sorgheae, genus Sorghum. *Cytologia, 23*(4), 395–418.

Chase, C. D. (2007). Cytoplasmic male sterility: A window to the world of plant mitochondrial–nuclear interactions. *Trends in Genetics, 23*(2), 81–90.

Chen, L., & Liu, Y.-G. (2014). Male sterility and fertility restoration in crops. *Annual Review of Plant Biology, 65*, 579–606.

Dahan, J., & Mireau, H. (2013). The *Rf* and *Rf*-like PPR in higher plants, a fast-evolving subclass of PPR genes. *RNA Biology, 10*(9), 1469–1476.

Desloire, S., Gherbi, H., Laloui, W., Marhadour, S., Clouet, V., Cattolico, L., Falentin, C., Giancola, S., Renard, M., & Budar, F. (2003). Identification of the fertility restoration locus, *Rfo*, in radish, as a member of the pentatricopeptide-repeat protein family. *EMBO Reports, 4*(6), 588–594.

Dixon, L., Leaver, C., Brettell, R., & Gengenbach, B. (1982). Mitochondrial sensitivity to Drechslera maydis T-toxin and the synthesis of a variant mitochondrial polypeptide in plants derived from maize tissue cultures with Texas male-sterile cytoplasm. *Theoretical and Applied Genetics, 63*(1), 75–80.

Elkonin, L., & Sarsenova, S. K. (2020). Development of fertility-restoring lines for 9E type CMS of sorghum using environmentally induced fertility revertants. *Russian Agricultural Sciences, 46*(6), 560–565.

Erichsen, A., & Ross, J. (1963). Irregularities at microsporogenesis in colchicine-induced male sterile mutants in *Sorghum vulgare* Pers. 1. *Crop Science, 3*(6), 481–483.

Food, Nations AOotU. (2019). *The State of Food Security and Nutrition in the World: Safeguarding against economic slowdowns and downturns*. Food and Agriculture Organization of the United Nations.

Fujii, S., & Toriyama, K. (2008). Genome barriers between nuclei and mitochondria exemplified by cytoplasmic male sterility. *Plant and Cell Physiology, 49*(10), 1484–1494.

Fukui, K., & Kuramitsu, S. (2011). Structure and function of the small MutS-related domain. *Molecular Biology International, 691735*. https://doi.org/10.4061/2011/691735. Epub 2011 Jul 19. PMID: 22091410; PMCID: PMC3200294.

Ganapathy, K., Gomashe, S., Rakshit, S., Prabhakar, B., Ambekar, S., Ghorade, R., Biradar, B., & Saxena, U. J. (2012). Genetic diversity revealed utility of SSR markers in classifying parental lines and elite genotypes of sorghum ('*Sorghum bicolor*' L. Moench). *Australian Journal of Crop Science, 6*(11), 1486–1493.

Garcia, L. E., Edera, A., Marfil, C. F., & Sánchez Puerta, M. V. (2019). Male sterility and somatic Hybridization in plant breeding. *Preprint* 2019070330. https://doi.org/10.20944/preprints201907.0330.v1

Greenberg, J. T., & Yao, N. (2004). The role and regulation of programmed cell death in plant–pathogen interactions. *Cellular Microbiology, 6*(3), 201–211.

Guo, J. X., & Liu, Y. G. (2012). Molecular control of male reproductive development and pollen fertility in rice F. *Journal of Integrative Plant Biology, 54*(12), 967–978.

Hanson, M. R., & Bentolila, S. (2004). Interactions of mitochondrial and nuclear genes that affect male gametophyte development. *The Plant Cell, 16*(suppl_1), S154–S169.

Hao, H., Li, Z., Leng, C., Lu, C., Luo, H., Liu, Y., Wu, X., Liu, Z., Shang, L., & Jing, H.-C. (2021). Sorghum breeding in the genomic era: Opportunities and challenges. *Theoretical and Applied Genetics*, *134* (7), 1899–1924.

Hashimoto, M., Endo, T., Peltier, G., Tasaka, M., & Shikanai, T. (2003). A nucleus-encoded factor, CRR2, is essential for the expression of chloroplast ndhBin Arabidopsis. *Plant Journal, 36*, 541–549.

Hattori, M., Miyake, H., & Sugita, M. (2007). A pentatricopeptide repeat protein is required for RNA processing of clpPpre-mRNA in moss chloroplasts. *Journal of Biological Chemistry, 282*, 10773–10782.

https://apps.fas.usda.gov/psdonline/circulars/production.pdf

https://phytozome-next.jgi.doe.gov/

https://www.districtsofindia.com/maharashtra/alldistricts/agriculture/index.aspx

https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi

Hu, J., Wang, K., Huang, W., Liu, G., Gao, Y., Wang, J., Huang, Q., Ji, Y., Qin, X., & Wan, L. (2012). The rice pentatricopeptide repeat protein $RF_5$ restores fertility in Hong-Lian cytoplasmic male-sterile lines via a complex with the glycine-rich protein GRP162. *The Plant Cell, 24*(1), 109–122.

Ingle, K., Gahukar, S., Moharil, M., Jadhav, P., Ghorade, R., Narkhede, G., & Penna, S. (2019). Validation of cytoplasmic genetic male sterility in *rabi sorghum hybrids and their parents using diagnostic set of microsatellite markers. Research Journal of Biotechnology.14(7), 67–73.*

Ingle, K. P. (2020). *Molecular studies on fertility restoration mechanism in rabi sorghum (Sorghum bicolor (L.) Moench)*. Dr. PDKV, Thesis unpublished, pp. 1–135.

Ingle, K. P., Santosh, G., Mangesh, M., Pravin, J., Rameshwar, G., Gopal, N., & Suprasanna, P. (2019). Validation of cytoplasmic genetic male sterility in *rabi* sorghum hybrids and their parents using diagnostic set of microsatellite markers. *Research Journal of Biotechnology, 14*(7), 67–73.

Ji, C., Li, H., Chen, L., Xie, M., Wang, F., Chen, Y., & Liu, Y.-G. (2013). A novel rice bHLH transcription factor, DTD, acts coordinately with TDR in controlling tapetum function and pollen development. *Molecular Plant, 6*(5), 1715–1718.

Jilani, S. K., Atale, S. B., & Wadhokar, R. S. (2000). *Heterosis in cytoplasmically diversed male sterile lines in sorghum (Sorghum bicolor (L.) Moench)*. In Vasantrao Naik Memorial National Agriculture Seminar Jan. College of Agriculture, Nagpur, 8:8.

Jordan, D., Mace, E. S., Henzell, R., Klein, P., & Klein, R. (2010). Molecular mapping and candidate gene identification of the $Rf_2$ gene for pollen fertility restoration in sorghum (*Sorghum bicolor* (L.) Moench). *Theoretical and Applied Genetics, 120*(7), 1279–1287.

Jordan, D., Klein, R., Sakrewski, K., Henzell, R., Klein, P., & Mace, E. (2011). Mapping and characterization of $Rf_5$: A new gene conditioning pollen fertility restoration in $A_1$ and $A_2$

cytoplasm in sorghum (*Sorghum bicolor* (L.) Moench). *Theoretical and Applied Genetics, 123*(3), 383–396.

Kante, M., Rattunde, H. F. W., Nébié, B., Weltzien, E., Haussmann, B. I., & Leiser, W. L. (2018). QTL mapping and validation of fertility restoration in West African sorghum $A_1$ cytoplasm and identification of a potential causative mutation for $Rf_2$. *Theoretical and Applied Genetics, 131*(11), 2397–2412.

Kaul, M. (1988). *Male sterility in higher plants*. Springer.

Kawanabe, T., Ariizumi, T., Kawai-Yamada, M., Uchimiya, H., & Toriyama, K. (2006). Abolition of the tapetum suicide program ruins microsporogenesis. *Plant and Cell Physiology, 47*(6), 784–787.

Kazama, T., & Toriyama, K. (2003). A pentatricopeptide repeat-containing gene that promotes the processing of aberrant *atp*6 RNA of cytoplasmic male-sterile rice. *FEBS Letters, 544*(1-3), 99–102.

Kim, D. H., Kang, J. G., & Kim, B.-D. (2007). Isolation and characterization of the cytoplasmic male sterility-associated *orf*456 gene of chili pepper (*Capsicum annuum* L.). *Plant Molecular Biology, 63*(4), 519–532.

Klein, R., Klein, P., Chhabra, A., Dong, J., Pammi, S., Childs, K., Mullet, J., Rooney, W., & Schertz, K. (2001). Molecular mapping of the $rf_1$ gene for pollen fertility restoration in sorghum (*Sorghum bicolor* L.). *Theoretical and Applied Genetics, 102*(8), 1206–1212.

Klein, R., Klein, P., Mullet, J., Minx, P., Rooney, W., & Schertz, K. (2005). Fertility restorer locus $Rf_1$ of sorghum (*Sorghum bicolor* L.) encodes a pentatricopeptide repeat protein not present in the colinear region of rice chromosome 12. *Theoretical and Applied Genetics, 111*(6), 994–1012.

Kofuji, K., Aoki, A., Tsubaki, K., Konishi, M., Isobe, T., & Murata, Y. (2012). Antioxidant activity of β-glucan. *International Scholarly Research Notices, 2012,* 1–5.

Koizuka, N., Imai, R., Fujimoto, H., Hayakawa, T., Kimura, Y., Kohno-Murase, J., Sakai, T., Kawasaki, S., & Imamura, J. (2003). Genetic characterization of a pentatricopeptide repeat protein gene, orf687, that restores fertility in the cytoplasmic male-sterile Kosena radish. *The Plant Journal, 34*(4), 407–415.

Kong, L., Dong, J., & Hart, G. E. (2000). Characteristics, linkage-map positions, and allelic differentiation of Sorghum bicolor (L.) Moench DNA simple sequence repeats (SSRs). *Theoretical and Applied Genetics, 101*, 438–448.

Koussevitzky, S., Nott, A., Mockler, T. C., Hong, F., & Sachetto-Martins, G. (2007). Signals from chloroplasts converge to regulate nuclear gene expression. *Science, 316*, 715–719.

Kramer, M. C., Anderson, S. J., & Gregory, B. D. (2018). The nucleotides they are a-changin': Function of RNA binding proteins in post-transcriptional messenger RNA editing and modification in Arabidopsis. *Current Opinion in Plant Biology, 45*, 88–95.

Kushalappa, K., Primomo, V., Tulsieram, L., Li, Z., Porter, K., Kebede, Y., Monk, R., & DeLong, R. (2015). *Sorghum fertility restorer genotypes and methods of marker-assisted selection*. Google Patents.

Lee, S. L. J., & Warmke, H. (1979). Organelle size and number in fertile and T-cytoplasmic male-sterile corn. *American Journal of Botany, 66*(2), 141–148.

Levings, C. (1993). Thoughts on cytoplasmic male sterility in cms-T maize. *The Plant Cell, 5*(10), 1285.

Liu, X., Kim, C. N., Yang, J., Jemmerson, R., & Wang, X. (1996). Induction of apoptotic program in cell-free extracts: Requirement for dATP and cytochrome c. *Cell, 86*(1), 147–157.

Liu, F., Cui, X., Horner, H. T., Weiner, H., & Schnable, P. S. (2001). Mitochondrial aldehyde dehydrogenase activity is required for male fertility in maize. *The Plant Cell, 13*(5), 1063–1078.

Luo, D., Xu, H., Liu, Z., Guo, J., Li, H., Chen, L., Fang, C., Zhang, Q., Bai, M., & Yao, N. (2013). A detrimental mitochondrial-nuclear interaction causes cytoplasmic male sterility in rice. *Nature Genetics, 45*(5), 573–577.

Madhusudhana, R., Rajendrakumar, P., & Patil, J. (2015). *Sorghum molecular breeding* (1st ed. ISBN 978-81-322-2421-1, p. 226). Springer.

Madugula, P., Uttam, A. G., Tonapi, V. A., & Ragimasalawada, M. (2018). Fine mapping of $Rf_2$, a major locus controlling pollen fertility restoration in sorghum $A_1$ cytoplasm, encodes a PPR gene and its validation through expression analysis. *Plant Breeding, 137*(2), 148–161.

Maunder, A., & Pickett, R. (1959). The genetic inheritance of cytoplasmic-genetic male sterility in grain sorghum 1. *Agronomy Journal, 51*(1), 47–49.

Mayr, E. (1986). Joseph Gottlieb Kolreuter's contributions to biology. *Osiris, 2*, 135–176.

Melonek, J., Duarte, J., Martin, J., Beuf, L., Murigneux, A., Varenne, P., Comadran, J., Specel, S., Levadoux, S., & Bernath-Levin, K. (2021). The genetic basis of cytoplasmic male sterility and fertility restoration in wheat. *Nature Communications, 12*(1), 1–14.

Miller, D., & Pickett, R. (1964). Inheritance of partial male-fertility in Sorghum vulgare Pers 1. *Crop Science, 4*(1), 1–4.

Mishra, S., & Kumari, V. (2018). A review on male sterility-concepts and utilization in vegetable crops. *International Journal of Current Microbiology and Applied Sciences, 7*(2), 3016–3034.

Morris, G. P., Ramu, P., Deshpande, S. P., Hash, C. T., Shah, T., Upadhyaya, H. D., Riera-Lizarazu, O., Brown, P. J., Acharya, C. B., & Mitchell, S. E. (2013). Population genomic and genome-wide association studies of agroclimatic traits in sorghum. *Proceedings of the National Academy of Sciences, 110*(2), 453–458.

Mukri, G., Biradar, B., & Sajjanar, G. (2010). Effect of temperature on seed setting behaviour in *rabi* sorghum (*Sorghum bicolor* (L). Moench). *Electronic Journal of Plant Breeding, 1*(4), 776–782.

Muleta, K. T., Pressoir, G., & Morris, G. P. (2019). Optimizing genomic selection for a sorghum breeding program in Haiti: A simulation study. *G3: Genes, Genomes, Genetics, 9*(2), 391–401.

Muller-McNicoll, M., Rossbach, O., Hui, J., & Medenbach, J. (2019). Auto-regulatory feedback by RNA- binding proteins. *Journal of Molecular Cell Biology, 11*(10), 930–939.

Okuda, K., Myouga, F., Motohashi, R., Shinozaki, K., & Shikanai, T. (2007). Conserved domain structure of pentatricopeptide repeat proteins involved in chloroplast RNA editing. *Proceedings of the National Academy of Sciences, 104*(19), 8178–8183.

Okuda, K., Chateigner-Boutin, A.-L., Nakamura, T., Delannoy, E., Sugita, M., Myouga, F., Motohashi, R., Shinozaki, K., Small, I., & Shikanai, T. (2009). Pentatricopeptide repeat proteins with the DYW motif have distinct molecular functions in RNA editing and RNA cleavage in Arabidopsis chloroplasts. *The Plant Cell, 21*(1), 146–156.

Pattanashetti, S. (2014). Inheritance pattern of fertility restoration on maldandi cytoplasm in *rabi* sorghum (*Sorghum bicolor* (L.) Moench). *Karnataka Journal of Agricultural Sciences, 27*(4), 522–523.

Praveen, M., Madhusudhana, R., & Anuraguttam, G. (2015). Selective genotyping for determining the linkage between SSR markers and a fertility restoration locus in *Sorghum bicolor* (L.) Moench. *International Journal of Current Research, 7*, 20459–20461.

Pring, D., Conde, M., & Schertz, K. (1982). Organelle genome diversity in sorghum: Male-sterile cytoplasms 1. *Crop Science, 22*(2), 414–421.

Quinby, J. (1981). Interaction of genes and cytoplasms in male sterility in sorghum. In *Proceedings of the annual corn and sorghum industry research conference American Seed Trade Association, Corn and Sorghum Division, Corn and Sorghum Research Conference*.

Rakshit, S., Gomashe, S. S., Ganapathy, K., Elangovan, M., Ratnavathi, C., Seetharama, N., & Patil, J. (2012). Morphological and molecular diversity reveal wide variability among sorghum Maldandi landraces from India. *Journal of Plant Biochemistry and Biotechnology, 21*(2), 145–156.

Rao, N., Tripathi, D., & Rana, B. (1984). Genetic analysis of cytoplasmic systems in sorghum. *Indian Journal of Genetics and Plant Breeding, 44*(3), 480–496.

Reape, T. J., & McCabe, P. F. (2010). Apoptotic-like regulation of programmed cell death in plants. *Apoptosis, 15*(3), 249–256.

Reddy, B. V., & Stenhouse, J. (1994). Sorghum improvement for semi-arid tropics region: Past current and future research thrusts in Asia. *PKV Research Journal, 18*(2), 155–169.

Reddy, B. V., Ramesh, S., Reddy, P. S., & Ramaiah, B. (2007). Combining ability and heterosis as influenced by male-sterility inducing cytoplasms in sorghum [Sorghum bicolor (L.) Moench]. *Euphytica, 154*(1), 153–164.

Reddy, B., Ashok Kumar, A., & Kaul, S. (2008). Alternative cytoplasmic male sterility systems in sorghum and their utilization. In *Sorghum improvement in the new millennium: ICRISAT, Patancheru, Andhra Pradesh, India* (pp. 132–144). ISBN 978-92-9066-512-0.

Reddy, P. S., Rao, D. M., Reddy, B., Kumar, A. A., Thakur, R., & Rao, V. (2011). Evaluation of $A_1$, $A_2$, $A_3$, $A_4$(M), $A_4$(G) and $A_4$(VZM) cytoplasms in iso-nuclear backgrounds for grain mold resistance. *Crop Protection, 30*(6), 658–662.

Reddy, P. S., Patil, J., Nirmal, S., & Gadakh, S. (2012). Improving post-rainy season sorghum productivity in medium soils: Does ideotype breeding hold a clue? *Current Science*, *102*(6), 904–908.

Rivals, E., Bruyere, C., Toffano-Nioche, C., & Lecharny, A. (2006). Formation of the Arabidopsis pentatricopeptide repeat family. *Plant Physiology, 141*(3), 825–839.

Ross, W., & Hackerott, H. (1972). Registration of seven isocytoplasmic sorghum germplasm lines 1 (Reg. Nos. GP 9 to GP 15). *Crop Science, 12*(5), 720–772.

Salone, V., Rudinger, M., Polsakiewicz, M., Hoffmann, B., Groth-Malonek, M., Szurek, B., Small, I., Knoop, V., & Lurin, C. (2007). A hypothesis on the identification of the editing enzyme in plant organelles. *FEBS Letters, 581*(22), 4132–4138.

Schertz, K., & Ritchey, J. (1978). Cytoplasmic-genic male-sterility systems in sorghum 1. *Crop Science, 18*(5), 890–893.

Schmitz-Linneweber, C., & Small, I. (2008). Pentatricopeptide repeat proteins: A socket set for organelle gene expression. *Trends in Plant Science, 13*(12), 663–670.

Schnable, P. S., & Wise, R. P. (1998). The molecular basis of cytoplasmic male sterility and fertility restoration. *Trends in Plant Science, 3*(5), 175–180.

Singh, S. P., Singh, S. P., Pandey, T., Singh, R. R., & Sawant, S. V. (2015). A novel male sterility-fertility restoration system in plants for hybrid seed production. *Scientific Reports, 5*(1), 1–14.

Stephens, J. (1937). Male sterility in sorghum: Its possible utilization in production of hybrid seed. *Journal of the American Society of Agronomy, 29*(8), 690–696.

Stephens, J. C., & Holland, R. F. (1954). Cytoplasmic male sterility for hybrid sorghum seed production. *Agronomy Journal, 46*, 20–23.

Tang, H. V., Pring, D. R., Shaw, L. C., Salazar, R. A., Muza, F. R., Yan, B., & Schertz, K. F. (1996). Transcript processing internal to a mitochondrial open reading frame is correlated with fertility restoration in male-sterile sorghum. *The Plant Journal, 10*(1), 123–133.

Taramino, G., Tarchini, R., Ferrario, S., & Enrico Pè, M. (1997). Characterization and mapping of simple sequence repeats (SSRS) in Sorghum bicolor. *Theoretical and Applied Genetics, 95*(1), 66–72.

Touzet, P., & Budar, F. (2004). Unveiling the molecular arms race between two conflicting genomes in cytoplasmic male sterility? *Trends in Plant Science, 9*(12), 568–570.

Upadhyaya, H. D., Vetriventhan, M., Asiri, A. M., Azevedo, V. C. R., Sharma, H. C., Sharma, R., Sharma, S. P., & Wang, Y.-H. (2019). Multi-trait diverse germplasm sources from mini core collection for sorghum improvement. *Agriculture, 9*(6), 121.

Van, K., Onoda, S., Kim, M., Kim, K., & Lee, S.-H. (2008). Allelic variation of the Waxy gene in foxtail millet [*Setaria italica* (L.) P. Beauv.] by single nucleotide polymorphisms. *Molecular Genetics and Genomics, 279*(3), 255–266.

Wang, Z., Zou, Y., Li, X., Zhang, Q., Chen, L., Wu, H., Su, D., Chen, Y., Guo, J., & Luo, D. (2006). Cytoplasmic male sterility of rice with boro II cytoplasm is caused by a cytotoxic peptide and is restored by two related PPR motif genes via distinct modes of mRNA silencing. *The Plant Cell, 18*(3), 676–687.

Wang, X., An, Y., Xu, P., & Xiao, J. (2021). Functioning of PPR Proteins in organelle RNA metabolism and chloroplast biogenesis. *Frontiers in Plant Science, 12*(1), 1-8 article ID 627501. https://doi.org/10.3389/fpls.2021.627501

Webster, O., & Singh, S. (1964). Breeding behavior and histological structure of a nondehiscent anther character in Sorghum vulgare Pers. 1. *Crop Science, 4*(6), 656–658.

Williams, P., & Barkan, A. (2003). A chloroplast-localized PPR protein required for plastid ribosome accumulation. *Plant Journal, 36*, 675–686.

Worstell, J., Kidd, H., & Schertz, K. (1984). Relationships among male-sterility inducing cytoplasms of sorghum 1. *Crop Science, 24*(1), 186–189.

Xin, Z., Huang, J., Smith, A. R., Chen, J., Burke, J., Sattler, S. E., & Zhao, D. (2017). Morphological characterization of a new and easily recognizable nuclear male sterile mutant of sorghum (*Sorghum bicolor*). *PLoS One, 12*(1), e0165195.

Yao, N., Tada, Y., Sakamoto, M., Nakayashiki, H., Park, P., Tosa, Y., & Mayama, S. (2002). Mitochondrial oxidative burst involved in apoptotic response in oats. *The Plant Journal, 30*(5), 567–579.

# In Vitro Embryo Rescue Techniques and Applications in Hybrid Plant Development


**Samuel Amiteye**

**Abstract** Embryo rescue is a tissue culture tool that is greatly used to facilitate breeding in plants. Embryo rescue provides an effective means for recovering hybrid embryos resulting from wide hybridizations, which often fail to develop in vivo into plants. Embryo rescue is mainly used to develop interspecific or intergeneric distant hybrids. This review elucidates the salient aspects of wide hybridizations toward plant improvement. The main causes of hybrid embryo failures in wide crosses that have been discussed are precocious seed germination, nutritional starvation of developing embryo, cytological aberrations in embryogenesis, endosperm balance number discrepancy, polar-nuclei activation hypothesis deviations, and post-zygotic barrier limitations in endosperm and embryo development. Wide hybridizations related to frequent embryo failures arising from pre-fertilization or postfertilization barriers are usually overcome through embryo rescue. In this chapter, various limitations of pre-fertilization and postfertilization barriers that are encountered in wide hybridizations have been reviewed. In addition, some significant factors that influence the success of embryo culture, such as embryo genotypic background, embryo developmental stage, nutrient media composition and growth temperature, and light conditions, have been elaborated. Furthermore, considerations such as the determination of appropriate embryo stage for rescue, nature of the embryo excision techniques, and media manipulations for efficient embryo culture are noteworthy for success in embryo rescuing. Discussed also are the following very useful embryo rescue techniques: embryo-nurse endosperm transplant method, in vitro ovary and ovule culture techniques, as well as the ovary and ovule slice or perforation procedure. Some important applications of the embryo rescue technique that have been mentioned include overcoming seed dormancy and embryo abortion, plants development in seedless varieties, germplasm conservation, and homozygous monoploid production. The most recent uses of embryo rescue in successful wide hybridiza-

S. Amiteye (✉)
Biotechnology Centre, Biotechnology and Nuclear Agricultural research Institute (BNARI), Ghana Atomic Energy Commission (GAEC), Accra, Ghana

Department of Nuclear Agriculture and Radiation Processing, School of Nuclear and Allied Sciences, College of Basic and Applied Sciences, University of Ghana, Accra, Ghana



A. Raina et al. (eds.), *Advanced Crop Improvement, Volume 2*,
https://doi.org/10.1007/978-3-031-26669-0_15

tions and the achieved improved agronomic traits in various plant genera or species have been highlighted.

**Keywords** Wide hybridization · Interspecific and intergeneric · Pre- and postfertilization barriers · Embryo rescue · Hybrid plant development

## 1 Introduction

Crop improvement is successfully achieved by the introduction of wide genetic variability through various plant breeding techniques. In this regard, the enhancement in the quality and yield of virtually all major crops has been realized through interspecific and intergeneric hybridizations, followed by selection (Araujo et al., 2021; Hristova-Cherbadzhi, 2020; Mahoney & Brand, 2021). Interspecific and intergeneric hybridization enables the transfer of desirable genes or traits from wild relatives to the respective domesticated plants through wide crosses between or among species. Unfortunately, in wide sexual crosses, most often than not, the endosperm fails to properly develop and causes majority of the embryos to abort in vivo or switch into dormancy for prolonged periods (Sahijram et al., 2013). It has been established that low fertility or poor survival of endosperm or hybrid embryos is largely due to the effects of pre-fertilization (pre-zygotic) and postfertilization (post-zygotic) physiological barriers (Okamoto & Ureshino, 2015; Sun et al., 2018). Quite frequently, zygotic development barriers prevent distant hybridizations from going through the normal sexual reproductive physiological processes to yield viable plants. Notably, inherent physiological incompatibility in wide hybridizations causes abortion of embryos at virtually any developmental stages (Okamoto & Ureshino, 2015; Sun et al., 2018). The achievement of successful wide hybridizations in various species or genera is, therefore, arduous due to hindering physiological barriers.

The most useful approach and widely employed method for overcoming postfertilization barriers has been the application of the technology of embryo rescue or culture (Hristova-Cherbadzhi, 2020). This in vitro tissue culture approach involves the removal of immature, mature, or defective hybrid embryo from ovule and nurturing it in culture into a whole plant. This practice releases the hybrid embryo obtained through wide crosses from the hindering influence of hybridization barriers and enables the embryo that hitherto would have aborted or degenerated to instead develop into a viable plant. The essence of embryo rescue is, therefore, to enable the limitations imposed by post-zygotic or fertilization barriers to be circumvented. The embryo rescue approach facilitates the development of interspecific and intergeneric plant hybrids.

The rescue and culture of embryos are relatively quite an easy technique to carry out and requires basically a simple agar-based nutrient medium incorporated with

sugar and minerals. Furthermore, using embryo rescue, embryonic development in relation to the physical and nutritional requirements has been elucidated through the study of the physiological processes involved in the growth of rescued young hybrid embryos. To a great extent, successful embryo rescue depends largely upon explant maturation, medium nutrient content, as well as the genotype (Okamoto & Ureshino, 2015; Sun et al., 2018). The embryo rescue technology aids effective rescue of hybrid embryos from wide hybridizations (Hu & Wang, 1986). The technique also enables haploid plant production and reduction in the duration of the breeding cycle, in cases of prolonged dormancy. Embryos from ripened seeds could be isolated and cultured to eliminate the influence of seed germination inhibitors in instances where dormancy is the identified constraint to hybrid embryo development. In addition, embryo culture presents a reliable means for testing seed viability and provides material for micro-propagation.

Using embryo rescue, young immature, mature, or weak defective embryos have been effectively rescued in a number of crops, forests, ornamentals, and wild plant species (Araujo et al., 2021; Buteme et al., 2021; Kuang et al., 2021; Mahoney & Brand, 2021). For example, embryo rescue and interspecific pollination techniques were combined in an attempt to produce doubled haploids in castor bean (*Ricinus communis*) (Baguma et al., 2019). In this instance, embryo rescue was used to save the resulting embryos that would have otherwise aborted. The procedure of embryo rescue has been extensively applied to achieve varied objectives for successful plant improvement. This review presents an update on the current knowledge and achievements made in the improvement of useful traits in plants through wide hybridizations mediated by embryo rescue.

## 2 Interspecific and Intergeneric Hybridization: Associated Constraints

Plant genetic hybridization is a breeding procedure in which parents derived from different species belonging to a genus (interspecific) or parents from different genera of a family (intergeneric) are crossed to combine their genomes through pollination, either naturally or by induction (Table 1). Hybridization in plant improvement also involves crosses between diploid and tetraploid species (Mwangangi et al., 2019). Furthermore, through backcrossing, successful gene transfer has been performed between two species of different genetic constitution leading to the development of cytoplasmic sterile male plants (Premjet et al., 2019). Wide hybridization is, therefore, a powerful approach used to facilitate gene transfer by overcoming the species barrier (Huylenbroeck et al., 2020). A summary of the main steps involved in hybrid plant generation through wide hybridizations and the validation processes commonly carried out for verifying the hybrid state is presented in Fig. 1a, b. The main target of such wide hybridization procedures is usually to successfully obtain interspecific or intergeneric gene transfer, with the aim of creating more variation in plants for desirable traits. Unfortunately, such crosses that involve distant genetic

**Table 1** Embryo rescue mediated wide hybridizations in various plants

| Interspecific and intergeneric hybridization | Outcome of hybridization | References |
|---|---|---|
| *Aronia melanocarpa* × *Pyrus communis* | *Hybrid* showed early-stage hybrid necrosis | Mahoney and Brand (2021) |
| *Sorbaronia dippelii* × *Pyrus communis* | Hybrids exhibited a late-stage hybrid necrosis | Mahoney and Brand (2021) |
| *Goyazia petraea* × *Mandirola hirsuta* | Structural abnormalities (monads, dyads, triads, and micronuclei) were observed at the final of the hybrid's meiosis | Araujo et al. (2021) |
| *Helianthus annuus* × *Carduus acanthoides* | $F_1$ plants strongly resembled the cultivated sunflower but had an intermediate type of heritability | Hristova-Cherbadzhi (2020) |
| *Jatropha curcas* × *Ricinus communis* | $F_1$ hybrid plant seeds were shrunken with slimmed embryos. | Premjet et al. (2019) |
| *Saccharum* spp. × *Erianthus arundinaceus* | $F_1$ hybrids were relatively weak had lower dry matter yield and lower millable stalk weight | Pachakkil et al. (2019) |
| *Manihot esculenta* × *Ricinus communis* | Haploids were induction of in cassava | Baguma et al. (2019) |
| *Oryza sativa* × *Leersia perrieri* | Plant height of the $F_1$ hybrids was very short | Ballesfin et al. (2018) |
| *Brassica oleracea* × *Sinapis alba* | Majority of $F_3$ plants obtained sufficient resistance to *Alternaria brassicae* | Li et al. (2017) |
| *Brassica napus* × *brassica rapa* | Pollen grains of *B. rapa* germinated well on the stigmas in crosses with *B. napus* as a maternal parent; crossability was relatively higher in crosses with *B. rapa* ssp. chinensis as the pistillate parent. | Niemannet al. (2015) |
| [*Capsicum baccatum* (♀) × *C. chinense* (♂)] (♀) *x C. annuum* (♂); [*Capsicum annuum* (♀) × *C. baccatum* (♂)] (♀) *x C. annuum* (♂) | Virus-like-syndrome or dwarfism was observed in $F_1$ hybrids when both *C. chinense* and *C. frutescens* were used as female parents | Manzur et al. (2015) |

backgrounds more often than not fail and do not produce viable plants (Premjet et al., 2019). There are several physiological barriers that cause endosperm and embryo development to fail at the pre- and postfertilization phases and, thus, hinder the introgression of genes from wild relatives to crops (Mahoney & Brand, 2021).

Pre-fertilization barriers comprise impediments that prevent successful fertilization. For instance, ineffective fertilization is usually caused by the prevention of proper pollen germination, tube growth and guidance, due to low pollen quality or lack of stigma receptivity (Buteme et al., 2021). Other factors that have been implicated to cause pre-fertilization limitations in wide crosses include dissimilarities in flower morphology, failure of pollen capture, adhesion or hydration, impaired pollen-pistil or pollen-ovule interactions, and fertilization failure (Köhler et al., 2021). Table 2 contains some recently identified pre-fertilization barriers encountered in wide hybridizations in various plants. Postfertilization barriers on the other hand arise due to ploidy differences, chromosome elimination, and seed dormancy

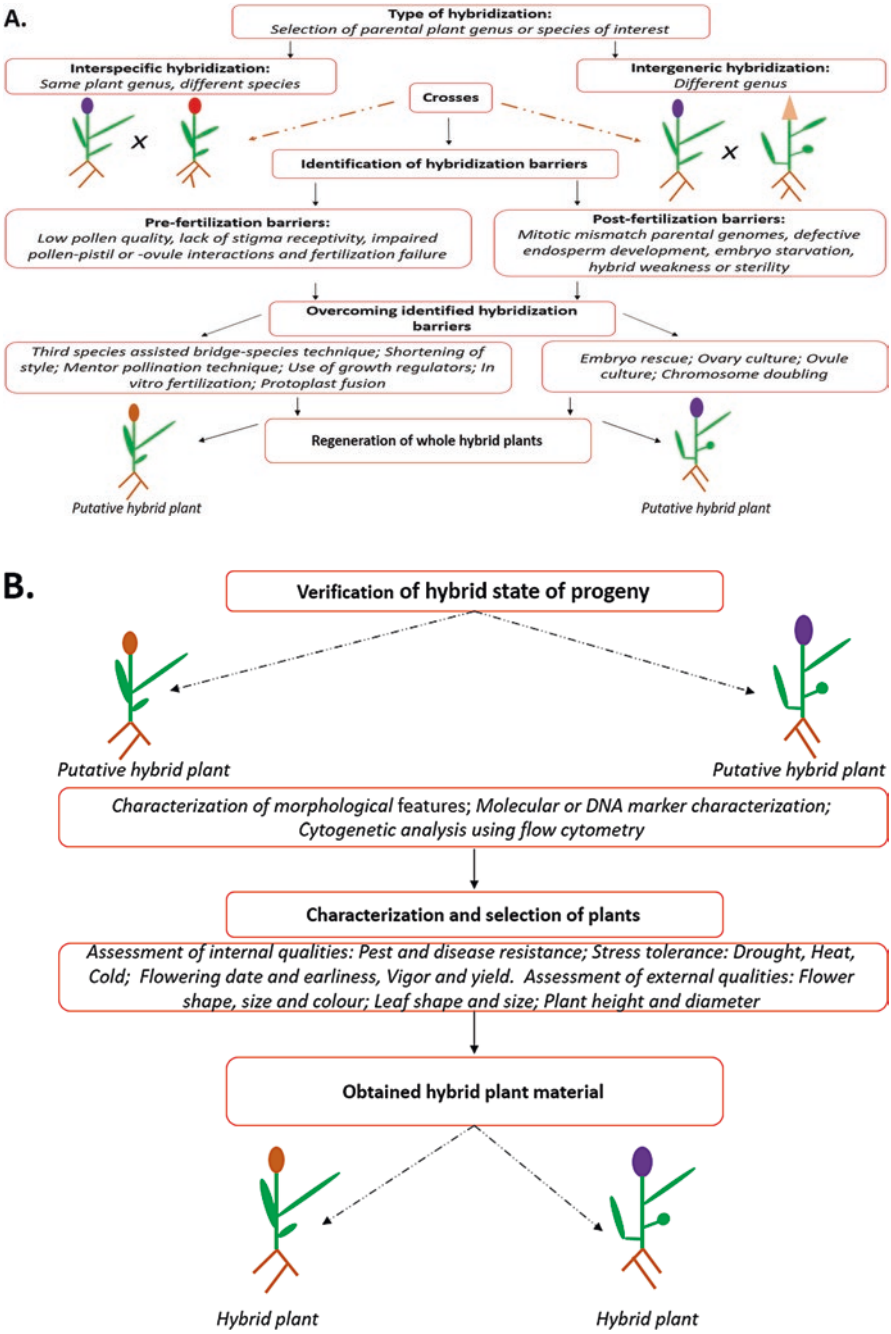



**Fig. 1** (**a**) Schematic presentation of the main steps involved in hybrid plant generation through wide hybridizations. (**b**) Validation processes for verifying the hybrid state of obtained putative hybrids from wide hybridizations

**Table 2** Identified pre-fertilization barriers in plant hybridizations

| Plant hybridization | Pre-fertilization barriers | References |
|---|---|---|
| *Kalanchoe garambiensis* × other long stem *Kalanchoe* species | Failure of pollen release from anther, pollen aggregation, and absence of pollen germination | Kuang et al. (2021) |
| Cross compatibility in *Solanum aethiopicum* | Pollen-pistil incompatibility occurred on the stigma, upper style, and lower style | Buteme et al. (2021) |
| *Brassica carinata* × *Brassica nigra*; *Brassica carinata* × *Brassica napus*; *Brassica napus* × *Brassica carinata*; *Brassica nigra* × *Brassica carinata; Brassica rapa* × *Brassica carinata* | Defect in pollen tubes, for example, coiling and bending of pollen tube, hairpin-shaped pollen tube, swelling of tube tip, tube bifurcation, more than one tube emerging from pollen, tubes growing in wrong direction | Pant et al. (2021) |
| *Nymphaea odorata* "Peter Slocum" × n*ymphaea gigantea* | Impaired pollen grains germination on the sigma | Sun et al. (2018) |
| Crosses among single-, double-, and multi-petal jasmine cultivars (*Jasminum sambac*) | Variation in pollen viability and stigma receptivity or incompatibilities; poor pollen quality caused defective pollen germination and growth and pollen tubes arrest in pistils | Deng et al. (2017) |
| Crosses with *Kalanchoë nyikae* as maternal plant × *K. blossfeldiana*, *K. blossfeldiana*, *K. marnieriana* | Inhibition of pollen germination on the stigma; abnormal growth of pollen tubes | Kuligowska et al. (2015) |
| Evergreen azalea species × *rhododendron uwaense* | Many pollen tubes stopped elongating in the style in crosses with azalea species as a seed parent; reverse crosses exhibited inhibition of pollen tube penetration into ovules | Okamoto and Ureshino (2015) |
| *Abelmoschus manihot* subsp. *Tetraphyllus* var. *pungens* × *Abelmoschus esculentus* | Delayed pollen tube; coupled with structural abnormalities such as twisting, swelling, high branching, bifurcated tip | Patil et al. (2013) |
| *Nelumbo nucifera cross* "Qinhuaihuadeng" × "Jinsenianhua" | Low pistil receptivity caused low seed set in "Qinhuaihuadeng" × "Jinsenianhua" | Wang et al. (2012) |
| *Juglans nigra* × *Juglans regia* | Disjunction in flowering time; differences in floral size; conspecific pollen advance | Pollegioni et al. (2013) |

(Premjet et al., 2019). Postfertilization barriers also occur as mitotic mismatch of parental genomes, defective endosperm growth and embryo malnutrition, as well as hybrid weakness or sterility, arising from ploidy or parental incompatibilities (Köhler et al., 2021).

Post-zygotic barriers inhibit zygote growth after fertilization has occurred, leading to abnormal seed formation. Seed malformation often emanates from physiologically defective hybrid endosperm, which causes acute failure in nutrient supply to the hybrid embryo (Okamoto & Ureshino, 2015; Sun et al., 2018). Moreover, endosperm or embryo failure could also be due to embryo-endosperm physiological mismatch induced by the exudation of lethal toxins from the endosperm to poison the embryo (Okamoto & Ureshino, 2015; Sun et al., 2018). In

principle, embryos of nonviable hybrids have an innate competence to transform into plantlets, but their development is hindered by failure to undergo normal differentiation.

## *2.1 Interspecific and Intergeneric Hybrid Failures: Main Causes*

### 2.1.1 Effects of Precocious Seed Germination

Precocious seed germination is characterized by the germination of seeds on the parent plant before the crop is harvested. In this abnormal form of seed germination, embryos initiate germination prior to full normal embryo development and maturity (Cota-Sanchez, 2018). Generally, precocious germination arises due to the elimination of the influence of endogenous germination inhibitors through the removal of the seed testa. Precocious germination is also induced by higher negative osmotic potential in vivo. Usually, precocious seed germination produces weak developing plantlets. This phenomenon has been observed to be generally widespread in plants that lack seed dormancy (Cota-Sanchez, 2018). This seed dormancy trait is generally characteristic of many wild plant species. Similarly, some crops inherently undergo short-duration seed dormancy. In some crops, the wild ancestors possessed seed dormancy traits; however, such plants lost the dormancy trait through the period of adaptation from the wild (Nakamura et al., 2017; Subburaj et al., 2016).

The absence of dormancy sometimes causes precocious seed germination particularly, in the form of preharvest sprouting of grains on the maternal plants when conditions of high humidity prevail (Cota-Sanchez, 2018; Subburaj et al., 2016). The culture or rescue of embryos could be employed to resolve and guide proper growth of embryos. The rescue and nurture of embryos can be achieved under different established culture conditions to stimulate embryological transformation into healthy plants. Ramming (1985) reported that the precocious germination of seeds can be circumvented through ovule rescue and culture in appropriate media. In *Prunus*, Ramming (1985) successfully used ovule culture to overcome the limitations posed by the integuments and, thus, prevent precocious germination.

### 2.1.2 Influence of Nutritional Starvation on Embryo Development

In making a transition to become a mature plant, the embryo first forms multiple tissues that subsequently result into a whole plant. Initially, an asymmetric zygotic differentiation produces the embryo and a suspensor, with both localized in the endosperm (Hristova-Cherbadzhi, 2020). The embryo is nourished with nutrients through the suspensor at the early developmental stages. It is known that the embryo and endosperm undergo parallel as well as interconnected developmental processes.

The hybrid embryo size, for example, is regulated by the endosperm enveloping it. Similarly, the embryo also influences the developmental faith of the endosperm. This interdependence is an indication that there might be an exchange or sharing of some metabolic factors between endosperm and embryo that tune their developmental processes. In instances of embryo development failure, therefore, nutritional starvation of the embryo has been implicated as the main cause of the failure, which then hinders the efficient development of both the endosperm and embryo in particularly interspecific hybrids (Dziasek et al., 2021). In *Capsella*, crosses of *Capsella rubella* and *C. grandiflora* produce unviable hybrids linked to chromatin abnormalities in the endosperm, which subsequently led to abortion (Dziasek et al., 2021). Hybrid seed collapse could also be due to nutrient starvation of the embryo arising from early retardation and disintegration of the endosperm as a result of overgrowth of the endothelium.

### 2.1.3 Implications of Cytological Aberrations in Embryogenesis

In eukaryotic organisms, it is essential that gene dosage exists in the right balance to enable normal physiological, biochemical, or gene function. Factors including ploidy level differences, chromosome structural changes that cause gene dosage disproportion in organisms, eventually lead to phenotypic abnormalities. Irregular cell divisions during mitosis are comparatively common and result in an increase or decrease in chromosomes during meiosis (Heslop-Harrison & Schwarzacher, 2011). Commonly, the phenomena of nondisjunction, aberrant spindles, lagging chromosomes, or chromosome breakages create mitotic abnormalities in in vitro culture that bring about the incidence of chromosomal variations and genome alterations. During in vitro culture, major genome changes produce karyotypic instability and cytogenetic irregularities such as ploidy level variation and chromosomal structure alterations due to chromosomal breakages that are often created in the course of in vitro multiplication (Neelakandan & Wang, 2012). Chromosome breakages cause rearrangements that induce direct mutational alterations in gene expression. In oat and maize, it was found that chromosome breakage occurred more frequently than ploidy variations (Kaeppler et al., 1998).

The introduction of polyploidy disparities during embryogenesis is more predominant compared to the occurrence of aneuploidy-chromosome karyotype deviation from the normal precise multiple of the haploid set (Kaeppler et al., 2000). Ploidy level variations in in vitro culture or embryogenesis are mostly associated with the phenomenon of endo-reduplication or nuclear fusion (Kaeppler et al., 2000). Lee et al. (2009) explained that endo-reduplication gives rise to polyploidy in instances where replication proceeds devoid of the successive cell division and eventually culminate in higher gene content in the nucleus. Aneuploidy manifests as monosomy or trisomy, which represents, respectively, the loss or gain of one or more specific chromosomes or, in some cases, large chromosomal fragments. Aneuploidy is generally observed during the initial stages of callus initiation and suspension cultures (Kaeppler et al., 1998, 2000). Aneuploidy is induced via nuclear

fragmentation occurring prior to mitosis or by irregular chromosome actions in the course of mitosis. In embryogenesis, long-term callus cultures are more frequently prone to cytogenetic abnormalities in especially regenerated plants of a variety of species (Rodriguez-López et al., 2010). Some cytological aberrations are usually checked at the initial phases of embryogenesis via the transfer of growth-regulating substances from the endosperm to the embryo.

### 2.1.4 Biological Significance of the Endosperm Balance Number

The entirety of the factors that drive successful seed development is still not fully understood and is very much speculative. Many different postulates or hypotheses have been put forward by some plant biologists to explain some likely factors responsible for the successful development of seeds in plants. One such hypothesis is the Endosperm Balance Number (Johnston et al., 1996). The Endosperm Balance Number hypothesis has been used to generally interpret and predict the success or failure of interspecific and interploidy hybridizations in plants (Carputo et al., 1999). Also, this hypothesis emphasizes the significance of a balanced parental genome mix that enables formation of physiologically normal endosperm. Some opinions state that a ploidy ratio of 2:3:2 of maternal tissue: endosperm: Embryo is an important consideration for viable seed formation. Others emphasize that the endosperm: embryo ratio is most important (Johnston et al., 1996; Katsiotis et al., 1995). Another school of thought considers the maternal tissue: endosperm ratio as the most crucial cytological balance ideal for successful seed development. Some plant biologists, however, suggest that endosperm function is autonomous and has no significant dependence on the maternal tissue and the embryo (Shukla, 2016).

Furthermore, other views stress the effectiveness of the 2 maternal: 1 paternal ratio for maternal and paternal genomes in the endosperm (Lester & Kang, 1998; Johnston, et al. 1996; Katsiotis et al., 1995). It has been emphasized that the Endosperm Balance 2: 1 ratio of maternal: paternal is an important prerequisite for successful interspecific crossability (Katsiotis et al., 1995). Despite the fact that such inconsistencies exist in opinion regarding the Endosperm Balance Number, the hypothesis has served as a useful measure toward the achievement of complex interspecific hybridizations involving various ploidy levels irrespective of the criterion relied on for selection (Hawkes & Jackson, 1992). Normal seeds have been obtained by some researchers without consideration for the Endosperm Balance Number (Katsiotis et al., 1995). The role of the histology of the endosperm in normal development must, therefore, be further investigated and better established.

### 2.1.5 Importance of the Polar-Nuclei Activation Hypothesis

The polar-nuclei activation hypothesis establishes the linkage of endosperm development to the activation of the two polar nuclei by fusion with a compatible male nucleus. The hypothesis is, therefore, dependent on the intensity of the

activating influence of the male nucleus and the reactive response of the female nucleus (Nishiyama et al., 1991; Nishiyama & Yabuno, 1978). The activation effect of the male nucleus is expressed as the *activating value*, and the reactive action of the female nucleus is expressed as the *response value*. Successful endosperm development or failure is closely linked to the difference in the Activation Index defined as the ratio of the activating value (AV) to the response value (RV). The physiological capability of the male nuclei to appropriately undergo mitotic divisions in the primary endosperm nucleus is dependent on a 2: 1 ratio between the AV and the RV (i.e., AV/2RV) (Nishiyama et al., 1991). The degree of endosperm development or failure is closely related to this ratio. Nishiyama and Yabuno (1978) recounted the achievement of crosses between various species of *Avena*, *Triticum*, and *Aegilops* based on the polar-nuclei activation hypothesis. Furthermore, in *Brassica* species, Nishiyama et al. (1991) estimated the relative activating value (AV) of diploid and tetraploid species to be, respectively, in the range 1.0–3.5 and 2.7–5.2. In addition, Nishiyama et al. (1991) found that hybridization based on the polar nuclei activation index of between 15% and 87% was effective, whereas crosses at activation index of less than 15% or more than 87% were incompatible.

### 2.1.6 Effects of Pre- or Post-Zygotic Barriers on Endosperm Development

Fertilization or zygotic barriers to endosperm development are broadly described as pre-zygotic (pre-fertilization) or post-zygotic (postfertilization). These barriers include factors such as pollen interactions with the pistil, hybrid zygote abnormality, and low hybrid fertility or sterility (Table 3). Lester and Kang (1998) described

**Table 3** Postfertilization barriers identified in plant hybridizations

| Plant hybridization | Postfertilization barriers | References |
|---|---|---|
| Wild diploid species × cultivated tetraploid cotton *Gossypium hirsutum* | Sterility of the triploid mainly due to ploidy | Konan et al. (2020) |
| *Nymphaea odorata* "Peter Slocum" × n*ymphaea colorata* | Defective embryos were observed in embryo development | Sun et al. (2018) |
| Crosses with *Kalanchoë nyikae* as maternal plant × *K. blossfeldiana*, *K. blossfeldiana*, *K. marnieriana* | Endosperm degeneration | Kuligowska et al. (2015) |
| Evergreen azalea species × *rhododendron uwaense* | Defective endosperm development; impaired photosynthetic activity of cotyledons due to chlorophyll deficiency; death of young seedlings | Okamoto and Ureshino (2015) |
| *Capsicum annuum* × *capsicum baccatum* | Embryo death; hybrid weakness (necrosis), lack of vigor; hybrid sterility | Martins et al. (2015) |
| *Nelumbo nucifera cross* "Jinsenianhua" × "Qinhuaihuadeng" | Low fecundity attributable to embryo abortion in "Jinsenianhua" × "Qinhuaihuadeng" | Wang et al. (2012) |
| Reciprocal crosses of *Nierembergia ericoides* × *Nierembergia linariifolia* | *N. ericoides* as pollen donor gave rise to normal gamete fusion but zygote abortions occurred | Soto et al. (2012) |

various postfertilization barriers to endosperm or seed development. Some of these barriers include embryo malformation or degeneration, endosperm and embryo death leading to abortion of ovules, early stage collapse of seeds during development, failure of hybridization between diploids, and their own autotetraploids as a result of embryo abortion. These barriers can be bypassed with the application of embryo rescue techniques (Okamoto & Ureshino, 2015; Sun et al., 2018). Appropriate pollinations or crosses within same species usually give rise to physiologically normal endosperm and embryo and, thus, result in viable seeds. Similarly, in some instances, no post-zygotic barriers arise in crosses between individuals of different species. In such cases also, the formation of normal zygotes or hybrids is achieved.

Nonetheless, instances arise where post hybrid zygote development and reproduction turn out unsuccessful. Gametes from different species can in some instances hybridize to produce hybrid zygotes, most of which turn out abnormal and never reach sexual maturity (Okamoto & Ureshino, 2015). Moreover, cases exist where reproduction is successful, and the resulting hybrids reach sexual maturity but are usually unable to reproduce. The reason is because an appreciable proportion of hybrid embryos turn out sterile and fail to produce viable gametes. In these examples, the reproduction of the different species fails because the offspring obtained are incapable of passing on their genes to the next generation. It is probable that the parents engaged in the hybridization have expended the energy involved in pollination and for producing hybrid offspring and yet end up with no transfer of their genetic materials to subsequent generations. The fitness of the hybrids is, therefore, considered to be zero due to the effect of zygotic barriers in reproduction.

## 3 Embryo Rescue Techniques and Essence of Application

An embryo in plants is a part of the seed that is formed after double fertilization and contains the preform of the plant organs. Embryo rescue and culture involve the nurturing of isolated defective hybrid embryos, under suitable sterile in vitro culture conditions in order to surmount the inhibiting effects of post-zygotic barriers on embryo initiation, growth, and development. Embryo rescue is, therefore, used to obtain fertile hybrid plants (Sahijram et al., 2013). Usually, the improper development of hybrid endosperm creates defective hybrid embryos from wide crosses. These failing embryos are saved from degenerating by isolating the embryo prior to its abortion and aseptically culturing the embryo under in vitro conditions. This strategy helps to circumvent the barriers that induce hybridization abortion (Konan et al., 2020; Okamoto & Ureshino, 2015; Sun et al., 2018).

In normal seed development, good physiological functioning of the embryo-nourishing tissue, the endosperm enables proper embryo development, which subsequently culminates into viable seeds. On the other hand, defective malfunctioning endosperm causes nutritional starvation which hinders proper embryo development and, consequently, gives rise to nonviable plants (Premjet

et al., 2019; Sun et al., 2018; Okamotoan Ureshino, 2015). Frequently, endosperm resulting from crosses between two distant species or diploids and tetraploids hybridizations more often than not fail to properly develop and thus cause embryo abortion, degeneration, or nonviable embryos (Konan et al., 2020; Sun et al., 2018). Improper development of hybrid embryo usually causes flowers to abort and drop due to distinct physiological differences in the parental embryos (Konan et al., 2020; Sun et al., 2018; Okamotoan Ureshino, 2015). Furthermore, the release of toxic inhibitory metabolic substances from the hybrid endosperm is also known to hinder embryo growth (Okamoto & Ureshino, 2015; Sun et al., 2018). Physiologically, however, the otherwise defective hybrid embryos indeed have inherent ability to start growth and subsequently develop into viable seeds if aided. The embryo rescue and culture technique are, therefore, carried out to save and aid the recovery of defective hybrid embryos which hitherto would have aborted.

The embryo rescue and culture technique generally involves a careful aseptic isolation of immature or mature embryos without injuring the embryos. The isolated embryos are then cultured in an appropriate nutrient medium supplemented with suitable carbon and inorganic nitrogen sources. The embryo is subsequently nurtured under suitable in vitro temperature, light, humidity, and osmotic conditions to induce continued embryogenic growth and seedling development into viable plants (Fathi & Jahani, 2012) and, thereby, circumventing the hindering influence of hybridization barriers. The embryo culture approach has proven very useful in the rescue of embryos that would normally abort or fail to follow the normal progressive sequence of ontogeny. The most valuable use of the embryo rescue technology has been the success in the development of interspecific and intergeneric hybrid plants (Pratap et al., 2021; Kaminski et al., 2020; Yin et al., 2020).

Through embryo rescue, it has been practicable to obtain viable seeds by circumventing most of the physiological barriers arising through wide crosses. Embryo rescue is used to successfully recover crosses between diploids and tetraploid species (Fathi & Jahani, 2012; Pachakkil et al., 2019). Furthermore, embryo culture has also proven to be an effective technique for resynthesizing some plant hybrids. The technology has, therefore, been the most effective method of valuable gene transfer from wild species. In addition, zygotic or seed embryos from embryo rescue have often been used as explants to initiate, for instance, callus cultures for crop improvement (Debnath & Arigundam, 2020; Koltunow et al., 1996). Over the years, embryo rescue and culture have become an attractive and valuable in vitro tool for plant tissue culture and breeding. Embryo culture has enabled the rescue of embryos from interspecific and intergeneric wide crosses as well as the achievement of seedless triploid embryos and haploids production. Embryo culture has also made it possible to circumvent the effect of germination inhibitors and thereby overcome seed dormancy and shortening the breeding cycle in some plant species (Fathi & Jahani, 2012). Embryo rescue has aided in bypassing germination inhibitions, for example, dormancy or sterility characteristics that are linked to the first filial or $F_1$ crossing generation in crop breeding (Pachakkil et al., 2019). The technique of embryo culture has also been effective in determining seed viability and development of plant variety from embryos that more often than not

fail to fully develop naturally or the embryo aborts under the inhibiting influence of physiological factors.

## *3.1 Historical Notes on the Embryo Rescue and Culture Technology*

Embryo rescue application began as early as the eighteenth century. This landmark in plant tissue culture is credited to Charles Bonnet who successfully regenerated whole plants from hybrid embryos obtained from crosses between *Phaseolus* and *Fagopyrum*. The procedure he carried out produced plants that were characteristically dwarf (Sharma et al., 1996). After that success by Charles Bonnet, many other plant scientists followed suit by culturing embryos in varied types of nutrient culture media. The period between 1890 and 1904 saw tremendous advancements in the efficiency of embryo rescue and culture techniques. Embryo culture became more systematic with the use of precise protocols of nutrient solutions supplemented with inorganic salts and carbon energy sources in the form of sugars and enhanced by aseptic manipulations (Amanate-Bordeos et al., 1992).

In 1904, Hanning became one of the first to succeed in obtaining viable plants in vitro from mature embryo culture (Hanning, 1904). He aseptically extracted mature embryos of two crucifers and cultured the embryos on a growth medium incorporated with minerals and sugar. Hanning (1904) described precocious germination tendencies in the embryos where he observed instantaneous initiation of growth in mature embryos, and thus, overcoming dormancy. The precocious germination characterized embryos developed into abnormal plantlets with small, weak architecture and nonviable (Mehetre & Aher, 2004). Subsequently, a successful culture of embryos of cherry by Tukey (1933) served as one of the very important advancements in the embryo rescue and culturing of fruit crops. Another authority in the applications of tissue culture whose work contributed greatly advanced the field was R. J. Gautheret. He was the first to obtain true plants from tissue cultures using cambial tissue of *Acer pseudoplatanus* (Gautheret, 1934, 1935). That feat at the time ushered in various different plant tissue culture procedures. Some of these culture methods include embryo culture, anther culture, pollen culture, shoot tip culture, root culture, and many others.

One of the early in vitro tissue culture methods that was effectively carried out to achieve efficient plant regeneration in crop improvement is embryo rescue. Yeung et al. (1981) indicated that embryo rescue is a useful tool for achieving a significant shortening in the breeding period by avoiding the delay that arises due to seed dormancy. In addition, embryo rescue is an appropriate approach to apply when the investigation of the endosperm and embryo germination involves a destructive analysis. Hu and Wang (1986) described several crosses that failed due to embryo abortion. Early embryo abortion is associated with failure of the endosperm to properly progress into physiological maturity or does not develop at all. The constraint of embryo failure or abortion is usually resolved by culturing the embryos in appropriate

nutrient medium to aid the embryo bypass post-zygotic barriers within the parental plant. Many successful examples of embryo rescue assisted interspecific and intergeneric generated hybrid plants have been described (Kaminski et al., 2020; Bridgen, 1994). Li et al. (2014) presented an exceedingly efficient procedure for hybrid embryo rescue from wide crosses that gave rise to important enhancement in effective breeding for disease-resistant trait in seedless grapes. During the past decades, embryo rescue or culture has enabled better appreciation of the physiology of embryonic development.

Furthermore, embryo rescue has been a very useful tool for bypassing seed dormancy to significantly reduce the duration of breeding, assessing seed viability, enhancing micro propagation efficiency, and rescuing undeveloped hybrid embryos from mismatched hybridizations (Caruso et al., 2020; Uma et al., 2011). To date, embryo rescue is extensively applied routinely in several fruit crops, for varied objectives, for example, breeding for seedless fruits, triploid plants, and interspecific hybridization. Some of the fruit crops in which embryo rescue has been successfully applied include banana (Uma et al., 2011), citrus (Caruso et al., 2020), persimmon (Hu et al., 2013), and watermelon (Taskın et al., 2013).

## *3.2 Types of Embryo Culture Technique*

Depending on the histological source from where the embryo explants were extracted for culture, two broad classes of embryo rescue techniques termed zygotic and somatic embryo culture are practiced. The embryo rescue technique is also classified as mature embryo culture and immature embryo culture based on the maturity level of the isolated explants. The mature embryo culture is used to circumvent seed dormancy in order to decrease duration of germination. Immature embryo culture on the other hand is carried out to achieve early embryo rescue. Practically, all the four broad types of embryo rescue or culture somewhat interconnect.

### 3.2.1 Mature Embryo Culture

Mature embryo rescue basically involves nurturing in vitro, the growth of mature embryos that are isolated out of ripe seeds. Mature embryo rescue or culture is carried out in instances where embryos fail to survive in vivo (Lentini et al., 2020). This in vitro procedure is also employed to remove restriction on seed germination, which causes seeds to stay dormant for protracted periods. In most plant species, seed dormancy is induced by chemical inhibitors in the embryonic tissue (Buteme et al., 2021). Another cause of seed dormancy is the effect of mechanical resistance created by structures casing the embryo. Some plant species also yield infertile seeds due to the defective physiological formation of embryos (Okamoto & Ureshino, 2015; Sun et al., 2018). Mature embryo rescue is done by surgically isolating the embryos that are autotrophic, out of the testa of the dormant seed that is

at the stage of maturity. In seeds with hard coats, the seeds are first disinfected by sterilization and then soaked in sterile distilled water for an appropriate duration, which could be a few hours or even days. The soaked seeds are cut open and appropriately dissected to remove the embryos. The isolated embryos are then cultured using basal inorganic medium with sucrose incorporated as energy source in order to circumvent seed dormancy and enable germination (Lentini et al., 2020). Embryo rescue protocols may culminate in viable plants. Mature embryo culture is employed in instances where seed dormancy of the hybrids is protracted. In cases of poor survival of embryos in vivo, the mature embryo culture technique is a useful tool for deriving viable seedlings.

### 3.2.2 Immature Embryo Culture

Embryo culture involving immature explants is also referred to as embryo rescue. The embryo rescue approach is also carried out as pre- or post-germinal immature embryo culture. Pre-germinal embryo culture is usually performed to regenerate plantlets. On the other hand, post-germinal embryo culture is done to boost embryo growth and development after germination. The embryo rescue approach fundamentally involves in vitro nurturing of immature embryos in order to rescue hybrid embryos prone to failure that result from wide hybridizations (Ren et al., 2019). Immature embryo rescue also aids to regenerate plantlets, in instances where parents are seedless, or in cases of heavy premature fruit fall during the initial stages of embryo development. In addition, the method of immature embryo rescue is applied to save seedless triploid embryos. The technique has also proven very valuable in the creation of haploids, bypassing of seed dormancy, and estimating of seed viability (Lentini et al., 2020). Immature embryo rescue is frequently used to overcome embryo abortion in order to create viable hybrid plants. Improper functioning of the endosperm usually culminates in malnourishment of the failing embryo. Ren et al. (2019) established an efficient immature embryo rescue protocol for the improvement of the plant *Ziziphus jujuba*.

## *3.3 Factors That Influence the Success of Embryo Culture*

### 3.3.1 Genotypic Background of Embryo

The genotype of the plant species involved in embryo rescue or culture is a key factor that determines the success or otherwise of the technique. In closely related or distant cultivars, embryos of some genotypes respond to in vitro culture conditions far more effectively and are easier to grow in culture than other genotypes whose culture in vitro is more daunting (Rangan, 1984). The ease of achievement of regeneration of whole physiologically normal plantlets through embryo culture, therefore, differs from genotype to genotype. Vidhanaarachchi et al. (2016) observed

significant differences in in vitro culture germination response in embryos of different selected genotypes of coconut. Lu and Bridgen (1996) also reported significant effect of parental genotypes on embryo germination, callus, and shoot induction in interspecific hybridization of *Alstroemeria*.

### 3.3.2 Developmental Stage of the Isolated Embryo

The growth of immature embryos in culture is quite daunting in spite of the remarkable progress made and the successes attained in the application of embryo culture (Pen et al., 2018). In instances where the abortion or degeneration of the embryo sets in very early, embryo rescue turns out to be extremely difficult and most often unsuccessful. Practically, an important strategy used to achieve effective rescue of immature embryos has been to carry out a technique referred to as the embryo-nurse endosperm transplant (Sect. 15.5.2). In this procedure, the immature embryo of a species is isolated and placed in the endosperm of a different seed of the same species. For instance, a 30–40% survival rate was achieved with the implantation method in the hybridization of *Hordeum x Secale*, compared to one percent survival rate using the traditional approach of embryo rescue (Kruse, 1974). Invariably, the more mature the isolated embryo, the easier it is to culture in vitro and more likely to successfully achieve regeneration of physiologically normal plants.

### 3.3.3 Composition of the Nutrient Media

One of the essential prerequisites toward any successful embryo rescue undertaking is the choice of suitable in vitro culture medium to nurture orderly differentiation and development of cultured embryos. The formulation of the culture medium used is determined primarily by the nature of embryo culture which could be either pre-germinal or post-germinal (Kumari et al., 2018). Pre-germinal immature embryo culture is purposely for obtaining plantlets regeneration. In such instances, the embryos require a complex nutrient medium. On the other hand, the aim of post-germinal immature embryo culture is usually carried out to hasten the development of the embryo after germination. Comparatively, post-germinal embryo culture is attained with less complex medium which could be as simple as just sucrose or glucose solution. Nonetheless, for embryo rescue generally, the culture media composition of mineral salts, organic nutrients, or growth regulators may be an important consideration and useful for the efficient culture of embryos (Lentini et al., 2020; Li et al., 2014). The formulation of the culture medium must take cognizance of the developmental phase of the isolated embryo to be cultured. Embryos at a heterotrophic phase of development depend on the endosperm and the surrounding maternal tissues for nutrients, whereas autotrophic stage embryos metabolically produce growth substances required for development (Lentini et al., 2020; Li et al., 2014).

### 3.3.4 Growth Temperature and Light Conditions

Light and temperature are environmental factors that have been identified to be very important for the efficient culture of embryos (Narayanaswamy and Norstog, 1964). Embryo rescue protocols usually have integrated in them a step of incubating cultures the in dark for the first 1 to 2 weeks until embryos or calluses appear. The embryos are subsequently transferred to appropriate light and temperature conditions to enable the embryo to begin synthesizing chlorophyll (Kumari et al., 2018). Compared to intact seeds, it has been realized that isolated embryos frequently germinate, grow, and develop better in a wider temperature range. Narayanaswamy and Norstog (1964) explained that the plant species from which the embryo explant was taken influences the optimum temperature depending on the plant species from which the embryo was obtained. Nonetheless, normally a temperature range as high as 25–30 °C is used (Brits et al., 2015). Cultured embryos usually germinate in a wider temperature range than whole seeds. Meanwhile, embryos from plant species such as *Allium* require a lower temperature of around 17 °C, whereas in some other plant species, cold treatment of 4 °C is usually essential for the establishment and growth of embryos in in vitro culture. For example, the growth and development of embryos of sweet cherry were significantly enhanced when immature and mature embryos were subjected to 40 and 60 days of cold treatment, respectively (Hajmansoor et al., 2009).

## *3.4 Salient Considerations Involved in Embryo Rescue and Culture*

### 3.4.1 Determination of Appropriate Embryo Stage for Rescue

Prior to carrying out embryo rescue, it is very useful to determine the physiological maturity stage of the embryos to be cultured. It is also helpful to know when abortion sets in for the particular plant species so that the dissection and isolation of the embryo can be carried out timely before the embryo degenerates or aborts. One of the recommended approaches for determining the stages of development of embryos is to make histological sections. Accurate establishment of the embryo development stage guides the appropriate medium formulation that is to be used (Haslam & Yeung, 2011). It is recommended that preliminary development of an efficient protocol for the selection of the right embryo development stage is very useful even though it could take appreciable time and some financial investment to achieve. However, such a tool, once in place, guides subsequent embryo isolation and collection at exactly the established most competent stage. This greatly enhances the success of the embryo rescue.

Another essential consideration in embryo culture is the condition under which the mother plant was growing prior to the isolation of the explant for culture initiation. The endosperm and the cotyledons have been found to develop more

efficiently in cases where the mother plant was maintained under strictly controlled environments. Consequently, the embryo growth and development is well promoted. It has been established that the younger the embryo chosen for rescue, the more complex the nutritional formulation that is used to sustain its culture and growth. Maturing embryos are subsequently transferred to less complex inorganic salt culture media (Yeung et al., 2001). Yan et al. (2014), for instance, intimated that in cassava, the rescue of embryos should be initiated at immature stages before 38 DAP, the time when the highest proportion of embryos is at the cotyledonary stage with an already fully developed endosperm. At the cotyledonary stage, the embryos can be seen and are easier to dissect and isolate from pollinated ovules without any damage or injury to the embryos. After 38 DAP, the seeds become too hard, and high rate of injury is usually caused to the embryo during the excision and isolation procedure (Lentini et al., 2020; Yan et al., 2014).

### 3.4.2 Embryo Excision Techniques

More often than not embryos are found within the ovule which presents a sterile environment. For this reason, carrying out surface sterilization to make the isolated embryos aseptic is of no practical value. Instead, in most protocols, the more common practice is that the florets are removed and the ovules are isolated from the ovaries. This is followed by surface sterilization of whole ovules or ovaries. The embryo is normally effectively protected from the usually severe surface sterilization procedures, by the bordering tissues. In plant species such as corn (*Zea mays* L.), to assess the embryo, it is required that hard seed coats are broken. Furthermore, in some cases, endophytic pathogens may be present in the seed coats. For plants with such type of seeds, direct disinfection of embryos by sterilization is necessary in order to establish an aseptic culture. The surface sterilization procedure of the entire ovules or ovaries is followed by aseptic excision and isolation of embryos from the ovules or ovaries and surrounding tissues. The dissection and excision of large embryos pose little difficulty.

However, to isolate small embryos without injury, appropriate tools are used to carry out micro-excision and isolation procedure aided by a dissecting microscope. It is precautionary worthy to note that embryos are quite fragile when the seed coat is broken. In addition, another essential precaution is that isolated embryos must not be subjected to desiccation during in vitro culture (Rangan, 1984). The technique or protocol for efficient isolation of immature embryos is usually tailored specific for the particular plant species. However, quite often, incision of the immature ovule is targeted at the micropylar end, and with exertion of appropriate pressure at the other end, the embryo is released through the incision. In applying the pressure, caution must be exercised not to injure the delicate embryonic tissue. Hu and Wang (1986) indicated that in the isolation of heart-stage or immature embryos, it is essential that the suspensors are not damaged or impaired to boost the growth and development of the embryos in culture. For the rescue of mature embryo, usually isolated seeds that are physiologically mature are decontaminated or disinfected by surface sterilization prior to embryo isolation.

### 3.4.3 Media Manipulations for Efficient Embryo Culture

Raghavan (1966) described the two main stages of embryo development as heterotrophic and autotrophic. In the heterotrophic phase, the immature embryo relies primarily on the endosperm and the neighboring maternal tissues. During this phase, the young embryo requires a medium with more complicated media composition and osmotic potential that is higher than required for mature embryos. As the initially immature embryo further grows in culture, its efficient development is promoted by culturing it on complex media augmented with appropriate combinations of amino acids, growth hormones or regulators, and vitamins. In addition, the incorporation of plant extracts, for example, coconut milk, also helps embryo development. At the autotrophic phase, the embryo is physiologically more mature. During this stage, the embryo is metabolically able to synthesize most essential biomolecules needed to support its growth and development using available salts and sugar. The embryos at this stage are now capable of germination and development on basic inorganic medium augmented with as sucrose a carbon source.

The choice of the appropriate culture medium and growth conditions is some of the most essential considerations for a successful embryo rescue procedure. The application of suitable culture conditions enables effective physiological growth of the embryo, its maturation, and regeneration into whole plants. A key factor in a successful embryo rescue is the optimal composition of the culture medium used. The optimal medium nutrient composition depends on the embryo stage, and the medium constituents vary during the development process of the embryo. The two extensively used basal media are the Murashige and Skoog (1962) and Gamborg's B5 medium (Gamborg et al., 1968), usually applied with some appropriate modifications to enhance efficient embryo culture growth. The required complexity or stringency of the media composition or growth conditions used is influenced by the level of maturity of the target embryo for rescue. In this regard, it has been successful to grow mature embryos using basal salt media with sucrose as an organic carbon source of energy. However, for immature embryos, in addition to the basal salt media and sucrose, different vitamins, amino acids, and growth regulators are incorporated in the culture medium. Varied compositions of mineral salts are incorporated in order to manipulate the growth of embryo cultures. In many protocols of embryo rescue and culture, amino acids and amino acid complexes such as casein hydrolysate as well as the vitamins, biotin, thiamine, pantothenic acid, and nicotinic have been widely used as additives in culture media to promote the development of the embryo. The various amino acids are also important components of culture media. Generally, the use of malic acid and trace amounts of organic nitrogen, for example, asparagine, glutamine, or casein hydrolysate, have often been observed to be useful. Pawar et al. (2015) reported that proline and glutamine improve in vitro callus induction and subsequent shooting in rice.

Sucrose is also a very important component in embryo culture media and serves two main purposes. The primary energy source in culture media is sucrose. Another important role of sucrose is that it stabilizes and maintains appropriate osmotic potential of the culture medium. Usually, mature embryos and immature embryos

are cultured on media with 2–3% sucrose concentration. Immature embryos are commonly cultured on media with high sucrose concentration between 8% and 12%, which in principle mimics the high osmotic potential of the embryo sac's intracellular environment and conditions. It has been found that generally, the more immature the isolated embryo, the higher the osmolarity condition required in the culture medium to promote growth and development of the embryo. Reinert et al. (1977) observed that high osmolarity inhibits precocious germination and prevents dividing cells from switching into a state of elongation. Moreover, the high osmotic environment together with the addition of hormones such as auxin and cytokinin in moderate amounts promotes the development of heart-stage embryos (Din et al., 2016). Mature embryos have been found to grow well on semisolid medium supplemented with only Knop's mineral salts and 2–5% sucrose. Generally, the most effective sources of inorganic N in media for embryo cultures have been ammonium nitrate and potassium nitrate. Essentially, ammonium boosts appropriate growth and differentiation of immature embryos in culture (Umbeck & Norstog, 1979). Ammonium is often incorporated together with mainly malate or citrate anions as a source of organic acid. The use of natural plant extracts as media constituents has also been found useful in attaining greater recovery of growth and development of embryos. The commonly used natural extract from plants is coconut milk. Similarly, tomato juice and extracts of banana are also useful in culture media.

The incorporation of plant growth regulators in embryo culture media generally plays significant role in boosting embryo growth and development (Ming et al., 2019). However, it must be noted that high concentrations of exogenous auxins appear to induce inhibitory effect on plant embryo growth in vitro (Din et al., 2016; Manzur et al., 2014). Therefore, the induction of somatic embryo is better stimulated rather by low concentrations of exogenous auxin in the culture media. Exogenous use of growth regulators such as kinetin (Kin), benzyl-amino-purine (BAP), and naphthalene acetic acid (NAA) or thidiazuron (TDZ) improved regeneration frequency (Din et al., 2016). Similarly, high levels of cytokinins, for example, zeatin, have been found to only slightly promote young embryo growth, when used as the sole media hormone additive (Manzur et al., 2014). Auxins and cytokinins are, therefore, not generally used for embryo culture except in cases where the aim is to induce callus. However, some cytokinins and auxins combinations show better performance in boosting the growth and differentiation of embryos (Din et al., 2016). It has been found that hormones induce plant architectural abnormalities when included in an embryo culture media. For example, gibberellins in some cases stimulate precocious germination (White et al., 2000). Therefore, the use of hormones in media for embryo cultures is generally avoided or used with precaution.

Commonly, in the preparation of embryo rescue media, agar at 0.5–1.5% is used as the main solidifying agent (Hu & Wang, 1986). Higher concentrations of agar hold less water and likely presence of higher contaminating salts. The use of higher concentrations of agar is, therefore, not recommended because that could inhibit growth. Alternatively, Pinto et al. (1994) successfully used a vermiculite support system for small embryos obtained at fruit maturity. As earlier explained in Sect. 3.4, light and temperature conditions are very essential considerations in the

technique of embryo culture. Light regulates cell division and rate of ethylene emission, a factor that affects caulogenesis—shoot initiation and rhizogenesis—root initiation. Timing and duration of exposure of explants to light play a vital role in the morphogenesis of embryos. In most species, the embryo stops growing between one and two weeks after inoculation. The embryos are then moved on to another medium with regular sucrose concentration as well as low levels of auxin and cytokinin in order to cause the embryo to regain growth and direct shoot initiation. In cases where the embryo fails to grow shoots directly, callus induction is performed followed by shoot induction and then eventually the development of plantlets in vitro. The plantlets are weaned on soil treated to become sterile and nurtured to develop under greenhouse conditions.

## *3.5 Major Embryo Rescue and Culture Procedures*

### 3.5.1 Rescue and Culture of Embryos

Usually, hybrid embryos obtained from interspecific and intergeneric crosses turn out to be defective and more often abort at a point during development or yield nonviable seeds (Araujo et al., 2021; Hristova-Cherbadzhi, 2020). Embryo rescue is used to resolve this constraint of failure in embryo development or abortion following hybridization. Embryo culture serves as a tool of great value to plant breeding, particularly in interspecific hybridization (Hristova-Cherbadzhi, 2020). The technique of embryo culture serves as a very effective and useful means of creating normal hybrid plants and producing viable seeds. Usually, seeds of fruits obtained from controlled pollination of plants are collected at an appropriate stage in order to avoid the period embryo abortion is known to set in. The embryo rescue procedure is achieved by the excision of the embryos from the harvested seeds and placing the embryos directly onto an appropriate culture medium (Buteme et al., 2021; Kuang et al., 2021).

Embryo culture is undertaken in various forms to achieve different objectives. The seeds of some plant species, for example, orchids, lack nutritious tissues and are without plumule and radicle. In such irregular type of seeds, the strategy has been to culture whole seeds with intact undifferentiated embryos. In a similar technique, intact mature embryo culture and manipulations are carried out to enhance embryonic growth and to track the metabolic and biochemical interactions involved in overcoming seed dormancy and inducing germination. Moreover, instead of directly culturing the intact mature embryo, the embryo could be surgically excised into various segments and cultured in vitro on suitable media to enable the monitoring of the physiological processes that come to play as well as the growth of the different parts of the mature embryo (Buteme et al., 2021; Konan et al., 2020). In addition, the culture of immature embryos is also a common procedure that involves mainly in vitro culture of globular or heart-shaped embryo development phases in appropriate nutrient medium to enable the differentiation and progressive

development of embryos (Buteme et al., 2021. Furthermore, in species such as lemons or oranges, embryos that result from nuclear tissue are more often defective and abortive. This challenge is overcome by culturing the embryos under artificial conditions and manipulated to attain clonal propagation. In such approach, adventitious embryos are in vitro cultured from polyembryonic seeds.

### 3.5.2 Embryo-Nurse Endosperm Transplant Method

It is often practically daunting to isolate defective immature or very tiny hybrid embryos that abort at the initial stages of growth. Besides, the initiation of such very small hybrid embryos for growth in vitro is quite challenging. Therefore, usually in handling very tiny or immature hybrid embryos, a specialized technique referred to as the embryo-nurse endosperm transplant method has often been used to improve success of the embryo culture (Shukla, 2016). In other instances, the hybrid embryo may be physiologically normal; however, the ovule may have a defective or immature endosperm which fails to serve as an important source of nutrition to the hybrid embryo. The embryo-nurse endosperm transplant technique involves a combination of a hybrid embryo resulting from a hybridization that is not compatible and a normal endosperm developed from a cross of related plant species that is compatible (Widiez et al., 2017). In this process, usually the small or very immature hybrid embryo is surgically inserted into an endosperm that has been extracted from a normal ovule of one of the parents crossed to produce the hybrid embryo, or alternatively, the normal endosperm is obtained from a different species (Shukla, 2016). The endosperm transplant technique is used mainly for rescuing immature embryos.

Basically, a hybrid embryo is excised from an ovule that is enclosed by an endosperm whose development has failed and degenerating (Fig. 2a). Next, from a normally developed endosperm, the ovule is dissected, and the normal embryo is taken out. This procedure creates a normal endosperm with an exit hole. The hybrid embryo is passed through the exit and placed into the endosperm (Fig. 2b). This procedure results in embryo-endosperm transplant that is subsequently transferred together and cultured on an appropriate in vitro medium (Williams et al., 1982). Typically, the embryo of an interspecific hybridization can be transplanted by inserting into an endosperm arising from an intraspecific hybridization that involves one of the parental species. Many interspecific and intergeneric plants have been obtained using the technique of embryo-endosperm transplant. Furthermore, modifications of the nurse endosperm technique, for example, embryo implantation or embryo transplantation, are employed in many different plant species. Embryo rescue via embryo-nurse endosperm transplants could enable about 30% recovery in wide hybridizations, compared to instances where the technique is not deployed (Shukla, 2016).

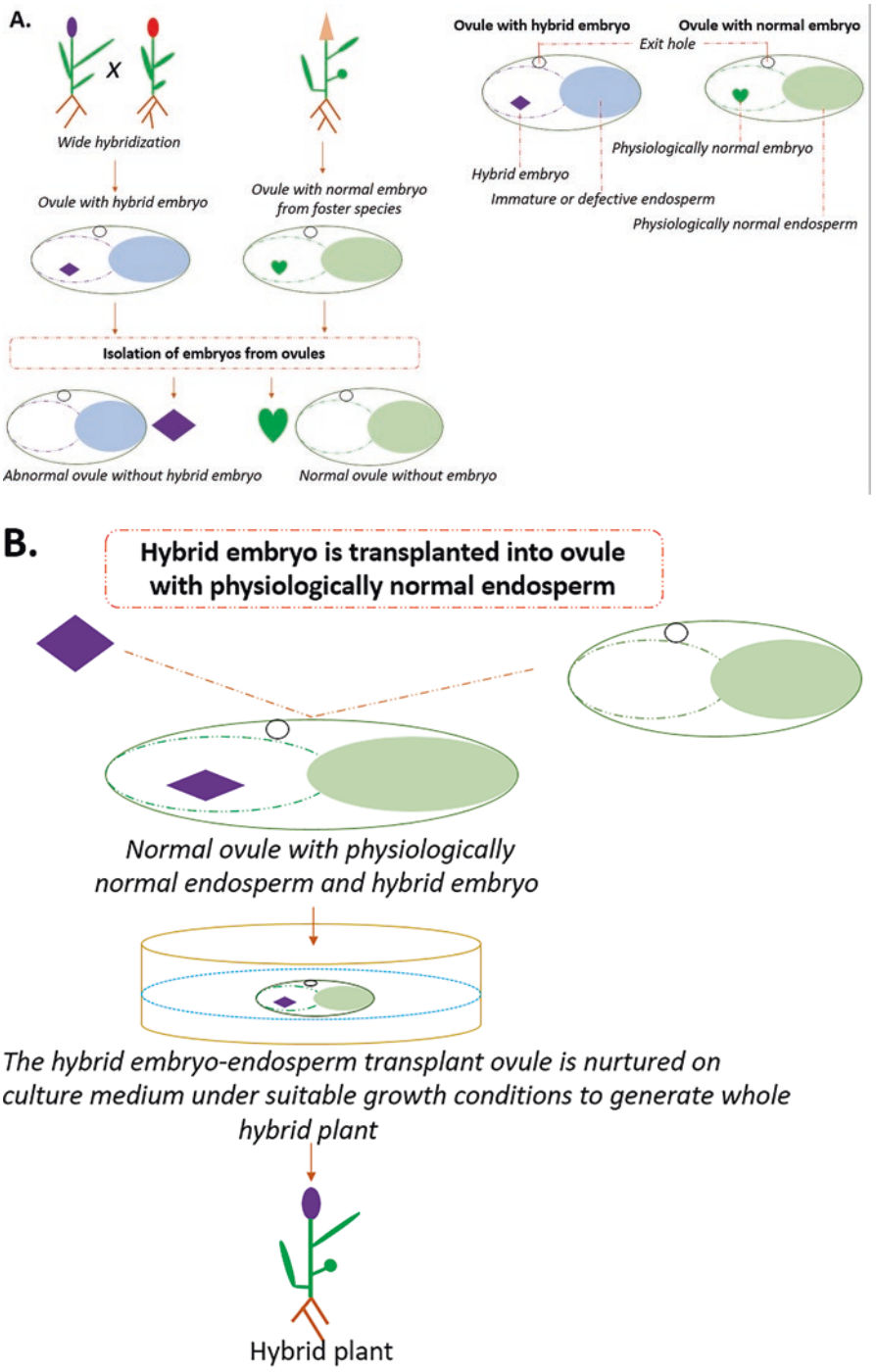



**Fig. 2** Steps involved in the embryo-nurse endosperm transplant technique. (**a**) Isolation of hybrid embryo from ovule with defective endosperm resulting from wide hybridization as well the isolation of normal embryo from normal ovule of foster plant species with physiologically functional endosperm. (**b**) Transplant of hybrid embryo into the normal ovule with physiologically normal endosperm and generation of hybrid plant through in vitro culture

### 3.5.3 In Vitro Ovary Culture

Ovary culture entails culturing the entire ovary in a culture medium. In an ovary culture procedure, ovaries are collected by isolating the ovaries followed by the removal of any remaining flower parts (Li et al., 2020). Pollinated ovaries are extracted by the removal of calyx, corolla, and stamens. The ovaries are then disinfested by surface sterilization to eliminate contaminants cautiously by avoiding damage to the ovaries. The ovaries are subsequently inoculated in culture medium and oriented such that the cut pedicle section is positioned direct contact with the culture nutrient medium (Ramming et al., 2003). The ovary culture is nurtured by varying in vitro conditions and monitoring the progress of culture growth toward eventually producing plants that will bear fully developed fruits with viable seeds. Typically, ovule culture involves surgically opening disinfected ovary to release the ovule. Comparatively, there is higher success rate of obtaining hybrid plants from the culture of ovary or ovule than from embryo culture (Lentini et al., 2020). The reason may likely be due to nutritional disparities and physical causes such as the protective influence of maternal or sporophytic tissues on the embryo.

### 3.5.4 Ovule Culture Technique

Ovule culture is an approach in which the entire ovule containing the ovaries is excised and placed onto an appropriate culture medium (Li et al., 2020). The advantage of this approach is that the likely damage to the embryo that may arise during the excision of the embryo is avoided (Lentini et al., 2020). Generally, ovule culture is either supported on filter paper and vermiculite support systems. In the filter paper technique, the ovule is cultured using filter papers positioned over liquid medium (Ramming et al., 2003). On the other hand, the vermiculite support method is carried out by placing in an orientation such that the micropylar section of the ovule is placed down making contact with a sterile vermiculite support.

### 3.5.5 Ovary and Ovule Slice or Perforation Procedure

In the ovary slice culture technique, transverse sections of ovaries are cut with a sterilized scalpel. The basal cut end of the sections is then placed in direct contact with the culture medium (Shukla, 2016). The ovary-slice culture technique is considered more efficient, less laborious, and takes less time than ovary culture or ovule culture. In the ovule perforation method, tiny holes are carefully created in the ovule using needles without damaging the embryos. The ovule perforation procedure is usually performed just before the ovule is placed on the culture medium. The tiny perforations enhance increased water and nutrient permeability and uptake by the ovule, thus stimulating embryo development (Pinto et al., 1994). It has been observed that surgically slicing the ovule also boosts embryo growth, probably because such a procedure enables better culture medium and embryo nutrient exchange.

## *3.6 Applications of the Embryo Rescue Technique*

### 3.6.1 Overcoming Seed Dormancy

Embryo rescue and culture serves as a very important strategic approach for overcoming seed dormancy (Pramanik et al., 2021). Seeds of several plant types undergo conventional dormancy where seed germination is inhibited by some hormones in seeds containing the embryo. In some plant species, the seeds remain dormant for a very long period without initiating germination. Some seeds may, however, germinate extremely slowly, or in some cases, the seeds fail completely to germinate even in normal conditions. A number of factors have been implicated in diverse plant species to be responsible for inducing seed dormancy. Some of these seed dormancy-inducing factors include influence of endogenous inhibitors, specific temperature, humidity, or light requirements during seed storage and state of embryo maturity (Brits et al., 2015). Seed dormancy-causing factors particularly endogenous inhibitors of seed germination, for example, certain plant hormones, may be contained within the seed coat, the endosperm, or present in both locations.

The embryo rescue and culture strategy for bypassing dormancy, therefore, involves the exclusion of the dormant embryos from the effects of the germination inhibitors in order for the embryos to sprout and develop rapidly. The embryo rescue protocol for inducing germination of dormant embryos is usually formulated to provide the appropriate culture media composition, growth hormone combinations and culture temperature, and light or humidity environment to enable embryo germination and proper growth (Mohapatra & Rout, 2005). Burgos and Ledbetter (1993) employed embryo rescue and culture successfully in an apricot to obtain higher proportion of seedlings. Similar result was achieved by Balla and Brozik (1993) to circumvent seed dormancy in sweet cherry. Efficient protocols have been established for successful embryo rescue in several plant species.

### 3.6.2 Shortening of the Breeding Cycle in Plants

In some plant species, the embryo requires sufficient time to reach physiological maturity in order to break seed dormancy which in some species could be so long as to cause significant extension in the breeding cycle. Generally, seedlings fail to develop immediately after fruit ripening. Examples of crops in which the seeds do not germinate soon after fruit ripening include apples and oil palm. In such fruit crops, the embryo rescue approach has been used to reduce the breeding period by circumventing germination seed dormancy. Dormancy-induced delay in germination has been significantly reduced and shortened the breeding cycle from years to a few months by extracting the embryos out of the control of dormancy-inducing factors which are localized in the seed coat and endosperm, or both. Embryo rescue has been the most effective practical approach to freeing the embryos from dormancy-inducing factors in various horticultural crops. Removal and in vitro

nurturing of immature embryos on appropriate culture medium enable germination in a short time and, therefore, reduce the breeding cycle (Fathi & Jahani, 2012). Shortening the breeding cycle enables the plant breeder to obtain many more generations of a crop per year.

Embryo rescue has been a very useful technique for shortening the breeding cycles in apple by achieving good germination via reduction in the duration of seed dormancy. The technology of rescuing embryos in culture has also aided a very effective and efficient increase in the germination rate of mature seeds in ripening fruits such as sweet cherry by between 30% and 60% (Fathi et al., 2002). Similarly, Tamaki et al. (2011) succeeded in shortening the duration of the breeding cycle of *Carica papaya* varieties by roughly three months, assisted by embryo rescue and culture breeding techniques.

### 3.6.3 Overcoming Embryo Abortion

Embryo abortion arises mainly through the malformation of the endosperm of the seed to properly develop into a physiologically normal nutritive tissue around the embryo (Berger et al., 2006). Embryo abortion is a major constraint that limits the effectiveness of conventional plant breeding and improvement in some plant species. This challenge to conventional breeding work exists because, more often than not, interspecific and intergeneric hybridization of diploids versus tetraploids results in endosperms that usually develop defectively or not at all. In this regard, embryo abortion is prevented by the application of the embryo rescue and culture approach to generate whole plants using in vitro culture protocols (Reed, 2005). The embryo rescue method has been very valuable in conventional breeding and crop improvement, in the effective rescue of young embryos resulting from intraspecific and intergeneric hybrids that usually yield seeds that are not viable (Table 4).

Generally, post-zygotic barriers such as developmental failure of defective endosperm are effectively circumvented by extracting the embryos out of the ovule and nursing them aseptically in culture on appropriate nutrient medium to develop and grow into whole physiologically normal plants. Yang et al. (2007) rescued triploid hybrid embryos from intraspecific hybridization in grape varieties using in vitro culture. Similarly, Guo et al. (2011) also used rescue of hybrid embryos in obtaining triploid grapes from the hybridization of diploid and tetraploid varieties. Furthermore, via embryo rescue procedures and manipulations, Zhiwu et al. (2009) obtained plants from young triploid hybrid embryos developed from intraspecific hybridization involving diploid and tetraploid crosses in daylily (*Hemerocallis*). In seedless mandarin oranges, embryo rescue and culture techniques were used to achieve the production of triploid plants (Aleza et al., 2012). Peach, cherry, apricot, and plum commonly yield nonviable seeds, and particularly, the early ripening varieties have been found to often fail to germinate even when exposed to natural or favorable conditions. Seed sterility which is usually caused by incomplete embryo development in the seeds of these crop varieties is often resolved by employing the technique of embryo rescue and culture to assist in germination and plant regeneration (Bohra et al., 2016).

**Table 4** Achieved improvement in various plants through distant hybridization

| Plants | Achieved improvement | Distant hybridization | References |
|---|---|---|---|
| Common bean | Disease-resistant germplasm | Crop wild relatives crosses with *Phaseolus vulgaris* | Pratap et al. (2021) |
| Blueberry | Marketable-related traits size, firmness, acidity, soluble solids, weight, and yield | *Vaccinium elliottiii* × commercial blueberry germplasm | Cabezas et al. (2021) |
| Chickpea | Three superior cultivars have been developed | *Cicer arietinum* × *Cicer reticulatum* | Pratap et al. (2021) |
| Brassica | Good seed set | *Brassica oleracea* × *brassica napus* | Kaminski et al. (2020) |
| Cotton | Tolerance resistance to the fungus, *Verticillium* wilt | *Gossypium herbaceum* × *Gossypium nelsonii* | Yin et al. (2020) |
| Mungbean and urdbean | Resistance to vagaries, biotic and abiotic, synchronous podding, non-shattering pods | *Vigna radiata* × *Vigna mungo* | Pratap et al. (2019) |
| Lentil | Short-duration biofortified pre-bred lines | Crop wild relatives crosses with *Lens culinaris* | Kumar et al. (2018) |
| Chickpea | Higher pod numbers and earliness | *Cicer reticulatum* and *Cicer echinospermum* hybridized with cultivated varieties | Singh et al. (2018) |
| Brassica | Black rot resistance | *Brassica carinata* × *brassica oleracea* | Sharma et al. (2017) |
| Rice | Improved yield component traits | *Crosses involving* six "AA" rice genome species: *Oryza glaberrima*, *Oryza barthii*, *Oryza nivara*, *Oryza rufipogon*, *Oryza longistaminata*, and *Oryza glumaepatula* | Bhatia et al. (2017) |
| Rice | Saline-tolerant Spartina rice crop | Rice♀ × Spartina ♂ | Chen et al. (2016) |
| Wheat | Enhanced the aluminum tolerance pentaploid hybrids | *Triticum aestivum* × *Triticum turgidum* spp. *durum* | Han et al. (2016) |
| Brassica | High yield and short duration | *Brassica oleracea* × *brassica rapa* | Karim et al. (2014) |
| Cowpea | Photo- and thermo-period insensitive | *Vigna umbellata* × *Vigna glabrescens* | Pratap et al. (2014) |
| Pigeon pea | Different cytoplasmic male sterility genes transferred | Crop wild relatives crosses with *Cajanus cajan* | Saxena et al. (2010) |
| Pepper | Anthracnose resistance | *Capsicum baccatum* × *capsicum annuum* | Yoon et al. (2006) |

### 3.6.4 Development of Plants in Seedless Varieties

In many seedless varieties, for example, grapes, the embryo ceases to develop postfertilization leading to failure of physiologically normal seed formation, a phenomenon known as stenospermocarpy (Picarella & Mazzucato, 2019). The constraints associated with stenospermocarpy render conventional breeding methods inefficient in the improvement of seedless varieties (Costantini et al., 2021; Picarella & Mazzucato, 2019). The technique of in ovulo embryo rescue was, therefore, developed and widely applied to rescue naturally immature, weak, or defective aborting embryos, with the ultimate aim of producing progeny from the hybridization of seedless parents. Typically, in ovulo embryo rescue is carried out by aseptically isolating the defective embryos surgically from ovules and manipulating the in vitro culture media and conditions until eventually plantlets are formed. However, in some cases, it has been practically very difficult to isolate the embryos out of the ovules. In such instances, the whole ovule containing the embryo is cultured (Sharma et al., 1996).

Seedless varieties in some crops are developed mainly via parthenocarpy and stenospermocarpy (Costantini et al., 2021; Picarella & Mazzucato, 2019; Pratt, 1971). In grapes, the large berried seedless ones are developed more often by manipulating stenospermocarpy and less from parthenocarpy (Costantini et al., 2021; Picarella & Mazzucato, 2019; Stout, 1936). Cain et al. (1983) were the first to employ embryo rescue in seedless grapes development. Later, the efficiency of the method was optimized for application in other crops (Kumari et al., 2018; Singh et al., 2011). It is now quite routine to grow rescued embryos into whole plants. Improvement in seedless lime has also been achieved with embryo rescue and culture techniques (Prasad et al., 1996). Embryo rescue has also enabled the generation of plants from triploid embryos obtained from diploids crossed with tetraploids of the same plant species.

### 3.6.5 In Vitro Vegetative Propagation of Plants

Embryo culture has also proven very valuable in vegetative propagation of plants. The embryos of some plant genera exhibit both juvenile and mature physiological characteristics. The embryos of such genera are usually used as initiation explants for vegetative propagation (Naing et al., 2019). The juvenile state of these embryos is more practically exploited because the embryos in this state are most responsive to manipulations for efficient vegetative propagation (Debnath & Arigundam, 2020). For instance, in the Poaceae, compared to mature callus tissue, juvenile callus gives rise to organogenesis easier. A similar observation was made in the propagation of conifers using immature calli produced via young embryos (Bornman, 2002). In this example also, comparatively, axillary shoot generation was found to be easier with juvenile calli. The major challenge associated with this approach, however, is that the resulting clones are more often not derived out of zygotic

materials. Nonetheless, in cases where embryos form from nucellar tissue, as observed in citrus, zygotic embryos could serve as the basis for generated clones (Koltunow et al., 1996).

### 3.6.6 Germplasm Conservation: Preservation of Embryos and Regrowth

Somatic embryos are a very convenient form of tissues for medium- and long-term in vitro conservation, preservation, and micropropagation (Danso & Elegba, 2017; Danso & Ford-Lloyd, 2002). Generally, in vitro conservation of embryos is achieved by inducing growth reduction in the tissues in order for the embryo to enter and stay in a dormant state for a period. Growth reduction for medium-term conservation is carried out via manipulating the growth temperature, humidity, and in vitro culture medium conditions (Cruz-Cruz et al., 2013). However, in long-term embryo preservation and conservation, reduction in the rate of embryo growth is achieved at very low temperature by storing the embryos in liquid nitrogen at −196 °C, a technique termed cryopreservation (Danso & Ford-Lloyd, 2002; Jaisankar et al., 2018). The successive actions of the process of cryopreservation of embryos are carried out under precise conditions which are usually determined for each type of material to be preserved and conserved. For the practical use of preserved embryos, after the required medium- or long-term period of storage, the preserved embryos are regrown into whole plants (Shukla, 2016).

In direct regrowth of the stored embryos in a nursery or through direct planting, the culture medium is manipulated essentially to bypass any secondary callogenesis or embryogenesis or both. The ability of the preserved embryos to grow into physiologically normal plants is dependent on the embryo size and the maturity of the mother plants that donated the preserved embryos (Tessereau et al., 1994). Protocols and procedures of cryopreservation have been established for many crops of food, medicinal, and industrial value (Cruz-Cruz et al., 2013; Reed, 2011). One of the pioneering extensive uses of cryopreservation involved the conservation of somatic embryos of oil palm (Palanyandy et al., 2020). In coconuts, it is very cumbersome to transport whole nuts due to the weight and requirement for huge cargo space on flights or ships. It is, therefore, mandatory to internationally exchange germplasm of coconut in the form of embryo cultures or embryos containing endosperm plugs (Lédo et al., 2017). Besides, embryos serve as disease free and safest materials for cryogenic storage and preservation. Development of seedlings from in vitro embryo culture prevents pests and diseases introduction and spread (Tegen, 2016). It has been possible to directly regrow frozen-thawed embryos into carrot and coffee plantlets. Large-sized cryopreserved embryos of some plants usually do not survive cryopreservation; however, Tessereau et al. (1994) found that large carrot embryos could survive cryopreservation. Utami et al. (2017) reported an efficient embryo rescue or culture procedure for obtaining plantlets in the medicinal orchid (*Dendrobium lasianthera*) using mature seed culture.

### 3.6.7 Homozygous Monoploid Production

Embryo culture is a useful tool for creating haploids via chromosome elimination after wide hybridization has been carried out. Usually, in some instances, fertilization takes place, but the chromosomes of the pollen-donating parent are later in the process, removed by the seed parent (Dresselhaus et al., 2016). Such cases give rise largely to nonviable haploid embryos. Viable haploid embryos and haploid plants production are, therefore, achieved through the in vitro rescue of haploid maternal embryos that have the paternal chromosomes removed (Seguí-Simarro et al., 2021). Subsequently, the maternal chromosomes are doubled using colchicine treatment of the rescued embryos to create homozygous monoploid embryos which eventually develop into monoploid plants (Chase, 1969; Mehetre & Thombre, 1980). Monoploid embryo induction and plant regeneration serve as a very useful tool in plant breeding. Monoploids are more often than not obtained through the manipulation of mainly embryo and anther cultures (Chaikam et al., 2019). Monoploids are considered to present valuable advantages and serve as one of the best materials for breeding-related studies and crop improvement (Hooghvorst et al., 2020).

## 4 Conclusion

The embryo rescue technology presents a huge potential for the generation of interspecific and intergeneric hybrids with desired traits. Various salient aspects of embryo rescue technique in plant breeding and improvement through wide hybridizations have been elucidated. In addition, some of the current successful achievements in the improvement of agronomic traits using embryo rescue or culture emphasize the usefulness of the technique. This informative review will serve as a valuable resource that will enable a better understanding and more effective use of the embryo rescue technology to quicken the development of superior-performing plants and boost the sustenance of food and nutritional security.

## References

Aleza, P., Juárez, J., Cuenca, J., et al. (2012). Extensive citrus triploid hybrid production by 2x × 4x sexual hybridizations and parent–effect on the length of the juvenile phase. *Plant Cell Reports, 31*(9), 1723–1735.

Amanate-Bordeos, A. D., Nelson, R. J., Oliva, N. P., Dalmacio, R. D., Leung, H., & Sitch, L. A. (1992). Transfer of blast and bacterial blight resistance from the tetraploid wild rice *Oryza minuta* to the cultivated rice, *O. sativa*. *Theoretical and Applied Genetics, 84*, 345–354.

Araujo, A. O. D., Peixoto, M., Souza, C. N. D., Gasparino, E. C., Faria, J. T., & Lombello, R. A. (2021). A natural intergeneric hybrid of Gesneriaceae from Brazil. *Phytotaxa, 497*(2), 20. https://doi.org/10.11646/phytotaxa.497.2.2

Baguma, J. K., Mukasa, S. B., Kawuki, R., Tugume, A. K., Buttibwa, M., et al. (2019). Fruit set and plant regeneration in cassava following interspecific pollination with castor bean. *African Crop Science Journal, 27*(1), 99–118.

Balla, I., & Brozik, S. (1993). *Embryo culture of sweet cherry hybrids* (pp. 385–386). In II International Cherry Symposium.

Ballesfin, M. L. E., Vinarao, R. B., Sapin, J., Kim, S. R., & Jena, K. K. (2018). Development of an intergeneric hybrid between *Oryza sativa* L. and *Leersia perrieri* (A. Camus) Launert. *Breeding Science, 68*(4), 474–480. https://doi.org/10.1270/jsbbs.18045. Epub. PMID: 30369822; PMCID: PMC6198897.

Berger, F., Grini, P. E., & Schnittger, A. (2006). Endosperm: An integrator of seed growth and development. *Current Opinion in Plant Biology, 9*(6), 664–670. https://doi.org/10.1016/j.pbi.2006.09.015

Bhatia, D., Joshi, S., Das, A., Vikal, Y., Sahi, G. K., Neelam, K., Kaur, K., & Singh, K. (2017). Introgression of yield component traits in rice (*Oryza sativa* ssp. *indica*) through interspecific hybridization. *Crop Science*. https://doi.org/10.2135/cropsci2015.11.0693

Bohra, A., Jha, U. C., Adhimoolam, P., Bisht, D., & Singh, N. P. (2016). Cytoplasmic male sterility (CMS) in hybrid breeding in field crops. *Plant Cell Reports, 35*(5), 967–993. https://doi.org/10.1007/s00299-016-1949-3

Bornman, C. H. (2002). Somatic seed in conifer biotechnology — A viable alternative to natural seed? *South African Journal of Botany, 68*, 119–126.

Bridgen, M. P. (1994). A review of plant embryo culture. *Horticultural Science, 29*, 1243–1246.

Brits, G. J., Brown, N. A. C., Calitz, F. J., & Van Staden, J. (2015). Effects of storage under low temperature, room temperature and in the soil on viability and vigour of *Leucospermum cordifolium* (Proteaceae) seeds. *South African Journal of Botany, 97*, 1–8. https://doi.org/10.1016/j.sajb.2014.11.003

Burgos, L., & Ledbetter, C. A. (1993). Improved efficiency in apricot breeding: Effects of embryo development and nutrient media on in vitro germination and seedling establishment. *Plant Cell, Tissue and Organ Culture, 35*(3), 217–222.

Buteme, R., Nakajiri, M., Kucel, N., Kabod, P. N., Sseremba, G., & Kizito, E. B. (2021). Intraspecific crossability and compatibility within *Solanum aethiopicum*. *Heliyon, 7*(7), e07645. https://doi.org/10.1016/j.heliyon.2021.e07645

Cabezas, D., de Bem, O. I., Acker, M., Lyrene, P., & Munoz, P. R. (2021). Evaluating wild germplasm introgression into autotetraploid blueberry. *Agronomy, 11*, 614. https://doi.org/10.3390/agronomy11040614

Cain, D. W., Emershad, R. L., & Tarailo, R. E. (1983). In-ovulo embryo culture and seedling development of seeded and seedless grapes (*Vitis vinifera* L.). *Vitis, 22*, 9–14.

Carputo, D., Monti, L., Werner, J. E., & Frusciante, L. (1999). Uses and usefulness of endosperm balance number. *Theoretical and Applied Genetics, 98*, 478–484.

Caruso, M., Smith, M. W., Froelicher, Y., Russo, G., & Gmitter, F. G., Jr. (2020). Traditional breeding. In M. Talon, M. Caruso, & F. G. Gmitter (Eds.), *The genus citrus* (1st ed., pp. 129–148). Elsevier.

Chaikam, V., Molenaar, W., Melchinger, A. E., et al. (2019). Doubled haploid technology for line development in maize: Technical advances and prospects. *Theoretical and Applied Genetics, 132*, 3227–3243. https://doi.org/10.1007/s00122-019-03433-x

Chase, S. S. (1969). Monoploids and monoploid-derivatives of maize (*Zea mays* L.). *The Botanical Review, 35*, 117–168. https://doi.org/10.1007/BF02858912

Chen, Q., Chen, B., Lu, H., Zhou, H., & Mei, Z. (2016). Innovation on distant hybridization of saline-tolerant mud flat Spartina and Rice germplasm. *Asian Agricultural Research, 08*(11), 87–94. https://doi.org/10.22004/ag.econ.253344

Costantini, L., Moreno-Sanz, P., Nwafor, C. C., et al. (2021). Somatic variants for seed and fruit set in grapevine. *BMC Plant Biology, 21*, 135. https://doi.org/10.1186/s12870-021-02865-2

Cruz-Cruz, C. A., González-Arnao, M. T., & Engelmann, F. (2013). Biotechnology and conservation of plant biodiversity. *Resources, 2*, 73–95. https://doi.org/10.3390/resources2020073

Danso, K., & Elegba, W. (2017). Optimization of somatic embryogenesis in cassava. *Biotechnologies for Plant Mutation Breeding*, 73–89. https://doi.org/10.1007/978-3-319-45021-6_5

Danso, K., & Ford-Lloyd, B. (2002). Induction of high-frequency somatic embryos in cassava for cryopreservation. *Plant Cell Reports, 21*, 226–232. https://doi.org/10.1007/s00299-002-0516-2

Debnath, S. C., & Arigundam, U. (2020). In vitro propagation strategies of medicinally important berry crop, lingonberry (*Vaccinium vitis-idaea* L.). *Agronomy, 10*, 744. https://doi.org/10.3390/agronomy10050744

Deng, Y., Sun, X., Gu, C., Jia, X., Liang, L., & Su, J. (2017). Identification of pre-fertilization reproductive barriers and the underlying cytological mechanism in crosses among three petal-types of *Jasminum sambac* and their relevance to phylogenetic relationships. *PLoS One, 12*(4), e0176026. https://doi.org/10.1371/journal.pone.0176026

Din, M. A. R. J., Ahmad, I. F., Wagiran, A., Samad, A. A., Rahmat, Z., & Sarmidi, M. R. (2016). Improvement of efficient *in vitro* regeneration potential of mature callus induced from Malaysian upland rice seed (*Oryza sativa* cv. Panderas). *Saudi Journal of Biological Sciences, 23*, S69–S77. https://doi.org/10.1016/j.sjbs.2015.10.022

Dresselhaus, T., Sprunck, S., & Wessel, G. M. (2016). Fertilization mechanisms in flowering plants. *Current Biology, 26*(3), 125–139. https://doi.org/10.1016/j.cub.2015.12.032

Dziasek, K., Simon, L., Lafon-Placette, C., Laenen, B., Wärdig, C., Santos-González, J., et al. (2021). Hybrid seed incompatibility in *Capsella* is connected to chromatin condensation defects in the endosperm. *PLoS Genetics, 17*(2), e1009370. https://doi.org/10.1371/journal.pgen.1009370

Fathi, H., & Jahani, U. (2012). Review of embryo culture in fruit trees. *Annals of Biological Research, 3*(9), 4276–4281.

Fathi, H., Arzani, K., Khalighi, A., & Ebadi, A. (2002). *The application of embryo culture in sweet cherry (Prunus avium L.) breeding*. Abstract Book of the Third Iranian Horticultural Congress. 31 August – 2 September, 355 pp.

Gamborg, O. L., Miller, R. A., & Ojima, K. (1968). Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research, 50*(1), 151–158. https://doi.org/10.1016/0014-4827(68)90403-5. PMID 5650857.

Gautheret, R. J. (1934). Culture de tissue cambial. *C. R. Hebd. Seances Acad. Sc, 198*, 2195–2196.

Gautheret, R. J. (1935). *Recherches sur la culture des tissues végétaux*. Ph. D. Thesis.

Guo, Y., Zhao, Y., Li, K., et al. (2011). Embryo rescue of crosses between diploid and tetraploid grape cultivars and production of triploid plants. *African Journal of Biotechnology, 10*(82), 19005–19010.

Hajmansoor, S., Bihamta, M. R., Nabipour, A., Mohammadi, A., Pirseyedi, S. M., & Nikkhah, H. R. (2009). Genetic diversity in barley genotypes: I. Seed storage proteins (hordeins) and agronomic traits. *Seed and Plant Improvement Journal, 25*(4), 51–64.

Han, C., Zhang, P., Ryan, P. R., Rathjen, T. M., Yan, Z., & Delhaize, E. (2016). Introgression of genes from bread wheat enhances the aluminium tolerance of durum wheat. *Theoretical and Applied Genetics, 129*, 729–739. https://doi.org/10.1007/s00122-015-2661-3

Hannig, E. (1904). Zur Physiologie Pflanzicher Embryonen I. Ueber die Cultur von Cruciferen-Embryonen ausserhalb des Embryosacks. *Bot Ztg, 62*, 45–80.

Haslam, T. M., & Yeung, E. C. (2011). Zygotic embryo culture: An overview. In T. A. Thorpe & E. C. Yeung (Eds.), *Plant embryo culture: Methods and protocols* (pp. 3–15). Springer. https://doi.org/10.1007/978-1-61737-988-8_1

Hawkes, J. G., & Jackson, M. T. (1992). Taxonomic and evolutionary implications of the endosperm balance number hypothesis in potatoes. *Theoretical and Applied Genetics, 84*, 180–185.

Heslop-Harrison, J. S., & Schwarzacher, T. (2011). Organization of the plant genome in chromosomes. *The Plant Journal, 66*, 18–33. https://doi.org/10.1111/j.1365-313X.2011.04544.x

Hooghvorst, I., Torrico, O., Hooghvorst, S., & Nogués, S. (2020). *In situ* parthenogenetic doubled haploid production in melon "Piel de Sapo" for breeding purposes. *Frontiers in Plant Science, 11*, 378. https://doi.org/10.3389/fpls.2020.00378

Hristova-Cherbadzhi, M. (2020). Intergeneric hybidization of sunflower (*Helianthus annuus* L.) with spiny plumeless thistle (*Carduus acanthoides* L.). *Helia, 43*(72), 83–98. https://doi.org/10.1515/helia-2020-0008

Hu, C., & Wang, P. (1986). Embryo culture: Technique and applications. In D. A. Evans, W. R. Sharp, & P. V. Ammirato (Eds.), *Handbook of plant cell culture, Vol 4, techniques and applications* (pp. 43–96). Macmillan Publishing.

Hu, D., Tang, X., Zhang, Q., & Luo, Z. (2013). Cross compatibilities of oriental persimmon 'Mopanshi' and 'Luotian-tianshi' with 'Zenjimaru'. *Acta Horticulturae, 996*, 165–170.

Hugo Cota-Sánchez, J. (2018). Precocious germination (Vivipary) in tomato: A link to economic loss? *Proceedings of the National Academy of Sciences, India Section B, 88*, 1443–1451. https://doi.org/10.1007/s40011-017-0878-4

Huylenbroeck, V. J., Eeckhaut, T., Leus, L., Laere, V. K., & Dhooghe, E. (2020). Bridging the gap: Tools for interspecific and intergeneric hybridization in ornamentals. In P. Franken, C. Tränkner, & U. Drüge (Eds.), *XXVI International Eucarpia Symposium Section Ornamentals: Editing Novelty* (Vol. 1283, pp. 161–168). Acta Horticulturae. International Society for Horticultural Science. https://doi.org/10.17660/ActaHortic.2020.1283.22

Jaisankar, I., Velmurugan, A., & Sivaperuman, C. (2018). Biodiversity conservation: Issues and strategies for the tropical islands. *Biodiversity and Climate Change Adaptation in Tropical Islands*, 525–552. https://doi.org/10.1016/B978-0-12-813064-3.00019-3

Johnston, S. A., & Hanneman, R. E. (1996). Genetic control of Endosperm Balance Number (EBN) in the Solanaceae based on trisomic and mutation analysis. *Genome, 39*, 314–321.

Kaeppler, S. M., Phillips, R. L., & Olhoft, P. (1998). Molecular basis of heritable tissue culture-induced variation in plants. In Jain et al. (Eds.), *Somaclonal variation and induced mutations in crop improvement. Current plant science and biotechnology in agriculture* (Vol. 32, pp. 465–484). Kluwer Academic Publishers.

Kaeppler, S. M., Kaeppler, H. F., & Rhee, Y. (2000). Epigenetic aspects of somaclonal variation in plants. *Plant Molecular Biology, 43*, 179–188.

Kaminski, P., Marasek-Ciolakowska, A., Podwyszynska, M., Starzycki, M., Starzycka-Korbas, E., & Nowak, K. (2020). Development and characteristics of interspecific hybrids between *Brassica oleracea* L. and *B. napus* L. *Agronomy, 10*, 1339. https://doi.org/10.3390/agronomy10091339

Karim, M. M., Siddika, A., Tonu, N. N., Hossain, D. M., Meah, M. B., Kawanabe, T., Fujimoto, R., & Okazaki, K. (2014). Production of high yield short duration *Brassica napus* by interspecific hybridization between *B. oleracea* and *B. rapa*. *Breeding Science, 63*, 495–502.

Katsiotis, A., Hanneman, R. E., Jr., & Forsberg, R. A. (1995). Endosperm balance number and the polar-nuclei activation hypotheses for endosperm development in interspecific crosses of Solanaceae and Gramineae, respectively. *Theoretical and Applied Genetics, 91*, 848–855.

Köhler, C., Dziasek, K., & León, G. D. (2021). Postzygotic reproductive isolation established in the endosperm: Mechanisms, drivers and relevance. *Philosophical Transactions of the Royal Society, B: Biological Sciences, 376*, 20200118. https://doi.org/10.1098/rstb.2020.0118

Koltunow, A. M., Hidaka, T., & Robinson, S. P. (1996). Accumulation of seed storage proteins in seeds and in embryos cultured *in vitro*. *Plant Physiology, 110*, 599–609.

Konan, N., Jacquemin, J., Baudoin, J., & Mergeai, G. (2020). Phenotypic, cytological and molecular (AFLP) analyses of the cotton synthetic allohexaploid hybrid (*G. hirsutum* × *G. longicalyx*)$^2$. *Open Journal of Genetics, 10*, 35–49. https://doi.org/10.4236/ojgen.2020.102004

Kruse, A. (1974). An *in vivo*/*vitro* embryo culture technique. *Hereditas, 77*(2), 219–224. https://doi.org/10.1111/j.1601-5223.1974.tb00935.x

Kuang, Y., Lu, C.-H., & Hsu, F.-C. (2021). Restoring fertility for novel interspecific hybrids between *Kalanchoe garambiensis* and *K. nyikae* using colchicine treatment. *Plants, 10*(2), 209. https://doi.org/10.3390/plants10020209

Kuligowska, K., Lütken, H., Christensen, B., et al. (2015). Evaluation of reproductive barriers contributes to the development of novel interspecific hybrids in the *Kalanchoë* genus. *BMC Plant Biology, 15*, 15. https://doi.org/10.1186/s12870-014-0394-0

Kumar, S., Choudhary, A. K., Rana, K. S., Sarker, A., & Singh, M. (2018). Bio-fortification potential of global wild annual lentil core collection. *PLoS One, 13*, e0191122. https://doi.org/10.1371/journal.pone.0191122

Kumari, P., Eshwari, T., & Hul, R. (2018). Embryo rescue in horticultural crops. *International Journal of Current Microbiology and Applied Sciences, 7*(6), 3350–3358. https://doi.org/10.20546/ijcmas.2018.706.393

Kumlehn, J., & Bradley, J. T. (2017). *Biotechnologies for plant mutation breeding protocols* (pp. 73–90). Springer Nature. https://doi.org/10.1007/978-3-319-45021-6

Lédo, A. S., Oliveira, L. A. A. R., Machado, C. A., Silva, A. V. C., & Ramos, S. R. R. (2017). Strategies for exchange of coconut germplasm in Brazil. *Biology Ciencia Rural, 47*, 3. https://doi.org/10.1590/0103-8478cr20160391

Lee, H. O., Davidson, J. M., & Duronio, R. J. (2009). Endoreplication: Polyploidy with purpose. *Genes & Development, 23*(21), 2461–2477. https://doi.org/10.1101/gad.1829209

Lentini, Z., Restrepo, G., Buitrago, M. E., & Tabares, E. (2020). Protocol for rescuing young cassava embryos. *Frontiers in Plant Science, 11*, 522. https://doi.org/10.3389/fpls.2020.00522

Lester, R. N., & Kang, J. H. (1998). Embryo and endosperm function and failure in Solanum species and hybrids. *Annals of Botany, 82*, 445–453. Article No. bo980695.

Li, G. R., Ji, W., Wang, G., Zhang, J. X., & Wang, Y. J. (2014). An improved embryo-rescue protocol for hybrid progeny from seedless *Vitis vinifera* grapes × wild Chinese *Vitis* species. *In Vitro Cellular & Developmental Biology. Plant, 50*, 110–120. https://doi.org/10.1007/s11627-013-9543-7

Li, J., Zhang, C., Guan, C., et al. (2017). Analysis of intergeneric sexual hybridization between transgenic *Brassica oleracea* and *Sinapis alba*. *Euphytica, 213*, 271. https://doi.org/10.1007/s10681-017-2063-5

Li, F., Cheng, Y., Zhao, X., et al. (2020). Haploid induction via un-pollinated ovule culture in *Gerbera hybrida*. *Scientific Reports, 10*, 1702. https://doi.org/10.1038/s41598-020-58552-z

Lu, C., & Bridgen, M. P. (1996). 1996. Effects of genotype, culture medium and embryo developmental stage of the *in vitro* responses from ovule culture of interspecific hybrids of *Alstroemeria*. *Plant Science, 116*, 205–212.

Mahoney, J. D., & Brand, M. H. (2021). Pre- and postzygotic barriers associated with intergeneric hybridization between *Aronia melanocarpa* (Michx.) Elliott x *Pyrus communis* L. and × *Sorbaronia dippelii* (Zabel) CK Schneid. *X Pyrus communis Hortscience, 56*(2), 177–184. https://doi.org/10.21273/HORTSCI15412-20

Manzur, J. P., Calvache-Asensio, M. N., & Rodriguez-Burruezo, A. (2014). Growth regulators and darkness increase efficiency in *in vitro* culture of immature embryos from peppers. *Genetics and Plant Breeding Sci agric (Piracicaba, Braz.), 71*, 488. https://doi.org/10.1590/0103-9016-2013-0230

Manzur, J. P., Fita, A., Prohens, J., & Rodríguez-Burruezo, A. (2015). Successful wide hybridization and introgression breeding in a diverse set of common peppers (*Capsicum annuum*) using different cultivated ají (*C. baccatum*) accessions as donor parents. *PLoS One, 10*(12), e0144142. https://doi.org/10.1371/journal.pone.0144142

Martins, K. C., Pereira, T. N. S., Souza, S. A. M., Rodrigues, R., & Junior, A. T. A. (2015). Crossability and evaluation of incompatibility barriers in crosses between capsicum species crop breed. *Applied Biotechnology, 15*(03). https://doi.org/10.1590/1984-70332015v15n3a25

Mehetre, S. S., & Aher, A. R. (2004). Embryo rescue: A tool to overcome incompatible interspecific hybridization in *Gossypium* Linn. – A review. *Indian Journal of Biotechnology, 3*, 29–36.

Mehetre, S. S., & Thombre, M. V. (1980). Meiosis in monoploid asiatic cotton (*Gossypium arboreum* L.). *Caryologia, 33*(3), 393–400. https://doi.org/10.1080/00087114.1980.10796852

Ming, N., Mostafiz, B. S., Johon, N. S., Zulkifli, A. N. S., & Wagiran, A. (2019). Combination of plant growth regulators, maltose, and partial desiccation treatment enhance somatic embryogenesis in selected Malaysian rice cultivar. *Plants (Basel), 8*(6), 144. https://doi.org/10.3390/plants8060144

Mohapatra, A., & Rout, G. R. (2005). Study of embryo rescue in floribunda rose. *Plant Cell Tiss Organ Cult, 81*, 113–117. https://doi.org/10.1007/s11240-004-3128-4

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum, 15*(3), 473–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Mwangangi, M., Muli, J. K., & Neondo, J. O. (2019). Plant hybridization as an alternative technique in plant breeding improvement immaculate. *Asian Journal of Research in Crop Science, 4*(1), 1–11. Article no. AJRCS.49096.

Naing, A. H., Kim, S. H., Chung, M. Y., et al. (2019). *In vitro* propagation method for production of morphologically and genetically stable plants of different strawberry cultivars. *Plant Methods, 15*, 36. https://doi.org/10.1186/s13007-019-0421-0

Nakamura, S., Pourkheirandish, M., Morishige, H., Sameri, M., Sato, K., & Komatsuda, T. (2017). Quantitative trait loci and maternal effects affecting the strong grain dormancy of wild barley (*Hordeum vulgare* ssp. *spontaneum*). Front. *Plant Science, 8*, 1840. https://doi.org/10.3389/fpls.2017.01840

Narayanaswami, S., & Norstog, K. (1964). Plant embryo culture. *The Botanical Review, 30*, 587–628.

Neelakandan, A. K., & Wang, K. (2012). Recent progress in the understanding of tissue culture-induced genome level changes in plants and potential applications. *Plant Cell Reports, 31*, 597–620. https://doi.org/10.1007/s00299-011-1202-z

Niemann, J., Olender, M., Wojciechowski, A., & Tomkowiak, A. (2015). Interspecific hybridization between *Brassica napus* and *Brassica rapa* ssp. chinensis genotypes through embryo rescue and their evaluation for crossability. *Biotechnologia, 96*, 184–191.

Nishiyama, I., & Yabuno, T. (1978). Causal relationships between the Polar nuclei in double fertilization and interspecific cross-incompatibility in *Avena*. *Cytologia, 43*(2), 453–466. https://doi.org/10.1508/cytologia.43.453

Nishiyama, I., Sarashima, M., & Matsuzawa, Y. (1991). Critical discussion on abortive interspecific crosses in Brassica. *Plant Breeding, 107*, 288. https://doi.org/10.1111/j.1439-0523.1991.tb00552.x

Okamoto, A., & Ureshino, K. (2015). Pre- and post-fertilization barriers in Iinterspecific hybridization between evergreen azalea species and *Rhododendron uwaense* H. Hara & T. Yamanaka. *The Horticulture Journal, 84*, 355. https://doi.org/10.2503/hortj.MI-036

Pachakkil, B., Terajima, Y., Ohmido, N., et al. (2019). Cytogenetic and agronomic characterization of intergeneric hybrids between *Saccharum* spp. hybrid and *Erianthus arundinaceus*. *Scientific Reports, 9*, 1748. https://doi.org/10.1038/s41598-018-38316-6

Palanyandy, S. R., Gantait, S., Subramaniam, S., & Sinniah, U. R. (2020). Cryopreservation of oil palm (*Elaeis guineensis* Jacq.) polyembryoids via encapsulation – Desiccation. *Biotech, 10*, 9. https://doi.org/10.1007/s13205-019-1997-9

Pant, U., Joshi, S., Lohani, P., & Dahiya, N. (2021). *Pre-fertilization barrier, crossability and meiotic behavior of interspecific hybrids among Brassica species Euphytica*. https://doi.org/10.21203/rs.3.rs-791559/v1

Patil, P., Malik, S. K., Negi, K. S., John, J., Yadav, S., Chaudhari, G., & Bhat, K. V. (2013). Pollen germination characteristics, pollen–pistil interaction and reproductive behaviour in interspecific crosses among *Abelmoschus esculentus* Moench and its wild relatives. *Grana, 52*(1), 1–14. https://doi.org/10.1080/00173134.2013.768699

Pawar, B., Kale, P., Bahurupe, J., Jadhav, A., Kale, A., & Pawar, S. (2015). Proline and glutamine improve *in vitro* callus induction and subsequent shooting in rice. *Rice Science, 22*, 283–289.

Pen, S., Nath, U. K., Song, S., Goswami, G., Lee, J.-H., Jung, H.-J., Kim, H.-T., Park, J.-I., & Nou, I. S. (2018). Developmental stage and shape of embryo determine the efficacy of embryo rescue in introgressing orange/yellow color and anthocyanin genes of Brassica species. *Plants, 7*, 99. https://doi.org/10.3390/plants7040099

Picarella, M. E., & Mazzucato, A. (2019). The occurrence of seedlessness in higher plants; insights on roles and mechanisms of parthenocarpy. *Frontiers in Plant Science, 9*, 1997.

Pinto, A. C., Rogers, S. M. D., & Byrne, D. H. (1994). Growth of immature peach embryos in response to media, ovule support method, and ovule perforation. *Horticultural Science, 29*(9), 1081–1083.

Pollegioni, P., Olimpieri, I., Woeste, K. E., Simoni, G. D., Gras, M., & Malvolti, M. E. (2013). Barriers to interspecific hybridization between *Juglans nigra* L. and *J. regia* L species. *Tree Genetics & Genomes, 9*, 291–305. https://doi.org/10.1007/s11295-012-0555-y

Pramanik, K., Sahoo, J. P., Mohapatra, P. P., Acharya, L. K., & Jena, C. (2021). Insights into the embryo rescue – a modern *in vitro* crop improvement approach in horticulture. *Plant Cell Biotechnology and Molecular Biology, 22*(15–16), 20–33. Retrieved from https://www.ikprress.org/index.php/PCBMB/article/view/6025

Prasad, M. B. N. V., Sahijram, L., & Rekha, A. (1996). *Role of embryo culture techniques in the improvement of seedless lime fruit quality* (pp. 28–30). Nat'l. Symp. Hortl. Biotech.

Pratap, A., Basu, P. S., Gupta, S., Malviya, N., Rajan, N., Tomar, R., et al. (2014). Identification and characterization of sources for photo- and thermo-insensitivity in *Vigna* species. *Plant Breeding, 133*, 756–764. https://doi.org/10.1111/pbr.12215

Pratap, A., Gupta, S., Basu, P. S., Tomar, R., Dubey, S., Rathore, M., et al. (2019). Towards development of climate smart mungbean: Challenges and opportunities. In C. Kole (Ed.), *Genomic designing of climate-smart pulse crops* (pp. 235–264). Springer. https://doi.org/10.1007/978-3-319-96932-9_5

Pratap, A., Das, A., Kumar, S., & Gupta, S. (2021). Current perspectives on introgression breeding in food legumes. *Frontiers in Plant Science, 11*. https://doi.org/10.3389/fpls.2020.589189

Pratt, C. (1971). Reproductive anatomy in cultivated grapes – A review. *American Journal of Enology and Viticulture, 22*, 92–109.

Premjet, D., Obeng, A. K., Kongbangkerd, A., & Premjet, S. (2019). Intergeneric hybrid from *Jatropha curcas* L. and *Ricinus communis* L.: Characterization and polyploid induction. *Biology, 8*(2), 50. https://doi.org/10.3390/biology8020050

Raghavan, V. (1966). Nutrition, growth and morphogenesis of plant embryos. *Biological Reviews, 41*, 1–58.

Ramming, D. W. (1985). *In ovulo* embryo culture of early maturing *Prunus*. *Horticultural Science, 20*, 419–420.

Ramming, D. W., Emershad, R. L., & Foster, C. (2003). *In vitro* factors during ovule culture affect development and conversion of immature peach and nectarine embryos. *HortScience: A publication of the American Society for Horticultural Science, 38*(3), 424. https://doi.org/10.21273/HORTSCI.38.3.424

Rangan, T. S. (1984). Culture of ovules. In I. K. Vasil (Ed.), *Cell culture and somatic cell genetics of plants. vol. 1. Laboratory procedures and their applications* (pp. 227–231). Academic.

Reed, S. M. (2005). Embryo rescue. In R. N. Trigiano & D. J. Gray (Eds.), *Plant development and biotechnology* (pp. 235–239). CRC Press.

Reed, B. M. (2011). Choosing and applying cryopreservation protocols to new plant species or tissues. *Acta Horticulturae, 908*(908), 363–372. https://doi.org/10.17660/ActaHortic.2011.908.48

Reinert, S., Bajaj, Y. P. S., & Zbell, B. (1977). Aspects of organization, organogenesis, embryogenesis, cytodifferentiation. In H. E. Street (Ed.), *Plant tissue and cell culture* (pp. 389–427). University of California Press.

Ren, H., Du, X., Li, D., Zhao, A., Wang, Y., Xue, X., Gong, G., & Du, J. (2019). An efficient method for immature embryo rescue and plant regeneration from the Calli of *Ziziphus jujube* 'Lengbaiyu'. *The Journal of Horticultural Science and Biotechnology, 94*(1), 63–69. https://doi.org/10.1080/14620316.2018.1446767

Rodriguez-Lopez, C. M., Wetten, A. C., & Wilkinson, M. J. (2010). Progressive erosion of genetic and epigenetic variation in callus-derived cocoa (*Theobroma cacao*) plants. *The New Phytologist, 186*, 856–868. https://doi.org/10.1111/j.1469-8137.2010.03242.x

Sahijram, L., Soneji, J. R., Naren, A., & Madhusudhana, B. R. (2013). Hybrid embryo rescue: A non-conventional breeding strategy in horticultural crops. *Journal of Horticultural Science, 8*(1), 1–20.

Saxena, K. B., Sultana, R., Mallikarjuna, N., Saxena, R. K., Kumar, R. V., Sawargaonkar, S. L., et al. (2010). Male-sterility systems in pigeonpea and their role in enhancing yield. *Plant Breeding, 129*, 125–134. https://doi.org/10.1111/j.1439-0523.2009.01752.x

Seguí-Simarro, J., Jacquier, N. M. A., & Widiez, T. (2021). Overview of *in vitro* and *in vivo* doubled haploid technologies. In J. M. Segui-Simarro (Ed.), *Doubled haploid technology* (Vol. 2287). Methods in Molecular Biology, Humana. https://doi.org/10.1007/978-1-0716-1315-3_1

Sharma, D. R., Daur, R., & Kumar, K. (1996). Embryo rescue in plants – A review. *Euphytica, 89*, 325–337. https://doi.org/10.1007/BF00022289

Shukla, S. (2016). Embryo rescue technology: An approach for varietal development and *in vitro* germplasm conservation. *International Journal of Tropical Agriculture, 34*(3), 841–847.

Singh, N. V., Singh, S. K., & Singh, A. K. (2011). Standardization of embryo rescue technique and bio-hardening of grape hybrids (*Vitis vinifera* L.) using arbuscular mycorrhizal fungi (AMF) under sub-tropical conditions. *Vitis, 50*(3), 115–118.

Singh, M., Rani, S., Malhotra, N., Katna, G., & Sarker, A. (2018). Transgressive segregations for agronomic improvement using interspecific crosses between *C. arietinum* L. × *C. reticulatum* Ladiz and *C. arietinum* L. × *C. echinospermum* Davis species. *PLoS One, 13*(9), e0203082. https://doi.org/10.1371/journal.pone.0203082

Soto, S., Bullrich, L., Bologna, P., Facciuto, G., & Borja, M. (2012). Post-fertilization barriers in interspecific crosses between *Nierembergia linariifolia* and *N. ericoides*. *Acta Horticulturae, 937*, 947–952. https://doi.org/10.17660/ActaHortic.2012.937.117

Stout, A. B. (1936). Seedlessness in grapes. *New York State Agricultural Experiment Station Technical Bulletin, 238*, 68.

Subburaj, S., Cao, S., Xia, X., & He, Z. (2016). Phylogenetic analysis, lineage-specific expansion and functional divergence of seed dormancy 4-like genes in plants. *PLoS One, 11*, e0153717. https://doi.org/10.1371/journal.pone.0153717

Sun, C., Ma, Z., Zhang, Z., Sun, G., & Dai, Z. (2018). Factors influencing cross barriers in interspecific hybridizations of water lily. *Journal of the American Society for Horticultural Science, 143*(2), 130–135. https://doi.org/10.21273/JASHS04302-17

Tamaki, M., Urasaki, N., Nakamura, I., Motomura, K., & Adaniya, S. (2011). Shortening the breeding cycle of papaya (*Carica papaya* L.) by culturing embryos treated with ethrel. *Plant Cell Tissue and Organ Culture, 106*(2), 225–233. https://doi.org/10.1007/s11240-010-9910-6

Taskın, H., Yücel, N. K., Baktemur, G., Çömlekçioğlu, S., & Büyükalaca, S. (2013). Effects of different genotypes and gamma ray doses on haploidization with irradiated pollen technique in watermelon (*Citrullus lanatus* L.). *Canadian Journal of Plant Science, 93*, 1165–1168.

Tegen, H. (2016). The role of plant tissue culture to supply disease free planting materials of major horticultural crops in Ethiopia. *Journal of Biology, Agriculture and Healthcare, 6*(1), 1–122.

Tessereau, H., Florin, B., Meschine, M. C., Thierry, C., & Pétiard, V. (1994). Cryopreservation of somatic embryos: A tool for germplasm storage and commercial delivery of selected plants. *Annals of Botany, 74*(5), 547–555.

Tukey, H. B. (1933). Artificial culture of sweet cherry embryos. *The Journal of Heredity, 24*, 7–12.

Uma, S., Lakshmi, S., Saraswathi, M. S., Akbar, A., & Mustaffa, M. M. (2011). Embryo rescue and plant regeneration in banana (*Musa spp*.). *Plant Cell, Tissue and Organ Culture, 105*, 105–111. https://doi.org/10.1007/s11240-010-9847-9

Umbeck, P. F., & Norstog, K. (1979). Effects of abscisic acid and ammonium ion on morphogenesis of cultured barley embryos. *Bulletin of the Torrey Botanical, 106*, 110–116.

Utami, E. S. W., Sri, Y., & Manuhara, W. (2017). In vitro propagation of the endangered medicinal orchid, *dendrobium lasianthera* J. J. Sm through mature seed culture. *Asian Pacific Journal of Tropical Biomedicine, 7*(5), 385–504. https://doi.org/10.1016/j.apjtb.2017.01.011

Vidhanaarachchi, V. R. M., Suranjith, W. C., & Gunathilake, T. R. (2016). Effect of genotype, embryo maturity and culture medium on *in vitro* embryo germination of Sri Lankan coconut (*Cocos nucifera* L.) varieties. *Journal of the National Science Foundation of Sri Lanka, 44*(3), 273–278.

Wang, Y.-L., Guan, Z.-Y., Chen, F.-D., Fang, W.-M., & Teng, N.-J. (2012). Pollen viability, pistil receptivity, and embryo development in hybridization of *Nelumbo nucifera* Gaertn. *The Scientific World Journal*. Article ID 678706, 8 pages. https://doi.org/10.1100/2012/678706

White, C. N., Proebsting, W. M., Hedden, P., & Rivin, C. J. (2000). Gibberellins and seed development in maize. I. Evidence that gibberellin/abscisic acid balance governs germination versus maturation pathways. *Plant Physiology, 122*(4), 1081–1088. https://doi.org/10.1104/pp.122.4.1081

Widiez, T., Ingram, G. C., & Gutierrez-Marcos, J. (2017). Embryo-endosperm-sporophyte interaction in maize seeds. In *Maize Kernel Development* (pp. 95–107). CABI.

Williams, E. G., Verry, I. M., & Williams, W. M. (1982). Use of embryo culture in interspecific hybridization. In I. K. Vasil, W. R. Scowcroft, & K. J. Frey (Eds.), *Plant improvement and somatic cell genetics* (pp. 119–128). Academic.

Yan, H., Lu, L., Alzate, A., Ceballos, H., Hershey, C., Chen, S., et al. (2014). Fruit, seed and embryo development of different cassava (*Manihot esculenta* Crantz) genotypes and embryo rescue. *African Journal of Biotechnology, 131*, 1520–1524. https://doi.org/10.5897/AJB2013.13180

Yang, D., Li, W., Li, S., Yang, X., et al. (2007). *In vitro* embryo rescue culture of $F_1$ progenies from crosses between diploid and tetraploid grape varieties. *Plant Growth Regulation, 51*(1), 63–71.

Yeung, E. C., Thorpe, T. A., & Jensen, C. J. (1981). *In vitro* fertilization and embryo culture. In T. A. Thorpe (Ed.), *Plant tissue culture: Methods and applications in agriculture* (pp. 253–271). Academic Press.

Yeung, E. C., Nickle, T. C., & Meinke, D. (2001). Embryology of flowering: An overview. *Phytomorphology, 51*, 289–304.

Yin, X., Zhan, R., He, Y., Song, S., Wang, L., Ge, Y., et al. (2020). Morphological description of a novel synthetic allotetraploid ($A_1A_1G_3G_3$) of *Gossypium herbaceum* L. and *G. nelsonii* Fryx. Suitable for disease-resistant breeding applications. *PLoS One, 15*(12), e0242620. https://doi.org/10.1371/journal.pone.0242620

Yoon, J. B., Yang, D. C., Do, J. W., & Park, H. G. (2006). Overcoming two post-fertilization genetic barriers in interspecific hybridization between *Capsicum annuum* and *C. baccatum* for introgression of anthracnose resistance. *Breeding Science, 56*(1), 31–38. https://doi.org/10.1270/jsbbs.56.31

Zhiwu, L., Pinkham, L., Campbell, N. F., et al. (2009). Development of triploid daylily (*Hemerocallis*) germplasm by embryo rescue. *Euphytica, 169*(3), 313–318.

# Proteomic and Biochemical Research for Exploring the Role of Plant-Derived Smoke in Food Crops

**Shafiq Ur Rehman, Amana Khatoon, Muhammad Mudasar Aslam, Muhammad Jamil, and Setsuko Komatsu**

**Abstract** Smoke derived during fire is well known as a growth regulator, and compounds derived from smoke solution have a role in plant physiological, biochemical, and molecular processes. The presence of karrikins in plant-derived smoke solution has established its positive effects on seed germination and seedling growth in different plant species. Plant-derived smoke positively triggers various growth characteristics such as photosynthesis, secondary metabolites, total soluble protein, and sugar content**.** Molecular processes in plants at the cellular level are regulated by plant-derived smoke under normal and stressed conditions, which highpoint the smoke as an effective growth regulator in optimal and suboptimal conditions. Plant-derived smoke is an important biological agent, likely to be used in weed control, crop improvement, habitat restoration, and establishment of new plant communities because of its eco-friendly nature. This review highlights the proteomic, biochemical, and molecular effects of plant-derived smoke on seed germination and plant growth.

**Keywords** Proteomics · Omics · Biochemical effect · Plant-derived smoke · Plant growth · Seed germination

S. U. Rehman
Department of Biology, University of Haripur, Haripur, Pakistan

A. Khatoon
Department of Botany, Kohat University of Science & Technology, Kohat, Pakistan

M. M. Aslam
Department of Botany, University of Science & Technology Bannu, Bannu, Pakistan

M. Jamil
Department of Biotechnology & Genetic Engineering, Kohat University of Science & Technology,
Kohat, Pakistan

S. Komatsu (✉)
Department of Applied Chemistry and Food Science, Fukui University of Technology,
Fukui, Japan
e-mail: skomatsu@fukui-ut.ac.jp



A. Raina et al. (eds.), *Advanced Crop Improvement, Volume 2*,
https://doi.org/10.1007/978-3-031-26669-0_16

## 1 Introduction

Fire has long been reported to increase seed germination and the regrowth of vegetation thus preserving and promoting the species diversity (de Lange & Boucher, 1990). There are many fire-related factors which are involved in the elicitation of seed germination process (van Staden et al., 2000). Of these, light, soil moisture, and nutrients availability in the soil are the indirect fire-related factors which enhanced see germination (van Staden et al., 2000). Fire and fire-related cues including smoke were considered as germination stimulants in postfire environments (Paul et al., 2007; Baldwin & Morse, 1994). Plant species belonging to fire-prone environment mostly showed positive responses to smoke, a postfire product (Todorovic et al., 2005). Seeds of *Audouinia capitate* were reported to show a remarkable enhancement in germination under natural conditions only after exposure to smoke in postfires (de Lange & Boucher, 1990). Seed germination process was also enhanced in various plant communities like the Mediterranean South Africa (Light et al., 2002; Brown et al., 2003) and Californian chaparral (Egerton-Warburton, 1998; Keely & Fotheringham, 1998) in response to smoke. The compounds in smoke were reported to be thermostable, water soluble, and active at low concentrations (Flematti et al., 2004). These findings initiated a new prospect to consider the postfire products as the germination cue and provided a baseline to isolate compounds from plant-derived smoke and determine the nature of compounds.

The mechanism of plant-derived smoke action is not fully known; however, based on existing investigations, it is elucidated that the intense chemical scarification at the seed surface may lead to the plasticization of external cuticle forming numerous small spheres thus altering the permeability of the internal cuticle (Egerton-Warburton, 1998). A significant increase in both the number and size of the channels in the sub-testa cuticle showed that these modifications are directly associated with the breaking of seed dormancy (Egerton-Warburton, 1998). Smoke mechanically influences the seed coat scarification, which facilitates imbibition process (Light et al., 2009). Taking plant-derived smoke as a vital seed germination agent having thousands of active compounds positively effecting plant growth, the actual mechanism couldn't be inferred at once. However, it is unanimously believed that plant-derived smoke enhance seed germination by (i) the compounds present in smoke solution soften seed coat, (ii) increase nutrients supply to seeds, and (iii) trigger the signals related with stimulation of seed germination (Roche et al., 1997). Highly complex and diverse nature of active molecules in smoke makes the response mechanism of treated plants even more multifaceted to be traced and explored. Numerous genomic and post-genomic profiling approaches are thought to be required to trace how plants perceive smoke signals and initiate the series of germination and post-germination associated responses (Bose et al., 2020). Keeping in view above facts, the secret behind these responses requires a "multi-omics" application and integrating the multi-omics datasets to understand KAR signaling associated with germination, post-germination, and stress tolerance responses of plants.

In addition, plant-derived smoke is also known for its stimulatory role in post-germination plant growth by positively affecting various physiological processes. The seedling of kangaroo grass treated with smoke grew more vigorously without any abnormalities (Baxter et al., 1994). Plant-derived smoke has a positive influence on seedling growth of several plants (Khatoon et al., 2020). Plant-derived smoke solution increased photosynthetic pigments (Akeel et al., 2019), ionic contents (Jamil et al., 2014), $CO_2$ concentration, and stomatal conductance (Zhou et al., 2013). Plant-derived smoke improved plant growth by mediating the total soluble sugar, nitrate, and flavonoid contents (Zhou et al., 2011). Plant-derived smoke upregulated the flavonoids-related genes (Soós et al., 2010), which are correlated with adventitious root formation in *Eucalyptus gunnii* by axillary bud stimulation (Curir et al., 1990). Additionally, the balance of glycolysis and redox homeostasis is related to growth of chickpea under plant-derived smoke treatment (Rehman et al., 2018). These results suggested that positive effects of plant-derived smoke are not limited to seed germination but can be observed at seedling stage also.

This review emphasizes the roles of plant-derived smoke in seed germination and plant growth by untying its involvement in physiological, biochemical, and molecular aspects of plants. The smoke derived from plant significantly enhanced germination and post-germination growth of plants by influencing various attributes at biochemical and molecular level. As plant-derived smoke is the mixture of thousands of active compounds, therefore it is believed that these active compounds are playing key role in seed germination and plant growth. Due to complex nature of plant-derived smoke, plant responses at molecular level are not fully understood yet. Therefore, the current review emphasizes the roles of smoke in seed germination and plant growth with the focus on its role in plant physiological, biochemical, and molecular aspects.

## 2 Chemistry of Plant-Derived Smoke Solution

Plant-derived smoke solution is an aqueous extract obtained by slow burning of semidried plant material and bubbling the smoke through distilled water. This aqueous extract is a mixture of various compounds, and several researches have been dedicated to isolate these compounds. This isolation process of compounds from plant-derived smoke is complicated due to the large number of compounds and very low concentration of the active compounds relative to the other components present in the smoke (Ma et al., 2018). Until now, seven different compounds have been identified in plant-derived smoke (Flematti et al., 2004, 2011a; van Staden et al., 2004; Light et al., 2010; Wang et al., 2017; Kamran et al., 2017; Burger et al., 2018) (Table 1 and Fig. 1). Among seven compounds, butenolide known as karrikins is the most active compound, which stimulates seed germination and plant growth (Commander et al., 2008; Dixon et al., 2009) (Fig. 1). Karrikins are water soluble, thermostable, long-lasting, and active at very low concentrations (van Staden et al., 2000). Karrikins contain a five-membered butenolide ring and combine to a

**Table 1** Compounds isolated from plant-derived smoke solution (PDS) of different species of plant

| PDS species | Compound name | References |
|---|---|---|
| *Passerina vulgaris, Themeda triandra* | 3-Methyle-2H-Furo [2,3-C] Pyran-2 One | Flematti et al. (2004) and van Staden et al. (2004) |
| *Passerina vulgaris, Themeda triandra* | 3,4,5-trimethyl- 2(5H)-furanone | Light et al. (2010) |
| *Anigozanthos manglesii* | Glyceronitrile, Cyanohydrin | Flematti et al. (2011a) |
| *Nicotiana attenuata* | Catechol | Wang et al. (2017) |
| *Ginkgo biloba* | Hydroqunone | Kamran et al. (2017) |
| *Passerina vulgaris*, *Themeda triandra* | 5,5-dimethyl-2(5H)- furanone, (5RS)-5-ethyl-2(5H)- furanone | Burger et al. (2018) |

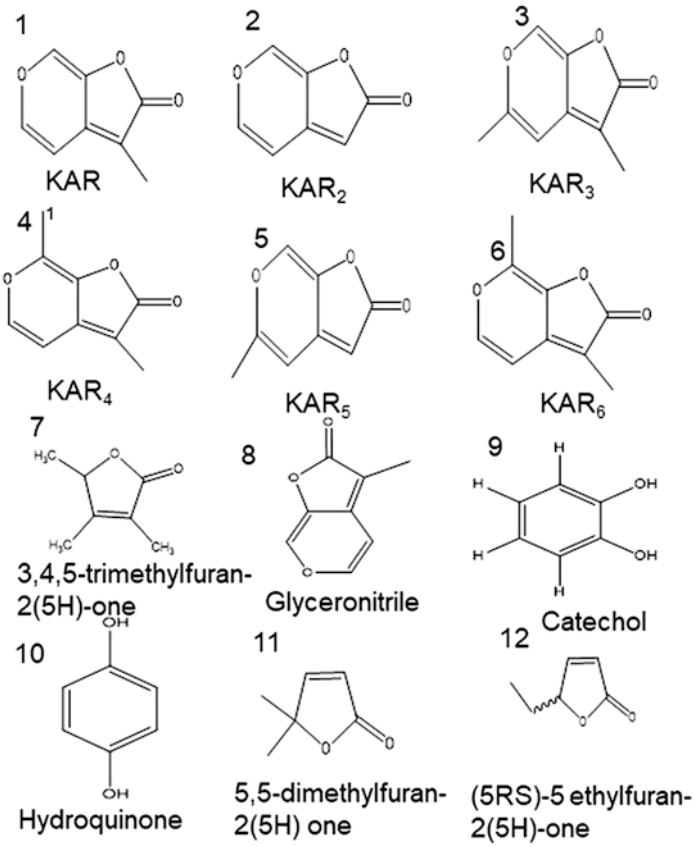



**Fig. 1** Different compound isolated from plant-derived smoke solution. Structure shows karrikins (1-6), 3,4,5-trimethylfuran-2(5H)-one (7), glyceronitrile (8), catechol (9), hydroquinone (10), 5,5-dimethylfuran-2(5H) one (11), (5RS)-5-ethylfuran-2(5H)-one (12)

six-membered pyran ring. There are six analogs of karrikins (KAR1-KAR6) differing with respect to methyl substitutions, in which KAR1 and KAR3 are the most active as seed germination stimulant (Flematti et al., 2007). KAR1 was isolated from cellulose or carbohydrates part of smoke-infused water prepared from the combustion of plant material (Flematti et al., 2011b). Based on combustion experiments, KAR1 is derived from a pyranose sugar (Flematti et al., 2011b; Nelson et al., 2012). KAR1 had a positive effect on root length (Waheed et al., 2016), number of leaves (Akeel et al., 2019), photosynthetic pigments, and phenolic compounds of banana seedlings (Aremu et al., 2012). Plant height, net photosynthetic rate, β-carotene, and ascorbic acid were enhanced in carrot by KAR1 treatments (Akeel et al., 2019). The mystery that how karrikins affect the plant growth, mechanism of their action, and whether this effect is regulated exogenously or endogenously is yet to be explored; however, they have been firmly recognized as an important family of naturally occurring plant growth regulators (Chiwocha et al., 2009).

## 3 Morphological and Physiological Responses of Plants to Plant-Derived Smoke Solution

### *3.1 Seed Germination*

Smoke solution positively affected seed germination of fire-free and fire-prone habitats species (Pierce et al., 1995) and plant species of the different regions of the world. Plant-derived smoke broke seed dormancy of California chaparral plants (Keely & Fotheringham, 1998), Southwestern Australian species (Tieu et al., 2001), celery (Thomas & van Staden, 1995), lettuce (Strydom et al., 1996), and wild oat (Kepczynski et al., 2010). Karrikins elicit germination of many plant species from fire-prone environment (Dixon et al., 2009), non-fire-prone (Merritt et al., 2007), and crops (Maurya et al., 2014). Another major germination stimulant was isolated from plant-derived smoke, known as cyanohydrin. Several related cyanohydrins, which are mandelonitrile, acetone cyanohydrin, glycolonitrile, and 2, 3, 4-trihydroxybutyronitrile, stimulated seed germination of different plant species (Flematti et al., 2013). The active nature of these compounds is due to the spontaneous release of cyanide, suggesting an ecological role of cyanide in the postfire revival of plant communities (Flematti et al., 2011a). These studies proved that cyanide is an important germination stimulant in postfire environments. 3, 4, 5-trimethyl-2(5H)-furanone significantly reduced germination-promoting nature of smoke-derived karrikins (Light et al., 2010). Additionally, 5, 5-dimethylfuran-2(5H)-one and (5RS)-5-ethylfuran-2(5H)-o isolated from *Passerina vulgaris* and *Themeda triandra* smoke solutions inhibited seed germination (Burger et al., 2018). Smoke has both promotive and inhibitory compounds, but overall impact of plant-derived smoke is positive on seed germination.


**Fig. 2** Effect of different concentration of plant-derived smoke solution on seed germination and seedling length of maize. Maize seeds were germinated in Petri plates and treated with 10,000, 2000, and 1000 ppm plant-derived smoke solution for 7 days

### *3.2 Plant Growth*

Plant-derived smoke solution is mostly studied at morphological and physiological level of plant growth and development. The morphological growth parameters are significantly enhanced by application of smoke or the derived active compounds from smoke. Positive effect of smoke solution is not dependent on seed size, plant species, genera, and families belonging to gymnosperms and angiosperms (van Staden et al., 2004), commercial crops, and different medicinal plants (Abdollahi et al., 2011). Beside these, few compounds, which are present in higher concentration in smoke solution, have inhibitory effects on plant growth (Daws et al., 2007). Plant-derived smoke has a positive influence on seedling growth of several plants including capsicum, salvia (Demir et al., 2012), wheat (Aslam et al., 2015), maize (Waheed et al., 2016; Aslam et al., 2017), rice (Jamil et al., 2014; Malook et al., 2017), chickpea (Rehman et al., 2018), sorghum (Pirzada et al., 2014), and soybean (Li et al., 2018; Otori et al., 2021). High concentration of smoke had inhibitory effects on maize seedling length, while seeds treated with 2000 ppm smoke solution had promotive effect on seed germination and seedling growth as compared to 1000 and 10,000 ppm (Fig. 2). These results highlight the positive role of plant-derived smoke solution on plant species belonging to different genera and families.

## 4 Biochemical Responses of Plants to Plant-Derived Smoke Solution

Smoke solution significantly enhanced biochemical characteristics of plants, which in turn increased vegetative and reproductive growth (Table 2).

**Table 2** Positive response of different biochemical parameters of various plant species to plant-derived smoke (PDS) solution

| Application type | Biochemical growth parameter studied | References |
|---|---|---|
| Tomato | | |
| Butenolide | Embryo, cotyledons, and seedling proteins | Jain et al. (2008) |
| Smoke and butenolide | Improved nutritional composition | Kulkarni et al. (2008) |
| PDS | Reduced abscisic acid content, enhanced α-amylase activity, ion contents, photosynthetic pigments | Elsadek and Yousef (2019) |
| Wild garlic | | |
| PDS | Stimulated secondary metabolites | Aremu et al. (2014) |
| Rice | | |
| PDS | Proline contents, photosynthetic pigments, zinc, lead, cadmium | Jamil et al. (2013) |
| PDS | $Na^+$ and $K^+$ ions | Malook et al. (2017) |
| PDS | Ions uptake, cellular injury, stability of plasma membrane, total soluble protein (TSP), and nitrogen contents | Malook et al. (2014) |
| PDS | Electrolytes ($Ca^{+2}$, $Na^{+2}$, $K^+$) uptakes, cell injury, protein and nitrogen (N) uptake, Pb uptake, TSP, total soluble sugar (TSS), proline analysis, antioxidant enzymes, glycine-betaine (GB), hydrogen peroxidase | Akhtar et al. (2017) |
| PDS | Chlorophyll contents, ionic contents, nitrogen and protein contents, | Jamil et al. (2014) |
| PDS and PGPR-solution | Chlorophyll contents, electrolyte ($Ca^{+2}$, $Na^{+2}$, $K^+$) uptake, TSP, proline content, TSS, peroxidases, and catalytic activities | Khan et al. (2017) |
| PDS | Carbohydrates contents, lipid and protein study, micro and macro elemental concentration | Jamil et al. (2020) |
| Wheat | | |
| PDS | Water potential, relative water content (RWC), osmotic potential, membrane stability index (MSI), chlorophyll contents, proline, free amino acids, sugar | Iqbal et al. (2018) |
| Kikuyu plant (grasses) | | |
| PDS/ butenolide | Uptake of Cd | Okem et al. (2015) |
| Red sage | | |
| PDS/butenolide | Accumulation of salvianolic acid and rosmarinic acid, biosynthesis of phenolic acids | Zhou et al. (2018a) |
| PDS/ karrikinolide | Tanshinone I, tanshinone IIA, cryptotanshinone, dihydrotanshinone I contents | Zhou et al. (2018b) |
| Dyer's woad | | |
| PDS | Amount of indigo | Zhou et al. (2011) |
| PDS | Photosynthate yield, photosynthetic pigment fluorescence | Zhou et al. (2014) |

(continued)

**Table 2** (continued)

| Application type | Biochemical growth parameter studied | References |
|---|---|---|
| Grapes | | |
| Pre- and post-smoke treatments | Photosynthesis rate, stomatal movement, rate of transpiration | Bell et al. (2013) |
| PDS/growth-promoting bacteria | Chlorophyll relative content, movement of stomata conductance, breakdown of lipids, sesquiterpenes biosynthesis | Salomon et al. (2017) |
| Papaya | | |
| PDS | Nitrogen, ion contents, iron, zinc, copper, chlorophyll content | Chumpookam et al. (2012) |
| Tree-aloe | | |
| PDS/ butenolide | Phytochemicals including flavonoid and total phenolic contents | Kulkarni et al. (2013) |
| Bone seed | | |
| PDS spray | Opening and closing of stomata, assimilation of $CO_2$, $CO_2$ levels in cellular spaces | Gilbert and Ripley (2002) |
| Banana | | |
| PDS/ karrikin | Chlorophyll contents, total phenolic contents, total flavonoid contents, proanthocyanidins | Aremu et al. (2012) |
| Earleaf nightshade, Fameflower, Horseweed, Flatweed | | |
| PDS/butenolide/ trimethyl butenolide | Activity of α–amylase | Papenfus et al. (2015a) |
| Okra | | |
| Smoke/ butenolide/ trimethyl butenolide | Bacterial abundance, α-amylase assay | Papenfus et al. (2015b) |
| Maize | | |
| Priming in smoke | Ion uptakes, antioxidant enzymes, chlorophyll contents | Waheed et al. (2016) |
| PDS | Photosynthetic contents, TSP | Aslam et al. (2017) |
| Lettuce | | |
| PDS | TSS | Jäger and van Staden (2002) |
| PDS/butenolide/ trimethyl butenolide | α-amylase assay, TSS, starch, TSP, lipase assay, lipid uptakes | Gupta et al. (2019) |
| Cockspur grass | | |
| PDS | ABA uptakes, α-amylase | Kamran et al. (2014) |
| Cucumber, Pot marigold, Hybrid gladiolus | | |
| PDS | Photosynthetic pigments, activity of α-amylase, ABA content, N, P, and K ions | Elsadek and Yousef (2019) |

(continued)

**Table 2** (continued)

| Application type | Biochemical growth parameter studied | References |
|---|---|---|
| Oat | | |
| PDS | α- and β-amylase assay, starch, β-tubulin concentration | Cembrowska-Lech and Kepczynski (2017) |
| Blue lupin | | |
| PDS at different temperature | Percentage of ion leakage, dehydrogenase, amylolytic activity, gibberellin content, conjugated, free abscisic acid content | Płazek et al. (2018) |
| Chickpea | | |
| PDS | TSP, TSS, number of rhizobial growth | Rehman et al. (2018) |
| Soybean | | |
| Flooding and PDS | ATPase abundance, ATP contents, ascorbate peroxidase, peroxiredoxin, glutathione reductase, nitric oxide contents | Otori et al. (2021) and Zhong et al. (2020) |

## *4.1 Photosynthesis*

Photosynthetic pigments are the key elements in the process of photosynthesis. Plant-derived smoke has positive effects on the health and growth of plant seedling resulting better photosynthetic phenomena (Baxter & van Staden, 1994). Plant-derived smoke increased the activities of enzymes related with photosynthesis process. Smoke water increased transpiration rate, photosynthetic rate, and stomatal movements in dyer's wood seedlings, suggesting that smoke-solution treatments could enhance the photosynthesis (Zhou et al., 2014). Plant-derived smoke treatments increased chlorophyll a/b and total carotenoids activities in rice seedling which are primarily involved in the process of photosynthesis (Jamil et al., 2013; Malook et al., 2014). These results indicate that plant-derived smoke is positively regulating the process of photosynthesis in different plant species.

## *4.2 Phenol and Flavonoids*

Phenolic compounds protect plants from the harmful effects of ultraviolet radiation (Bieza & Lois, 2001). Presence of flavonoids and phenolic compounds is necessary for plant protection against stresses (Pourmorad et al., 2006; De Klerk et al., 2011) and helpful in hormonal transport (Peer & Murphy, 2007). Flavonoids have important role in the rhizobium legume development and different soil-plant microbe interactions (Taylor & Grotewold, 2005). Amount of secondary metabolites such as flavonoid and total phenolic content increased in plant after the application of smoke

solution (Aremu et al., 2014). Smoke solution stimulated phenyl propanoid pathway and flavonoid-related genes by enhancing phenolic biosynthesis (Soós et al., 2010; Rehman et al., 2018; Savio et al., 2011). Furthermore, compounds including indigo phenolic, flavonoids, and condensed tannins significantly increased by plant-derived smoke solution treatments in different plants including dyer's wood (Zhou et al., 2011; Aremu et al., 2012), krantz aloe (Kulkarni et al., 2013), and wild garlic (Jamil et al., 2013). Plant-derived smoke treatment increased phenolic and flavonoids contents in wild garlic (Aremu et al., 2014). These results provide clear evidence about the involvement of plant-derived smoke treatment in enhancement of different secondary metabolites in plants.

### *4.3 Other Biochemical Changes*

Smoke dilution significantly increased levels of nitrogen contents in seedling and magnesium in shoot (Chumpookam et al., 2012). Karrikins influenced the metabolism and production of plant hormones (Flematti et al., 2004; van Staden et al., 2004; Chiwocha et al., 2009). Burned soil has more nitrate than unburned soil, but nitrate is not sufficient to reproduce smoke solution effects (Thanos & Rundel, 1995). Nitrite and nitrate are useful in breaking dormancy of whispering bells at acidic pH levels, and it is concluded that oxidizing gases in smoke and/or acids play a role in germination of postfire annuals in chaparral (Keeley & Fotheringham, 1998). Smoke solution treatments did not change size, weight, and nutritional composition of ascorbic acid, lycopene, β-carotene, and total soluble solids in tomato fruit (Kulkurni et al., 2008). Furthermore, they are associated with developmental processes as pollen germination, auxin transport, root hair growth, and allelopathic responses (Taylor & Grotewold, 2005). Plant-derived smoke solution enhanced the absorption of growth nutrients (Jamil et al., 2013), leading to an accelerated protein biosynthesis and other growth-related phenomena, which ultimately resulted in a move on plant growth. Different plant species are tested for biochemical growth parameter and have shown positive responses to plant-derived smoke solution. The results provide a clear statement about the enhancement of biochemical growth parameters of different plant species in response to plant-derived smoke solution.

## 5 Molecular Responses of Plants to Plant-Derived Smoke Solution

Plant growth processes are controlled by signaling molecules which in turn may act singly or in coordination with other molecules, both endogenously and exogenously, which resulted in variety of responses (Table 3).

**Table 3** Molecular response of different plant species to plant-derived smoke solution (PDS) solution

| Application type | Response of plants species to PDS solution at molecular level | References |
|---|---|---|
| Tomato | | |
| Butenolide | Butenolide has no negative effects on the structure configuration gene, DNA, RNA, and proteins profile | Jain et al. (2008) |
| Lettuce | | |
| PDS | Genes associated to seed germination, metabolic reactions, and hormone (ABA) were altered by PDS | Soos et al. (2009a) |
| Karrikins | Smoke-derived compound karrikins suppressed seed dormancy and ABA associated genes while expressed by trimethyle butenolide | Soos et al. (2012) |
| Maize | | |
| PDS | PDS treatments increased growth and resistance against stresses in plants | Soos et al. (2009b) |
| PDS and karrikins | Genes related to aquaporins and proteins degradation were triggered by PDS solution | Soos et al. (2010) |
| PDS | PDS has positive effects on genes associated with metabolic processes | Aslam et al. (2019) |
| Arabidopsis | | |
| Karrikin | Karrikins have an exciting ecological role on post-germination attributes of plants | Nelson et al. (2010) |
| Karrikin and strigolactone | Mechanistic and genetic links between karrikin and strigolactone signals | Nelson et al. (2011) |
| Karrikin and strigolactone | D14 and KAI2 proteins allow plants to recognized/ karrikins and strigolactones to elicit appropriate/ developmental responses | Waters et al. (2012) |
| Karrikin | Elucidation of the KAI2 structure, which is valuable insight into its involvement in different pathways; structure of karrikin insensitive protein KA12 | Bythell-Douglas et al. (2013) |
| Karrikin | KAI2c proteins recognize KAI2 ligand | Conn and Nelson (2016) |
| Karrikins | Structural basis for placing KAI2 within the signal transduction pathway associated with the perception of smoke-derived chemical signals such as KAR 1 | Guo et al. (2013) |
| Karrikins and strigolactones | HY5 is not essential for the perception of strigolactones or karrikins. Different butenolides are functionally discriminated by KAI2 and AtD14. | Waters and Smith (2013) |
| Karrikins | Deeper study of KAI2 function help to understand karrikin action and evolution | Waters et al. (2013) |
| Strigolactones/ karrikins | Signal transduction pathways and downstream targets | Smith and Li (2014) |
| Karrikins | Analysis provided novel indications of a chloroplast-based signaling pathway that works in parallel to KAI2-mediated karrikin signaling | Baldrianova et al. (2015) |
| Red sage | | |
| PDS and karrikin | PDS and KAR1 treatments have positive effects on secondary metabolites and help in the biosynthesis of liposoluble tanshinones | Zhou et al. (2018b) |

(continued)

**Table 3** (continued)

| Application type | Response of plants species to PDS solution at molecular level | References |
|---|---|---|
| Rice | | |
| Strigolactone and karrikins | D14 and D14L gene structure analyses | Kagiyama et al. (2013) |
| Chickpea | | |
| PDS | Activation of metabolic pathways by PDS to enhance growth | Rehman et al. (2018) |
| Soybean | | |
| PDS | PDS reduces flooding stress by accumulating structural and metabolic proteins after flooding stress in soybean | Li et al. (2018) |
| | PDS promotes soybean root growth by eliminating ROS and produces ATPs under flooding stress | Otori et al. (2021) |
| | PDS normalizes nitrogen-carbon conversion by regulating ornithine synthesis pathway | Zhong et al. (2020) |

## *5.1 Plant-Derived Smoke*

Plant-derived smoke application altered cell wall expansion, seed germination, cell division, carbohydrates metabolism, translation regulation, and abscisic acid regulation genes in lettuce seeds (Soos et al., 2009b). They concluded that smoke solution increase cell division and food mobilization (Soos et al., 2009b). Inanother experiment, they observed that stress and abscisic acid responsive genes were overexpressed in smoke solution-treated maize seedlings (Soos et al., 2009a). It is possible that higher concentration of abscisic acid may result in a better adaptation to abiotic stress factors during seed germination (Soos et al., 2009a).

The proteomic results depicted a significant increase in proteins related to signaling and transport; however, protein metabolism, cell, and cell wall-related proteins decreased in chickpea (Rehman et al., 2018). Furthermore, fructose bisphosphate aldolase increased, and genes related to phosphor-glyceraldehyde 3 phosphate dehydrogenase and glutamine synthetase were upregulated. This increase in plant growth-related proteins and genes is strengthening the fact that plant-derived smoke improves early stage of growth in chickpea with the balance of many cascades such as glycolysis, redox homeostasis, and secondary metabolism (Rehman et al., 2018). Proteomic analyses confirm that signaling, and nucleotide binding proteins were decreased while sucrose synthase, glutathione, and nucleotides related proteins were significantly increased in chickpea by plant-derived smoke. A decrease in cell wall, lipids, photosynthesis, and amino acid degradations related proteins was observed which supports the molecular-level involvement of plant-derived smoke in plants. Proteomic analyses confirmed that proteins related to sucrose synthase, nucleotides, signaling, and glutathione were significantly increased whereas lipids, photosynthetic, cell wall, and amino acid degradation proteins were decreased by plant-derived smoke treatments (Aslam et al., 2019). These results suggest that the key processes in plant life, including photosynthesis, protein synthesis, nucleotide synthesis, and food mobilization, are enhanced by plant-derived smoke, which may lead to better plant growth.

Plant-derived smoke is known to alleviate the negative effects of different stresses (Malook et al., 2017). Soybean seedling growth was inhibited under flooding stress but recovered after treatment with plant-derived smoke (Li et al., 2018). Sucrose/starch metabolism and glycolysis-related protein were suppressed, while proteins related to the cell wall and peptidyl-prolyl cis-trans isomerase were higher in smoke-treated flooded soybean (Li et al., 2018). These results suggest that plant-derived smoke enhances soybean growth during recovery from flooding stress through the balance of sucrose/starch metabolism, glycolysis, and cell wall-related protein (Li et al., 2018). Furthermore, plant-derived smoke altered metabolites related to amino acid, carboxylic acids, and sugars. It regulated nitrogen-carbon transformation through ornithine synthesis pathway and promoted soybean normal growth (Zhong et al., 2020). These changes in proteins and accumulation of metabolites enabled lateral root development during soybean recovery after flooding (Zhong et al., 2020). Additionally, plant-derived smoke treatment improves soybean root growth under flooding stress through energy production and reactive oxygen scavenging even under flooding stress (Otori et al., 2021). These results suggested that plant-derived smoke has the potential to protect plant from stress condition.

## *5.2 Karrikins*

In *Arabidopsis* seeds, KAR1 treatment enhanced the expression of light-responsive genes including ABRE-like promoter motif and putative HY5-binding targets (Nelson et al., 2010). These evidence clarify the role of KAR1 in light responses in a synergistic way so as to affect postfire ecology. The pattern of protein ubiquitination and gene expression was noted to be different in response to KAR1 and smoke treatment (Soós et al., 2010). Protein ubiquitination was enhanced, and protein degradation-related genes were activated in smoke solution treatments, while KAR1 significantly upregulated an aquaporin gene (Soós et al., 2010). Plant species have shown different growth responses to karrikins because karrikins required specific conditions for action such as continuous intensity of light and the presence of phytochromes (Nelson et al., 2010). These results suggest that karrikins improved seed germination and seedling growth with varying level of response in different plants species.

Karrikins present in smoke derived from burned plant materials promoted seed germination, but the mechanism of karrikins action is still unclear (Nelson et al., 2011; Waters et al., 2012). Proteomic approach was used to identify early karrikin-responsive proteins and presented clear model of karrikin action in *Arabidopsis* seedlings (Baldrianova et al., 2015). All of the identified proteins were karrikin-responsive except HSP70-3, and most of the proteins were located in chloroplast (Baldrianova et al., 2015). These karrikin-response specific proteins were photosynthetic, carbohydrate metabolism, redox homeostasis, transcription control, protein transport, and processing/protein degradation-related proteins (Baldrianova et al., 2015). These findings strengthen the view point that karrikins affect the growth

processes in plant through regulation of various molecular level responses, which ultimately lead to quickening of plant growth.

## 6 Concluding Remarks

Plant-derived smoke induces various cellular and molecular level responses in plants. After the perception of karrikins by its receptors, the α/β-hydrolase KAI2 (Karrikin Insensitive 2) results in a conformational change in KAI2. Subsequently, the activated KAI2 interacts with MAX2 (*MORE AUXILIARY GROWTH2*), which is an F-box protein, resulting in a Skp-Cullin-F-box (SCF) ubiquitin ligase complex containing SMAX1 (SUPPRESSOR OF MAX2 1). SMAX1 is considered as a putative substrate in the KAI2-SCFMAX2 complex, which is degraded in 26S proteasome after polyubiquitination (Guo et al., 2013). These changes lead to the enhancement of seed germination, hypocotyl elongation, and other growth processes (Table 3 and Fig. 3). The cell membrane stability increased through decreased nutrient leakage from the cell membrane and increased hydraulic conductivity, further equipping the cell against abiotic stress (Akhtar et al., 2017). Photosynthesis-related proteins increased (Rehman et al., 2018), resulting in increased growth of

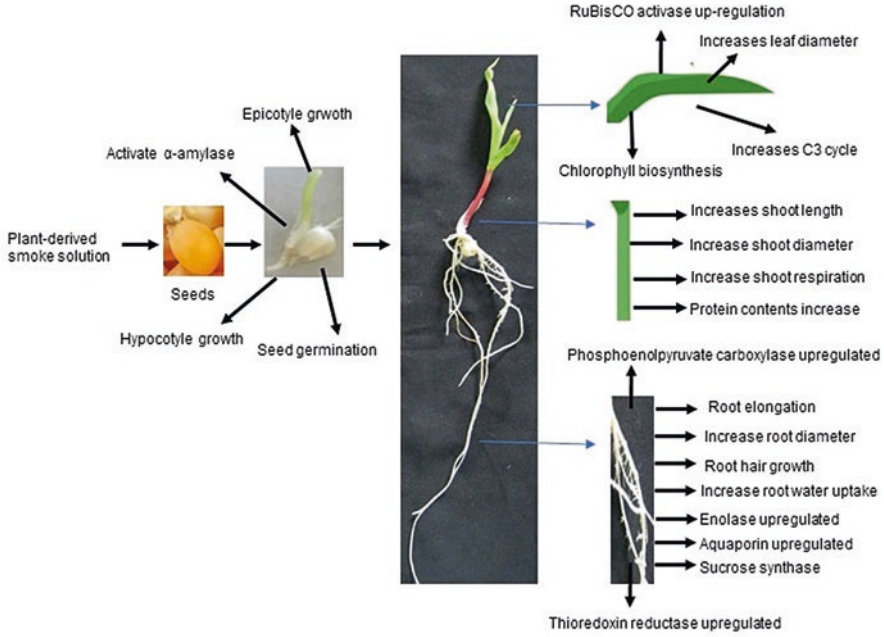



**Fig. 3** A summary of physiological, biochemical, and molecular responses of various organs of plant toward plant-derived smoke solution. Various biochemical and molecular changes finally lead to increased growth and vigor of plant

plants (Akeel et al., 2019; Iqbal et al., 2018). This research identifies that plant-derived smoke promotes the plant-growth processes. This is done by not only influencing the growth itself but also strengthening defenses against abiotic stresses and by affecting the cell membrane and its hydraulic conductivity. These factors are enhanced in plants treated with plant-derived smoke.

## References

Abdollahi, M. R., Mehrshad, B., & Moosavi, S. S. (2011). Effect of method of seed treatment with plant derived smoke solutions on germination and seedling growth of milk thistle (*Silybum marianum* L.). *Seed Science and Technology, 39*, 225–229.

Akeel, A., Khan, M. M. A., Jaleel, H., & Din, U. M. (2019). Smoke-saturated water and karrikinolide modulate germination, growth, photosynthesis and nutritional values of carrot (*Daucus carota* L.). *Journal of Plant Growth Regulation, 38*, 1387–1401.

Akhtar, N., Khan, S., Malook, I., Rehman, S. U., & Jamil, M. (2017). Pb-induced changes in roots of two cultivated rice cultivars grown in lead-contaminated soil mediated by smoke. *Environmental Science and Pollution Research, 24*, 21298–21310.

Aremu, A. O., Bairu, M. W., Finnie, J. F., & van Staden, J. (2012). Stimulatory role of smoke-water and karrikinolide on the photosynthetic pigment and phenolic contents of micro-propagated 'Williams' bananas. *Plant Growth Regulation, 67*, 271–279.

Aremu, A. O., Masondo, N. A., & van Staden, J. (2014). Smoke–water stimulates secondary metabolites during in vitro seedling development in *Tulbaghia species*. *South African Journal of Botany, 91*, 49–52.

Aslam, M. M., Jamil, M., Khatoon, A., El-Hendawy, S. E., Al-Suhaibani, N. A., Shakir, S. K., Malook, I., & Rehman, S. U. (2015). Does weeds-derived smoke improve plant growth of wheat? *Journal of Bio-Molecular Sciences, 3*, 86–96.

Aslam, M. M., Jamil, M., Khatoon, A., Hendawy, S. E., AL-Suhaibani, N. A., Malook, I., & Rehman, S. U. (2017). Physiological and biochemical responses of maize (*Zea mays* L.) to plant derived smoke solution. *Pakistan Journal of Botany, 49*, 435–443.

Aslam, M. M., Rehman, S. U., Khatoon, A., Jamil, M., Yamaguchi, H., Hitachi, K., Tsuchida, K., Sunohara, Y., Matsumoto, H., & Komatsu, S. (2019). Proteomic analysis of the effect of plant-derived smoke on maize growth. *International Journal of Molecular Sciences, 20*, 1319.

Baldrianova, J., Cerny, M., Novák, J., Jedelsky, P., & Brzobohaty, B. (2015). Arabidopsis proteome responses to the smoke-derived growth regulator karrikin. *Journal of Proteomics, 120*, 7–20.

Baldwin, I. T., & Morse, L. (1994). Up in smoke. 2. Germination of Nicotiana attenuata in response to smoke-derived cues and nutrients in burned and unburned soils. *Journal of Chemical Ecology, 20*, 2373–2391.

Baxter, B. J. M., & van Staden, J. (1994). Plant-derived smoke: An effective seed pre-treatment. *Plant Growth Regulation, 14*, 279–282.

Baxter, B. J. M., van Staden, J., Granger, E. J., & Brown, N. A. C. (1994). Seed germination of the fire-climax grass *Themeda triandra*. *Environmental and Experimental Botany, 34*, 217–223.

Bell, T. L., Stephens, S., & Moritz, M. A. (2013). Short-term physiological effects of smoke on grapevine leaves. *International Journal of Wildland Fire, 22*, 933–946.

Bieza, K., & Lois, R. (2001). An Arabidopsis mutant tolerant to lethal ultraviolet-B levels shows constitutively elevated accumulation of flavonoids and other phenolics. *Plant Physiology, 126*, 1105–1115.

Bose, U., Juhász, A., Broadbent, J. A., Komatsu, S., & Colgrave, M. L. (2020). Multi-omics strategies for decoding smoke-assisted germination pathways and seed vigour. *International Journal of Molecular Sciences, 21*, 7512. https://doi.org/10.3390/ijms21207512

Brown, N. A. C., van Staden, J., Daws, M. I., & Johnson, T. (2003). Patterns in the seed germination response to smoke in plants from the Cape Floristic Region, South Africa. *South African Journal of Botany, 69*, 514–525.

Burger, B. V., Poštac, M., Lightb, M. E., Kulkarni, M. G., Viviersa, M. Z., & van Staden, J. (2018). More butenolides from plant-derived smoke with germination inhibitory activity against karrikinolide. *South African Journal of Botany, 115*, 256–263.

Bythell-Douglas, R., Waters, M. T., Scaffidi, A., Flematti, G. R., Smith, S. M., & Bond, C. S. (2013). Structure of karrikin insensitive protein KA12 in *Arabidopsis thaliana*. *PLoS One, 8*, e54758.

Cembrowska-Lech, D., & Kępczyńsk, J. (2017). Plant-derived smoke induced activity of amylases, DNA replication and β-tubulin accumulation before radicle protrusion of dormant Avena fatua L. caryopses. *Acta Physiologiae Plantarum, 39*, 39–50.

Chiwocha, S. D. S., Dixon, K. W., Flematti, G. R., Ghisalberti, E. L., Merritt, D. J., Nelson, D. C., Riseborough, J. M., Smith, S. M., & Stevens, J. C. (2009). Karrikins: A new family of plant growth regulators in smoke. *Plant Science, 177*, 252–256.

Chumpookam, J., Lin, H. L., Shiesh, C. C., & Ku, K. L. (2012). Effect of smoke-water on seed germination and resistance to *Rhizoctonia solani* inciting papaya damping-off. *Horticultural Science, 37*, 13–29.

Commander, L. E., Merritt, D. P., Flematti, G. R., & Dixon, K. W. (2008). Seed germination of *Solanum species* (Solanaceae) for use in rehabilitation and commercial industries. *Australian Journal of Botany, 56*, 333–341.

Conn, C. E., & Nelson, D. C. (2016). Evidence that KARRIKIN-INSENSITIVE2 (KAI2) receptors may perceive an unknown signal that is not Karrikin or Strigolactone. *Frontiers in Plant Science, 6*, 1219.

Curir, P., Vansumere, C. F., Termini, A., Barthe, P., Marchesini, A., & Dolci, M. (1990). Flavonoid accumulation is correlated with adventitious roots formation in *Eucalyptus gunnii* Hook micropropagated through axillary bud stimulation. *Plant Physiology, 92*, 1148–1153.

Daws, M. I., Davies, J., Pritchard, H. W., Brown, N. A. C., & van Staden, J. (2007). Butenolide from plant-derived smoke enhances germination and seedling growth of arable weed species. *Plant Growth Regulation, 51*, 73–82.

De Klerk, G. J., Guan, H., Huisman, P., & Marinova, S. (2011). Effects of phenolic compounds on adventitious root formation and oxidative decarboxylation of applied indoleacetic acid in Malus 'Jork9'. *Plant Growth Regulation, 63*, 175–185.

de Lange, J. H., & Boucher, C. (1990). Auto ecological studies on *Audinia capitata* (Bruniaceaae), plant-derived smoke as a germination cue. *South African Journal of Botany, 56*, 188–202.

Demir, I., Ozuaydin, F., Yasar, J., & van Staden, J. (2012). Effect of smoke-derived butenolide priming treatment on pepper and salvia seeds in relation to transplant quality and catalase activity. *South African Journal of Botany, 78*, 83–87.

Dixon, K. W., Merritt, D. J., Flematti, G. R., & Ghisalberti, E. L. (2009). Karrikinolide-a phytoreactive compound derived from smoke with applications in horticulture, ecological restoration and agriculture. *Acta Horticulturae, 813*, 155–170.

Egerton-Warburton, L. (1998). A smoke-induced alteration of the sub-testa cuticle in seeds of the post-fire recruiter, *Emmenanthe penduliflora* Benth. (Hydrophyllaceae). *Journal of Experimental Botany, 49*, 1317–1327.

Elsadek, M. A., & Yousef, E. A. A. (2019). Smoke-water enhances germination and seedling growth of four horticultural crops. *Plants, 8*, 104. https://doi.org/10.3390/plants8040104

Flematti, G. R., Ghisalberti, E. L., Dixon, K. W., & Trengove, R. D. (2004). A compound from smoke that promotes seed germination. *Science, 305*, 977.

Flematti, G. R., Goddard-Berg, E. D., Merritt, D. J., Ghisalberti, E. L., Dixon, K. W., & Trengove, R. D. (2007). Preparation of 3-methyl-2H-furo[2,3-c]pyran-2-one derivatives and evaluation of their germination-promoting activity. *Journal of Agricultural and Food Chemistry, 55*, 2189–2194.

Flematti, G. R., Merritt, D. J., Piggott, M. J., Trengove, R. D., Smith, S. M., Dixon, K. W., & Ghisalberti, E. L. (2011a). Burning vegetation produces cyanohydrins that liberate cyanide and promote seed germination. *Nature Communications, 2*, 360.

Flematti, G. R., Scaffidi, A., Dixon, K. W., Smith, S. M., & Ghisalberti, E. L. (2011b). Production of the seed germination stimulant karrikinolide from combustion of simple carbohydrates. *Journal of Agricultural and Food Chemistry, 59*, 1195–1198.

Flematti, G. R., Watersb, M. T., Scaffidia, A., Merrittc, M. T., Emilio, D., Ghisalbertia, L., Dixon, K. D., & Smith, S. M. (2013). Karrikin and cyanohydrin smoke signals provide clues to new endogenous plant signaling compounds. *Molecular Plant, 6*, 29–37.

Gilbert, M. E., & Ripley, B. S. (2002). The effect of smoke on the photosynthetic gas exchange of *Chrysanthemoides monilifera*. *South African Journal of Botany, 68*, 525–531.

Guo, Y., Zheng, Z., Clair, J. J. L., Chory, J., & Noel, J. P. (2013). Smoke-derived karrikin perception by the α/β-hydrolase KAI2 from Arabidopsis. *Proceedings of the National Academy of Sciences, 110*, 8284–8289.

Gupta, S., Plačková, L., Kulkarni, M. G., Dolezal, K., & van Staden, J. (2019). Role of smoke stimulatory and inhibitory biomolecules in phytochrome-regulated seed germination of *Lactuca sativa*. *Plant Physiology, 181*, 458–470.

Iqbal, M., Asif, S., Ilyas, N., Hassan, F. U., Raja, N., Hussain, M., Ejaz, M., & Saira, H. (2018). Smoke produced from plants waste material elicits growth of wheat (*Triticum aestivum* L.) by improving morphological, physiological and biochemical activity. *Biotechnology Reports, 17*, 35–44.

Jäger, A. K., & van Staden, J. (2002). Soluble sugars in light-sensitive Grand Rapids lettuce seeds treated with red light, gibberellic acid and a plant-derived smoke extract. *South African Journal of Botany, 68*, 404–407.

Jain, N., Soos, V., Balazs, E., & van Staden, J. (2008). Changes in cellular macromolecules (DNA, RNA and protein) during seed germination in tomato, following the use of a butenolide, isolated from plant derived smoke. *Plant Growth Regulation, 54*, 105–113.

Jamil, M., Malook, I., Parveen, S., Naz, T. M., Ali, A., Jan, S. U., & Rehman, S. U. (2013). Smoke priming, a potent protective agent against salinity: Effect on proline accumulation, elemental uptake, pigmental attributes and protein banding patterns of rice (*Oryza sativa*). *The Journal of Stress Physiology & Biochemistry, 9*, 169–183.

Jamil, M., Kanwal, M., Aslam, M. M., Khan, S. S., Malook, I., Tu, J., & Rehman, S. U. (2014). Effect of plant-derived smoke priming on physiological and biochemical characteristics of rice under salt stress condition. *Australian Journal of Crop Science, 8*, 159–170.

Jamil, M., Jahangir, M., & Rehman, S. U. (2020). Smoke induced physiological, biochemical and molecular changes in germinating rice seeds. *Pakistan Journal of Botany, 52*, 865–871.

Kagiyama, M., Hirano, Y., Mori, T., Kim, S. Y., Kyozuka, J., Seto, Y., Yamaguchi, S., & Hakoshima, T. (2013). Structures of D14 and D14L in the strigolactone and karrikin signaling pathways. *Genes to Cells, 18*, 147–160.

Kamran, M., Khan, A. L., Waqas, M., Imran, Q. M., Hamayun, M., Kang, S. M., Kim, Y. H., Kim, M. J., & Lee, I. J. (2014). Effects of plant-derived smoke on the growth dynamics of Barnyard Grass (*Echinochloa crusgalli*). *Acta Agriculturae Scandinavica Section B Soil and Plant Science, 64*, 121–128.

Kamran, M., Khan, A. L., Ali, L., Hussain, J., Waqas, M., Al-Harrasi, A., Imran, Q. M., Kim, Y. H., Kang, S. M., Yun, B. K., & Lee, I. J. (2017). Hydroquinone; A novel bioactive compound from plant-derived smoke can cue seed germination of lettuce. *Frontiers in Chemistry, 5*, 30.

Keeley, J. E., & Fotheringham, C. J. (1998). Mechanism of smoke-induced seed germination in a post-fire chaparral annual. *Journal of Ecology, 86*, 27–36.

Keely, J. E., & Fotheringham, C. J. (1998). Smoke-induced seed germination in *Californian chaparral*. *Ecology, 79*, 2320–2336.

Kepczynski, J., Cembrowska, D., & van Staden, J. (2010). Releasing primary dormancy in *Avena fatua* L. caryopses by smoke-derived butenolide. *Plant Growth Regulation, 62*, 85–91.

Khan, M. H. U., Khattak, J. Z. K., Jamil, M., Malook, I., Khan, S. U., Jan, M., Din, I., Saud, S., Kamran, M., Alharby, H., & Fahad, S. (2017). *Bacillus safensis* with plant-derived smoke stimulates rice growth under saline conditions. *Environmental Science and Pollution Research, 24*, 23850–23863.

Khatoon, A., Rehman, S., Aslam, M. M., Jamil, M., & Komatsu, S. (2020). Plant-derived smoke affects biochemical mechanism on plant growth and seed germination. *International Journal of Molecular Sciences, 21*, 7760.

Kulkarni, M. G., Amoo, S. O., Kandari, L. S., & van Staden, J. (2013). Seed germination and phytochemical evaluation in seedlings of *Aloe arborescens* Mill. *Plant Biosystems, 148*, 460–466.

Kulkurni, M. G., Ascough, G. D., & van Staden, J. (2008). Smoke-water and smoke-isolated butenolide improve growth and yield of tomatoes under greenhouse conditions. *Horticultural Technology, 18*, 449–454.

Li, X., Rehman, S. U., Yamaguchi, H., Hitachi, K., Tsuchida, K., Yamaguchi, T., Sunohara, Y., Matsumoto, H., & Komatsu, S. (2018). Proteomic analysis of the effect of plant-derived smoke on soybean during recovery from flooding stress. *Journal of Proteomics, 181*, 238–248.

Light, M. E., Gardner, M. J., Jager, A. K., & van Staden, J. (2002). Dual regulation of seed germination by smoke solution. *Plant Growth Regulation, 37*, 135–141.

Light, M. E., Daws, M. I., & van Staden, J. (2009). Smoke-derived butenolide: Towards understanding its biological effects. *South African Journal of Botany, 70*, 1–7.

Light, M. E., Burger, B. V., Staerk, D., Kohout, L., & van Staden, J. (2010). Butenolides from plant-derived smoke: Natural plant growth regulators with antagonistic actions on seed germination. *Journal of Natural Products, 73*, 267–269.

Ma, H., Erickson, T. E., & Merritt, D. J. (2018). Seed dormancy regulates germination response to smoke and temperature in a rhizomatous evergreen perennial. *AoB Plants, 10*, 42.

Malook, I., Itlas, A., Rehman, S. U., Weng, W., & Jamil, M. (2014). Smoke alleviates adverse effects induced by stress on rice. *Toxicological and Environmental Chemistry, 96*, 755–767.

Malook, J., Shah, G., Jan, M., Shinwari, K. I., Aslam, M. M., Rehman, S. U., & Jamil, M. (2017). Smoke priming regulates growth and the expression of myeloblastosis and zinc-finger genes in rice under salt stress. *Arabian Journal for Science and Engineering, 42*, 2207–2215.

Maurya, S. K., Srivastava, A., & Garg, S. K. (2014). Karrikin: A seed germination stimulant. *Life Sciences Leaflets, 57*, 86–94.

Merritt, D. J., Turner, S. R., Clark, S., & Dixon, K. W. (2007). Seed dormancy and germination stimulation syndromes for Australian temperate species. *Australian Journal of Botany, 55*, 336–344.

Nelson, D. C., Flematti, G. R., Riseborough, J. A., Ghisalberti, E. L., Dixon, K. W., & Smith, S. M. (2010). Karrikins enhance light responses during germination and seedling development in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences, 2010*(107), 7095–7100.

Nelson, D. C., Scaffidi, A., Dun, E. A., Waters, M. T., Flematti, G. R., Dixon, K. W., Beveridge, C. A., Ghisalberti, E. L., & Smith, S. M. (2011). F-box protein MAX2 has dual roles in karrikin and strigolactone signaling in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences, 108*, 8897–8902.

Nelson, D. C., Flematti, G. R., Ghisalberti, E. L., Dixon, K. W., & Smith, S. M. (2012). Regulation of seed germination and seedling growth by chemical signals from burning vegetation. *Annual Review of Plant Biology, 63*, 107–130.

Okem, A., Kulkarni, M. G., & van Staden, J. (2015). Enhancing phytoremediation potential of *Pennisetum clandestinum* Hochst in cadmium contaminated soil using smoke-water and smoke isolated karrikinolide. *International Journal of Phytoremediation, 17*, 1046–1052.

Otori, M., Murashita, Y., Rehman, S. U., & Komatsu, S. (2021). Smoke on soybean (*Glycine max* L.) root growth under flooding stress. *Plant Molecular Biology Reporter, 39*, 24–33.

Papenfus, H. B., Kulkarni, M. G., Jeffrey, M. P., Finnie, F., & van Staden, J. (2015a). Smoke-isolated trimethyl butenolide inhibits seed germination of different weed species by reducing amylase activity. *Weed Science, 63*, 312–320.

Papenfus, H. B., Kulkarni, M. G., Stirk, W. A., Rengasamy, K. R. R., Salomon, M. V., Piccoli, P., Bottini, R., & van Staden, J. (2015b). Interactions between a plant growth-promoting rhizobia

bacterium and smoke-derived compounds and their effect on okra growth. *Journal of Plant Nutrition and Soil Science, 178*, 741–747.

Paul, B. T., Charles, E. M., & Tony, D. A. (2007). Response surfaces for the combined effects of heat shock and smoke on germination of 16 species forming soil seed bank in South- East Australia. *Austral Ecology, 32*, 605–616.

Peer, W. A., & Murphy, A. S. (2007). Flavonoids and auxin-transport: Modulators or regulators? *Trends in Plant Science, 12*, 556–563.

Pierce, S. M., Esler, K., & Cowling, R. M. (1995). Smoke-induced germination of succulents (*Mesembry anthemaceae*) from fire-prone and fire-free habitats in South Africa. *Oecologia, 102*, 520–522.

Pirzada, K., Shafiq, U. R., Jamil, M., Irfan, S., Waheed, M. A., Aslam, M. M., Kanwal, M., & Shakir, S. K. (2014). Alleviation of boron stress through plant derived smoke extracts in *Sorghum bicolor*. *The Journal of Stress Physiology & Biochemistry, 10*, 153–165.

Płazek, A., Dubert, F., Kopec, P., Dziurka, M., Kalandyk, A., Pastuszak, J., & Wolko, B. (2018). Seed hydro-priming and smoke water significantly improve low-temperature germination of *Lupinus angustifolius* L. *International Journal of Molecular Sciences, 19*, 992.

Pourmorad, F., Hosseinimehr, S. J., & Shahabimajd, N. (2006). Antioxidant activity, phenol and flavonoid contents of some selected Iranian medicinal plants. *African Journal of Biotechnology, 5*, 1142–1145.

Rehman, A., Rehman, S. U., Khatoon, A., Qasim, M., Itohd, T., Iwasakid, Y., Wang, X., Sunohara, Y., Matsumoto, H., & Komatsu, S. (2018). Proteomic analysis of the promotive effect of plant-derived smoke on plant growth of chickpea. *Journal of Proteomics, 176*, 56–70.

Roche, S., Dixon, K. W., & Pate, J. S. (1997). Seed aging and smoke: Partner cue in amelioration of seed dormancy in selected Australian native species. *Australian Journal of Botany, 45*, 783–815.

Salomon, M. V., Piccoli, P., Pinter, I. F., Stirk, W. A., Kulkarni, M., van Staden, J., & Bottini, R. (2017). Bacteria and smoke-water extract improve growth and induce the synthesis of volatile defense mechanisms in *Vitis vinifera* L. *Plant Physiology and Biochemistry, 120*, 1–9.

Savio, L. E. B., Astarita, L. V., & Santarem, E. R. (2011). Secondary metabolism in micropropagated *Hypericum perforatum* L. grown in non-aerated liquid medium. *Plant Cell, Tissue and Organ Culture, 108*, 465–472.

Smith, S. M., & Li, J. (2014). Signaling and responses to strigolactones and karrikins. *Current Opinion in Plant Biology, 21*, 23–29.

Soos, V., Sebestyen, E., Juhasz, A., Pinter, J., Light, M. E., van Staden, J., & Balazs, E. (2009a). Stress-related genes define essential steps in the response of maize seedlings to smoke-water. *Functional & Integrative Genomics, 9*, 231–242.

Soos, V., Juhasz, A., Light, M. E., van Staden, J., & Balazs, E. (2009b). Smoke-water-induced changes of expression pattern in Grand Rapids lettuce achenes. *Seed Science Research, 19*, 37–49.

Soós, V., Sebestyén, E., Juhász, A., Light, M. E., Kohout, L., Szalai, G., Tandori, J., van Staden, J., & Balázs, E. (2010). Transcriptome analysis of germinating maize kernels exposed to smoke-water and the active compound KAR1. *BMC Plant Biology, 10*, 236.

Soos, V., Sebestyen, E., Posta, M., Kohout, L., Light, M. E., van Staden, J., & Balazs, E. (2012). Molecular aspects of the antagonistic interaction of smoke-derived butenolides on the germination process of Grand Rapids lettuce (*Lactuca sativa*) achenes. *The New Phytologist, 196*, 1060–1073.

Strydom, A., Jager, A. K., & van Stade, J. (1996). Effect of a plant-derived smoke extract, N6-benzyladenineand gibberellic acid on the thermo-dormancy of lettuce seeds. *Plant Growth Regulation, 19*, 97–100.

Taylor, L. P., & Grotewold, E. (2005). Flavonoids as developmental regulators. *Current Opinion in Plant Biology, 8*, 317–323.

Thanos, C. A., & Rundel, P. W. (1995). Fire-followers in chaparral: Nitrogenous compounds trigger seed germination. *Journal of Ecology, 83*, 207–216.

Thomas, T. H., & van Staden, J. (1995). Dormancy break of celery (*Apium graveolens* L.) seeds by plant derived smoke extract. *Plant Growth Regulation, 17*, 195–198.

Tieu, A., Dixon, K. W., Menny, K. A., & Sivasithamparam, K. (2001). The interaction of heat and smoke in the release of seed dormancy in seven species from South Western Australia. *Annals of Botany, 88*, 259–265.

Todorovic, S., Giba, Z., Zivkovic, S., Grubisic, D., & Konjevic, R. (2005). Stimulation of empress tree seed germination by liquid smoke. *Plant Growth Regulation, 47*, 141–148.

van Staden, J., Brown, N. A. C., Jäger, A. K., & Johnson, T. A. (2000). Smoke as a germination cue. *Plant Species Biology, 15*, 167–178.

van Staden, J., Jager, A. K., Light, M. E., & Burger, B. V. (2004). Isolation of the major germination cue from plant-derived smoke. *South African Journal of Botany, 70*, 654–659.

Waheed, M. A., Jamil, M., Khan, M. D., Shakir, S. K., & Rehman, S. U. (2016). Effect of plant-derived smoke solutions on physiological and biochemical attributes of maize (*Zea mays* L.) under salt stress. *Pakistan Journal of Botany, 48*, 1763–1774.

Wang, M., Schoettner, M., Xu, S., Paetz, C., Wilde, J., Baldwin, I. T., & Groten, K. (2017). Catechol, a major component of smoke, influences primary root growth and root hair elongation through reactive oxygen species-mediated redox signaling. *New Phytologist, 213*, 1755–1770.

Waters, M. T., & Smith, S. M. (2013). KAI2- and MAX2-mediated responses to karrikins and strigolactones are largely independent of HY5 in Arabidopsis seedlings. *Molecular Plant, 6*, 63–75.

Waters, M. T., Nelson, D. C., Scaffidi, A., Flematti, G. R., Sun, Y. M., Dixon, K. W., & Smith, S. M. (2012). Specialisation within the DWARF14 protein family confers distinct responses to karrikins and strigolactones in Arabidopsis. *Development, 139*, 1285–1295.

Waters, M. T., Scaffidi, A., Flematti, G. R., & Smith, S. M. (2013). The origins and mechanisms of karrikin signaling. *Current Opinion in Plant Biology, 16*, 667–673.

Zhong, Z., Kobayashi, T., Zhu, W., Imai, H., Zhao, R., Ohno, T., Rehman, S. U., Uemura, M., Tian, J., & Komatsu, S. (2020). Plant-derived smoke enhances plant growth through ornithine-synthesis pathway and ubiquitin-proteasome pathway in soybean. *Journal of Proteomics, 221*, 103781.

Zhou, J., van Staden, J., Guo, L. P., & Huang, L. Q. (2011). Smoke-water improves shoot growth and indigo accumulation in shoots of *Isatis indigotica* seedlings. *South African Journal of Botany, 77*, 787–789.

Zhou, J., Fang, L., Wang, X., Guo, L., & Huang, L. (2013). Effects of smoke-water on photosynthetic characteristics of *Isatis indigotica* seedlings. *Sustainable Agriculture Research, 2*, 24. https://doi.org/10.5539/sar.v2n2p24

Zhou, J., Da-Silva, J. A. T., & Ma, G. (2014). Effects of smoke water and karrikin on seed germination of 13 species growing in China. *Central European Journal of Biology, 9*, 1108–1116.

Zhou, J., Xu, Z., Rani, Z., Fang, L., & Guo, L. (2018a). Effects of smoke-water and smoke-derived butenolide on accumulation of phenolic acids in cultured hairy roots of salvia miltiorrhiza bung. *Bangladesh Journal of Botany, 47*, 479–485.

Zhou, J., Ran, Z., Xu, Z., Liu, Q., Huang, M., Fang, L., & Guo, L. (2018b). Effects of smoke-water and smoke-isolated karrikinolide on tanshinones production in *Salvia miltiorrhiza* hairy roots. *South African Journal of Botany, 119*, 265–270.

# Genome-Wide Association Study (GWAS): Concept and Methodology for Gene Mapping in Plants


**Tanmaya Kumar Sahu, Monika Singh, Sanjay Kalia, and Amit Kumar Singh**

**Abstract** The concept of genome-wide association studies (GWAS) was originally developed in humans where it is very successful and has led to identification of key genes for disease and many other traits. With the advent of genotyping technologies and advancement in the field of high-throughput phenotyping, GWAS is becoming a popular approach to uncover the genomic regions/candidate genes governing desirable traits in plants. In the past 10 years, hundreds of association studies have been conducted for a number of economically useful and development related traits in plants. Contrary to the widely used conventional QTL mapping, GWAS can be conducted using natural populations, and thus, it has potential to greatly accelerate mainstreaming of genetic diversity in crop improvement. GWAS can also take advantages of genomic tools, especially the "Omics"-based tools, to accelerate candidate gene discovery in plants. We have described here the detailed procedures, such as preprocessing of genotype and phenotype data and marker-trait association analyses, along with the tools used for GWAS analysis. Finally, we have also discussed how the integration of GWAS with QTL mapping can enhance mapping resolution and accelerate discovery of candidate genes for quantitative traits.

**Keywords** GWAS · Association mapping · Candidate genes · Quantitative trait loci · Multi-locus multi-trait models

T. K. Sahu · M. Singh · A. K. Singh (✉)
Division of Genomic Resources, ICAR-National Bureau of Plant Genetic Resources, New Delhi, India
e-mail: Monika.Singh@icar.gov.in; Amit.Singh5@icar.gov.in

S. Kalia
Department of Biotechnology, Ministry of Science and Technology, Government of India, New Delhi, India
e-mail: sanjay.kalia@nic.in



A. Raina et al. (eds.), *Advanced Crop Improvement, Volume 2*,
https://doi.org/10.1007/978-3-031-26669-0_17

## 1 Introduction

Understanding the genetic and molecular basis of a trait is important for its improvement through conventional or modern breeding approaches. Most of the agriculturally important traits are complex, governed by a number of loci and also influenced by the genotype environment interactions (James et al., 2006; Chaurasia et al., 2020). Therefore, it is extremely important to dissect the genetic basis and identify the genes and alleles controlling complex traits in plants. The identification of genes/genomic regions/quantitative trait loci (QTLs) would not only facilitate precision breeding of such traits but it would also reduce the time involved in the development of improved varieties. Conventionally, in plants, traits have been mapped using QTL mapping approach which uses biparental or multi-parent mapping populations. The QTL mapping approach has led to identification of many important genes/QTLs for various important traits in crops including biotic and abiotic stress tolerance, grain yield, grain and fruit quality, etc. (reviewed by Yang et al., 2017). However, this approach has many limitations: (1) requires generation of mapping population, which is a laborious, time-consuming process involving skilled person for attempting crosses and also to take care of generation advancement; (2) provides low-resolution mapping, i.e., to the order of 10–20 cM, as biparental populations are results of few crossover events; and (3) in case RIL population is used for the genetic mapping, it may take many years to identify genomic regions for the complex traits.

In the past decade, association mapping which exploits linkage disequilibrium (LD) has emerged as a very powerful approach for trait mapping in plants (Ingvarsson & Street, 2011). Currently, it is becoming increasingly popular for genetic dissection of complex traits because of continuous improvement in single nucleotide polymorphism (SNP) genotyping technologies and availability of various next-generation sequencing (NGS)-based genotyping approaches, such as genotyping by sequencing (GBS) and whole-genome re-sequencing (WGRS). Moreover, the increasing interest toward utilizing genetic diversity and improvement in statistical methods has also contributed to wide-scale adoption of genome-wide association studies (GWAS) for genetic dissection of complex traits. Besides, GWAS approach has many advantages over conventional QTL mapping approach, such as the following: (1) provides higher mapping resolution even up to the gene level; (2) allows survey of large number of alleles present in the diversity panel; (3) does not involve generation of mapping population and, thus, saves time and resources; (4) uses historical phenotyping data of the genotypes to conduct GWAS; and (5) enables the use of genetically diverse panel, once genotyped, to map many traits (Korte & Farlow, 2013). The above said advantages make GWAS a method of choice for trait mapping in plants. Furthermore, GWAS can also be integrated with voluminous multidimensional data generated using other genomics technologies such as epigenomics, metabolomics, transcriptomics, proteomics, and ionomics to identify the true causal variant and accelerate the discovery of candidate genes

controlling various agronomically useful traits in plants. In this chapter, we have presented an overview of GWAS methodology, statistical models, tools used, databases related to GWAS. Additionally, we have briefly described various limitations of GWAS and some important GWA studies in crop plants.

## 2 History of GWAS

Klein et al. (2005) introduced GWAS for the first time while working on age-related macular degeneration by identifying a variant of the Complement Factor H gene. Later on, GWAS was carried out for 7 common human diseases by the Wellcome Trust Case-Control Consortium (WTCCC), where approximately 2000 individuals were examined using Affymetrix GeneChip 500 K Mapping Array Set (The Wellcome Trust Case Control Consortium, 2007). Since then, the GWAS was employed in the area of understanding the genetics of several diseases.

In crop plants, the genetic architecture of complex traits can be well understood with references to the human diseases. The complex traits of agricultural and evolutionary importance are generally influenced by multiple genetic loci and their interaction, diversified environmental conditions and the interaction between loci and the environment (Holland, 2007; Mackay et al., 2009). Linkage analysis and association mapping [LD mapping] are being commonly employed for dissecting complex traits.

In comparison to linkage analysis, GWAS offers advantages of high mapping resolution, low research time, and higher allele number (Yu & Buckler, 2006). The advancement in high-throughput genomic technologies, methodology development, improvements in statistical tools, and the desire of identification of novel/superior alleles has enhanced the application of association mapping in model plant and crop species (Zhu et al., 2008). Further, the continued efforts in the sequencing technologies have made GWAS to be emerged as an important tool for such studies.

In the plants, GWAS was initially applied to the modal plant *Arabidopsis*, focusing single feature polymorphism studies, recombination, and linkage disequilibrium (Borevitz et al., 2007; Kim et al., 2006). Later on, the GWAS research has been expanded to the studies on growth, metabolism, defense, and evolution of tolerance to abiotic stress in *Arabidopsis* (Chan et al., 2010b; Wu et al., 2016, 2018; Fusari et al., 2017; Exposito-Alonso et al., 2019). Further, GWAS has been adopted in cereal crops (rice, maize, wheat, and barley), legumes (soybean, peanut), horticultural crops (cucumber, melon, peach, tomato), and other crops such as cotton, lettuce, tea, and sesame. A detailed review of these studies for biotic and abiotic stress tolerance, yield-associated traits, and metabolic composition has been reported recently by Alseekh et al. (2021). A pictorial representation showing the timeline of GWAS research in plant is well depicted in Fig. 1.

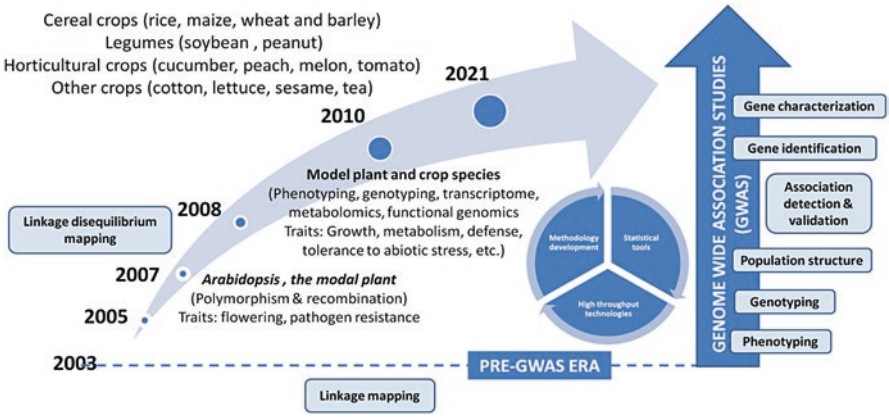



**Fig. 1** Timeline for GWAS research in plants

## 3 Principle and Basic Procedure for Conducting GWAS in Plants

GWAS exploits the phenomenon of linkage disequilibrium (LD) to identify QTLs/genomic regions controlling a target trait. In this approach, QTLs and genomic regions controlling a target trait are identified based on the strength of correlation between thousands of genome-wide markers and the targeted phenotype (Mackay & Powell, 2007). In fact, association mapping, like QTL mapping, also assumes that recombination breaks up the genome into pieces which can be correlated with phenotype to detect genomic regions for complex traits. The main advantage of LD mapping is that it can be applied on natural population and thus very helpful in accelerating the utilization of ex situ germplasm collection of crops maintained in genebanks around the word in crop breeding programs.

### *3.1 Linkage Disequilibrium*

The concept of LD was first put forth about 100 years ago by Jennings (1917), which refers to nonrandom association of the alleles at different loci on the same chromosome. Since then, the topic has been well studied with a greater focus on quantification of LD. LD is now a widely used concept by population biologists and also used as the basis genetic mapping in animal, humans, as well as in plants. Lewontin and Kojima (1960) were among the first to give the measure of LD ($D$); thereafter, many other statistics for LD were developed, each having own advantages and disadvantages. However, the most common statistics employed to measure LD are $D'$ and $r^2$. LD is influenced by many factors including mutation, recombination, selection, position of loci on a chromosome, population structure, genetic drift, gene flow, etc. In plants, LD varies greatly, from few 100 bases to

kilobases and also between different regions of genomes and genes. Generally, self-pollinated crops have higher level of LD as compared to cross-pollinated crops.

LD is generally expressed as the coefficient of linkage disequilibrium (D), which measures a deviation of the observed haplotype frequencies from its corresponding expected allele frequencies on the assumption that the alleles at two loci are associating independently. For an example, we may consider two different loci where each locus has two alleles. Suppose one locus has ***M*** and ***m*** alleles with frequencies *pM* and *1-pM*, respectively, and the other locus has alleles ***N*** and ***n*** with frequencies *pN* and *1-pN*, respectively, then at the equilibrium, although the loci are linked, the expected haplotype frequencies will be the product of the constituent allele frequencies. Thus, *pMN* being the frequency of the two locus haplotype containing alleles *M* and *N* for the *MN* haplotype can be calculated as:

$$pMN = pM \times pN$$

Any deviation from this state is known as the coefficient of linkage disequilibrium (*D*), which is given by the following equation:

$$D = pMN\text{-}(pM \times pN)$$

At equilibrium, *D* is 0 and always defined in context to a pair of alleles at two loci. This type of situation would be difficult to interpret as the range LD depends on allele frequency, and it is not symmetrical about zero. Therefore, it is generally rescaled to a value ranging between 0 and 1 (Mackay & Powell, 2007).

## 3.2 Linkage Disequilibrium Decay

LD is always in the context of a population and depends on recombination rate and times. If the recombination rate is higher, LD decay will be very faster in short time. In a random mating population, without any selection, mutation, random drift, and chance effects, LD value over the successive generations would be $D_{t+1} = D_t\,(1-c)$. Hence, $D_t$ (the LD decay at generation $t$) is calculated as $D_0\,(1-c)^t$, where $D$ is coefficient of LD, $c$ is the recombination frequency, and $t$ is the number of generations of random mating. At higher recombination frequencies, LD decays rapidly and $D$ decreases only by a factor of a half each generation for unlinked loci ($c = 0.5$).

## 3.3 Steps in GWAS Analysis

Various steps followed in GWAS analysis are represented in Fig. 2. However, detailed description on various steps is given in the following subsections.

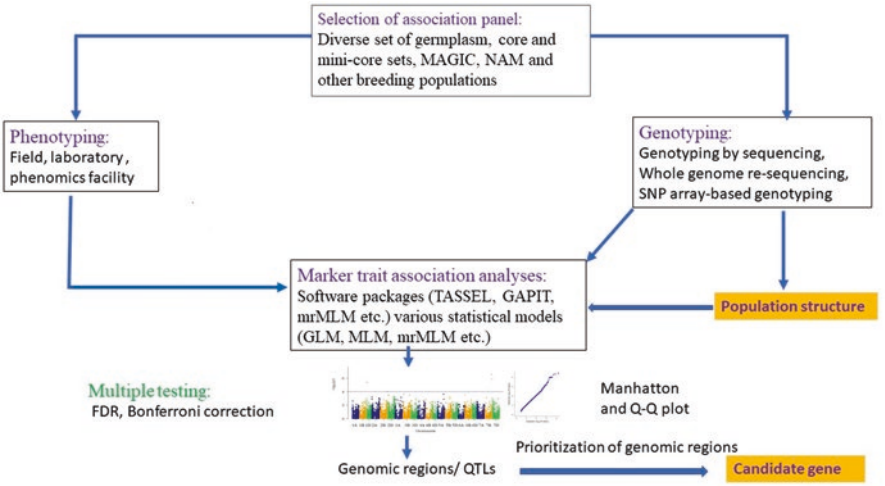



**Fig. 2** Steps of GWAS analysis

### 3.3.1 Selection of Diverse Panel of Individuals

To achieve high-resolution mapping of a target trait, it is very important that association panel has genetically diverse set of individuals. The association panel for GWAS can be constituted by assembling individuals (landraces, varieties) from various geographical regions within a country or from various regions of the world. The core and mini-core sets constituted from the large germplasm collection of crops conserved in the national and international genebanks are also considered as very good association panels for GWAS. In addition, multi-parent populations such as nested association mapping (NAM), multi-parent advanced generation intercross (MAGIC), etc. can be also used for trait mapping using GWAS.

Furthermore, association panel size is a very important factor that determines mapping resolution. Generally, an association panel with around 300 individuals is considered good for trait mapping using GWAS, provided it has sufficient variability for the trait under consideration. However, in case core or mini-core sets are used as association panel in any study, a smaller number than 300 may be also considered sufficient as these are genetically very diverse and capture total diversity available in collection of a crop species.

### 3.3.2 Phenotyping of the Association Panel for the Trait of Interest

Accurate phenotyping is critical for identification of reliable genomic regions/QTLs controlling a trait, using GWAS. Phenotyping methods should provide minimum scope for errors both in data generation and in data acquisition. Moreover, it is very

important that complex quantitative traits should be phenotyped over multiple locations and/or multiple years to get reliable data for association analysis. For reliability of GWAS results, means estimates that have low variance such as best linear unbiased prediction (BLUP) and best linear unbiased estimates (BLUE), which consider fixed and random effects, respectively, should be derived for multilocation/multiyear phenotypic evaluation data. As compared to field phenotyping, the dedicated phenomics facility developed by various institutions across the world may be highly useful for complex traits including abiotic stresses, nutrient deficiency, etc.

### 3.3.3 Genotyping of the GWAS Association Panel

The association panel should be densely genotyped for high-resolution trait mapping. As a thumb rule, at least one marker should be present at every linkage decay distance for the reasonable mapping resolution using GWAS. In the last few years, with the advancement of SNP genotyping methods, it is feasible to generate genotypic information of the association panel at a low cost. High-density SNP arrays have been developed in many crops, such as wheat, rice, pigeon pea, chickpea, and soybean (Varshney et al., 2021). Besides SNP arrays, next-generation-based approaches such as GBS and WGRS can also be used for the genotyping.

### 3.3.4 Population Structure Analysis Based on the Genotyping Information

The number of populations in association panel can be inferred based on the genotyping data using software tools. STRUCTURE v2.2.3 (Pritchard et al., 2000) is the most widely used program for population structure analysis and visualization. The structure results are then used to estimate most probable number of populations using the method of Evanno et al. (2005). Population structure results are integrated in GWAS analysis to help minimize chances of spurious associations.

### 3.3.5 Marker-Trait Association Analysis

The genotyping and respective phenotypic data is analyzed using appropriate statistical model to detect QTLs/genomic regions for the target trait. Description of various GWAS models and software programs is provided in the subsequent sections. Most of the GWAS analysis program generates a scatterplot popularly known as Manhattan plot which depicts chromosomal positions of markers on x-axis and the corresponding *p*-values on the y-axis. The lower *p*-value on the Manhattan plot shows that corresponding marker has strong association with the trait (Fig. 2).

## 4 Data Generation and Quality Filtrating for GWAS

The collection of genotypic and phenotypic data and their quality check is an important step in GWAS analysis. Analysis of low-quality data lead to biased or erroneous results that in turn draw a misleading inference. Thus, proper care has to be taken to collect or generate high-quality data, and appropriate filtration criteria have to be imposed on the data for quality improvement. Description of GWAS data along with quality parameters has been described in detail under this section.

### *4.1 Genotypic Data*

The genotypic data is generated by genotyping of a DNA sample of interest. Through genotyping techniques, the alleles of an individual at different locus are uncovered. Genotyping is carried out using DNA microarrays, GBS, and other technologies based on restriction enzymes and polymerase chain reactions (PCR). Among the promising and widely used methods, genotyping by microarrays has gained considerable reputation. Microarrays are preferred to identify common variants, whereas rare variants are identified using next-generation sequencing technologies.

Two common genotypic data formats, i.e., haplotype map (Hapmap) and Variant Call Format (VCF), are used for GWAS. Hapmap and VCF formats were created to manage and distribute the information on polymorphisms in the genomes of different organisms. The Hapmap Project was taken up by International HapMap Consortium in 2001. As a product of this project, the dbSNP of NCBI was developed and made freely available to public (The International HapMap Consortium, 2003). The Hapmap file format contains 11 columns followed by 1 column each for genotyped samples. Different column identifiers are described in Table 1.

**Table 1** Description of Hapmap file format

| Col. no. | Column name | Column description |
|---|---|---|
| 1 | rs# | SNP identifier |
| 2 | alleles | SNP alleles as per NCBI dbSNP |
| 3 | chrom | Chromosome number where SNP is located |
| 4 | pos | Chromosomal location of SNP |
| 5 | Strand | Orientation of the SNP in the DNA strand, i.e., forward (+) or in the reverse (−) orientation |
| 6 | assembly# | Version of reference sequence assembly |
| 7 | center | Name of genotyping center |
| 8 | protLSID | Identifier for HapMap protocol |
| 9 | assayLSID | Identifier for HapMap assay |
| 10 | panelLSID | Identifier for panel of individuals genotyped |
| 11 | QCcode | Quality control code for all entries |
| 12 | Sample accessions/ names/IDs | The sample accession/name/ID that contain marker genotype in each row |

**Table 2** Description of VCF file format

| Attribute | Description |
|---|---|
| *Meta information* | |
| ##INFO | Information on variables, their type, and values |
| ID | Name of the variable |
| Number | Number of values |
| Type | Type of variable |
| Description | Description of the variable |
| *Header* | |
| #CHROM | Chromosome number |
| POS | Position on the chromosome |
| ID | Identifier |
| REF | Reference nucleotide base(s) |
| ALT | Alternate/non-reference nucleotide base(s) |
| QUAL | Phred quality score for the statement made in ALT |
| FILTER | PASS if it has passed in all filters, if not, contains a reason |
| INFO | Additional information separated by semicolons |
| FORMAT | Format IDs separated by colon |
| IDs | Tab delimited list of sample identifiers |

The VCF data format is a product of 1000 Genomes Project led by the International 1000 Genomes Consortium (Abecasis et al., 2010). This type of genotypic file format includes lines of meta-information, header lines, followed by the data lines. All the meta-data about the variable are provided under the meta-information lines. Header line contains tab limited columns where variant information of each sample follows eight mandatory columns. The data line(s) contains the value of each variant under columns defined in the header line. A dot (.) represents missing data in the data line. A clear description of VCF file format is represented in Table 2.

## *4.2 Phenotypic Data*

The process of determining the phenotype is called as phenotyping, which involves the measurement of complex traits related to biotic and abiotic stress tolerance, yield, growth, etc. (Fiorani & Schurr, 2013). Plant phenotyping is concerned, and basic measurement of quantitative properties associated with complex traits (Li et al., 2014a, b) is carried out, which may include quantification of plant biomass, root morphology, leaf characteristics, and fruit traits (Costa et al., 2019). The phenotypic information obtained from phenotyping contains the morphological characteristics represented in columns for different individuals (as rows) of the sample in excel or tab delimited text format. The phenotypic data can be qualitative or

quantitative or both. As some of the morphological measurements are prone to data handling errors, the data should be collected carefully and processed properly based on all quality parameters before its use in GWAS analysis.

## *4.3 Quality Control*

High-quality data always give accurate and reliable result. Hence, quality control for both phenotypic and genotypic data is highly essential before GWAS analysis. Various protocols and guidelines have been established for data quality control for GWAS analysis (Köttgen et al., 2010; Teslovich et al., 2010). These protocols or guidelines focus on data normality, outlier detection, imputation quality, missing data, minor allele frequency (MAF), Hardy-Weinberg's equilibrium, etc. The major parameters for data quality control are discussed below:

### 4.3.1 Outlier Detection

In a dataset, an outlier is referred to an observation that is not in agreement with distribution of data points (Barnett & Lewis, 1994). An outlier is a biological replicate in a plant dataset that deviates from the overall distribution of measured plant variables. Different methods exist for outlier detection from the datasets based on either single traits (Grubbs, 1950; Utz, 2003) or multiple traits (Reimann et al., 2008; Rousseeuw & Hubert, 2011). Grubbs test (Grubbs, 1950) and Bonferroni outlier tests are among the most commonly used methods for outlier detection (Camargo et al., 2014). To consider a QTL significant in GWAS, the values of the variable of interest should differ significantly between genotypes with different alleles for a marker of interest (Alvarez-Prado et al., 2019).

### 4.3.2 Data Normality

Normality of data is a common assumption that is considered in many statistical methods. If data is not normal, any comparison of the data to the population parameters may not be valid. Normality in the data allows the parametric statistical tests to be applied on the data. Therefore, data normality tests are required to enhance the quality of the data. Shapiro normality and Bonferroni outlier tests are very often used to ensure the data normality (Camargo et al., 2014).

### 4.3.3 Imputation Quality

Genotype imputation is a powerful tool for handling missing data. It estimates missing genotypes from the genotype reference panel and enhances the power of SNP detection in GWAS (Shi et al., 2018). However, the performance of imputation

depends on reference selection, sample size, sequencing coverage, etc. Thus, care should be taken while selecting the method and reference for imputation. A high-quality imputation contributes to reliable results.

### 4.3.4 Missing Data

The missing genotype data refers to the genotypes where one or more marker information is missing. The incidence of missing information in genotype data is due to unsuccessful assay of markers on genotyping platforms (Lin et al., 2008). Further, genotypes with missing information on high number of markers can often lead to a biased analysis. Thus, either the missing values are imputed computationally or genotypes/markers with missing values beyond a threshold (usually 5%) are removed from the analysis.

### 4.3.5 Minor Allele Frequency

Very often, errors in large genotypic datasets are reduced by eliminating the markers having an MAF below a predetermined threshold. The rare genetic variants have a low MAF, which is usually less than 5 or 1% (Lasky-Su, 2017). Therefore, the SNPs having MAF greater than 0.05 (5%) are considered in many GWA studies.

### 4.3.6 Linkage Disequilibrium (LD) Pruning

A marker subset can be chosen through LD pruning based on detectable linkage equilibrium (Joiret et al., 2019). LD pruning filters genetic markers by selecting only markers that are representatives of the genetic haplotype blocks.

### 4.3.7 Hardy-Weinberg's Equilibrium (HWE)

HWE law indicates whether the observed genotypic frequencies are significantly different expected frequencies or not (Wigginton et al., 2005; Marees et al., 2019). If the genotypic data in GWAS analysis deviates from HWE law, then it could be due to genotyping errors.

### 4.3.8 Marker Heterozygosity

The marker loci having high heterozygosity indicate technical artifact or paralogous/repetitive regions, which could not be discriminated through genotyping (Glaubitz et al., 2014). Natural populations of self-pollinating crops and inbred lines are highly homozygous, where a marker loci with modest heterozygosity rate is also doubtful (Pavan et al., 2020). Further, extremely heterozygous markers are

introduced when homeolog detecting probes fail to distinguish between two highly similar sub-genome sequences (Otyama et al., 2019). So, highly heterozygous markers can be filtered out by using a suitable heterozygosity threshold.

## 5 Statistical Models for GWAS

Several statistical models exist for GWAS data analysis which are applied based on the nature of data and the method of data collection. The statistical models for GWAS are categorized into single locus models, multi-locus models, multi-trait models, and multi-locus multi-trait models, which are described in the subsequent subsections.

### *5.1 Single-Locus Models*

Most of the GWAS analysis used earlier was based on single-locus single trait (SLST) approach. However, SLST is known to yield biased results plausibly because of confounding effect, multiple testing problem, inability to identify pleiotropic effects, and observed LD not due to linkage (Jaiswal et al., 2016). The single-locus mixed models consider both fixed and random effects (Yu et al., 2006) and deal fairly with the problem of false positives. The single-locus methods are categorized into *exact* and *approximate* methods. The exact methods provide exact estimates of marker effects but are comparatively more time-consuming (Zhou & Stephens, 2012; Lipka et al., 2015), Whereas the approximate methods provide approximate estimates and faster than exact methods as population parameters are not estimated for every marker.

#### 5.1.1 ANOVA: Analysis of Variance

ANOVA is a standard statistical for estimating the association between markers and phenotypes under study in a quantitative phenotype association study (Pagano & Gauvreau, 2000). ANOVA determines significant difference in the group means while considering within group variances. It partitions the total variance of data into within-group and between-group variances where the difference between the groups becomes significant if between-group variance is considerably higher than the within-group variance (Zhang et al., 2008).

#### 5.1.2 GLM: General Linear Model

Generalized linear models (GLM) are based on the concept of linear regression model. In GLM, principal components are used as covariates to minimize the number of false positives that could be only because of population structure (Price et al., 2006).

### 5.1.3 MLM: Mixed Linear Model

Mixed linear model (MLM; Yu et al., 2006) methods are advantageous, as these models are able to efficiently manage the population structure as well as relatedness within GWAS. In MLM, population structure is considered as fixed effect, whereas the random effect includes the kinship among individuals as variance-covariance structure (Zhang et al., 2010). The incorporation of these covariates in MLM controls the number of false positives (Gupta et al., 2019). However, these methods suffer from the problem of overfitting, leading to false negatives, and are computationally intensive for large datasets.

### 5.1.4 CMLM: Compressed MLM

Compressed MLM is the extreme cases of GLM and MLM (Zhang et al., 2010). MLM is comparable to CMLM while considering each individual as a single group. On the other hand, GLM becomes equivalent to CMLM when all individuals are in one group.

### 5.1.5 ECMLM: Enriched Compressed MLM

ECMLM is an extension of CMLM that calculates kinship using group kinship as well as clustering algorithms. Subsequently, best combination of kinship and clustering algorithms is chosen. ECMLM includes an additional parameter to CMLM-GWAS by exploring every alternative for computing the kinship between groups by averaging the pair-wise individual kinships (Li et al., 2014a, b).

### 5.1.6 SUPER: Settlement of MLM Under Progressively Exclusive Relationship

SUPER reduces number of genetic markers to a greater extent that characterize individual relationships as well as considerably enhances the statistical power. The whole genome is divided into smaller bins representing most significant markers, and subsequently, the most influential bin is selected. Further, optimization of the size and number of selected bins is carried out using maximum likelihood method (Wang et al., 2014). Further, relationship among the individuals is defined by using a small set of markers. This small marker set is obtained by eliminating the markers, which are in LD to test marker, independent of the local distance.

### 5.1.7 EMMA: Efficient Mixed-Model Association

EMMA is a mixed GWAS model that includes two variance components. EMMA corrects population structure and genetic relatedness via a kinship matrix generated from genome-wide markers. It greatly reduces the number of false positives under

a structured population. The model takes advantages of the nature of optimization problem that significantly increases the accuracy and reliability of the results as well as consumes low computational time (Kang et al., 2008).

#### 5.1.8 GEMMA: Genome-Wide Efficient Mixed-Model Association

GEMMA is reported to tackle the issue of exact computation of the standard test statistics that are not feasible computationally even with a moderate-scaled GWAS (Zhou & Stephens, 2012). GEMMA is *s* times faster than EMMA, where *s* is the number of individuals in the sample.

#### 5.1.9 FaST-LMM: Factored Spectrally Transformed Linear Mixed Models

FaST-LMM is a reformulation of linear mixed models where it produces precisely the same results like standard linear mixed models but scales linearly with the cohort size (number of SNPs to be tested) in both computational speed and memory use (Lippert et al., 2011). It assumes that the number of markers used to approximate the genetic similarity is smaller than size of the cohort, and the similarities have been determined using the realized relationship matrix.

### *5.2 Multi-locus Models*

Multi-locus models are characterized by higher statistical power as well as lesser false-positive rate than single-locus models as these models are thought to be true representative of plant and animal genetic models (Wang et al., 2016a, b). These types of GWAS models help identify more marker-trait associations as they don't require the Bonferroni correction. Further, multi-locus models also address the issues of pleiotropy and the background noise (Segura et al., 2012; Korte et al., 2012). Multi-locus models involve polygenic effect and population structure additionally to reduce bias in the estimation of effects (Yu et al., 2006; Zhang et al., 2005, 2010). However, undertaking the correction for population structure reduces confounding effects, which frequently generate false positives/false negatives (Klasen et al., 2016). Implementation of principal components analysis (PCA), multidimensional scaling (MDS), STRUCTURE, and/or other methods provides estimates of global ancestry that are required for adjusting confounding due to population structure in linear models (Hellwege et al., 2017). Considering the between-marker correlations, Klasen et al. (2016) developed the Quantitative Trait Cluster Association Test (QTCAT), which is capable of detecting multi-locus associations concurrently and eliminates the need of correcting the population structure and genetic background.

### 5.2.1 MLMM: Multiple Loci MLM

MLMM is used as a standard method to map complex traits in a structured population (Segura et al., 2012). It handles the confounding effects of a scattered background of large numbers of loci of small effect as well as accounts for loci of larger effect. MLMM treats the genetic background comparable to composite interval mapping (Segura et al., 2012).

### 5.2.2 FarmCPU: Fixed and Random Model Circulating Probability Unification

In FarmCPU (Liu et al., 2016), the fixed effect model is used along with a random effect model iteratively to completely abolish the confounding effect. The fixed effect model tests markers one by one and considers multiple associated markers as covariates to control the rate of false positives. The random effect model avoids overfitting and estimates the associated markers by employing them in defining the kinship. The unification of *p*-values per iteration was done corresponding to the testing and associated markers.

### 5.2.3 mrMLM: Multi-locus Random-SNP-Effect MLM

The mrMLM being a multi-locus model avoids Bonferroni correction for multiple tests. Further, it also includes markers selected from the random-SNP-effect MLM method with a less stringent selection criterion. SNP effect is treated as random in mrMLM, and this model is reported to be powerful in detecting the quantitative trait nucleotides (QTN) and estimating the QTN effects more accurately than EMMA (Wang et al., 2016a, b).

### 5.2.4 FASTmrMLM: Fast Multi-locus Random-SNP-Effect MLM

FASTmrMLM is more than 50% computationally faster than mrMLM. FASTmrMLM detects and estimates QTNs with high accuracy and less false positives than mrMLM, FarmCPU, and GEMMA. Among multi-locus GWAS, FASTmrMLM has considerable reliability as well as accuracy and low execution time (Tamba & Zhang, 2018).

### 5.2.5 FASTmrEMMA: Fast Multi-locus Random-SNP-Effect EMMA

FASTmrEMMA model is based on random SNP effects. Initially, putative QTNs with *p*-values less than equal to 0.005 are selected and subsequently included in a multi-locus model to detect the true QTNs. Here, a less stringent selection criterion

is employed instead of the Bonferroni correction. FASTmrEMMA is reported as more powerful in QTN detection as it estimates the QTN effect estimation with low bias and takes low computational time as compared to single-locus and other multi-locus methods like SUPER, empirical Bayes, EMMA, CMLM, and ECMLM (Wen et al., 2018).

## *5.3 Multi-trait Models*

More than one trait can possibly be controlled by a single genomic region (QTLs/genes) (Zhan et al., 2017). Based on this fact, the multi-trait MLM (MTMM) has been developed that accommodates pairs of correlated traits and increases the number of tests as compared to that of single-trait analysis (Gupta et al., 2019). MTMM also addresses the issues of background noise and pleiotropy (Segura et al., 2012; Korte et al., 2012). Multiple traits are often transformed into a group of pseudo-principal components based on a residual covariance matrix for improvement of accuracy and reliability. The matrix-variate linear mixed model (mvLMM; Furlotte & Eskin, 2015) is a variation of MTMM that reduces the execution time for maximum-likelihood inference by using data transformation in a multi-trait model. Multi-trait analysis of GWAS (MTAG; Turley et al., 2018) model can handle the sample overlap and can estimate the trait-specific effect for individual SNP rapidly. MTAG is benefited by the between-trait and between-error correlations.

## *5.4 Multi-locus, Multi-trait Models*

If the multi-trait GWAS is carried out for multiple loci as a combined analysis, then it involves multi-locus multi-trait models which are computer intensive. However, these models are more advantageous than either multi-locus models or multi-trait models separately (Lippert et al., 2014) and have the ability to unravel the complex and significant associations. Recently, Igolkina et al. (2020) proposed a multi-trait multi-locus model based on structural equation modeling, i.e., mtmlSEM (Wright, 1918, 1921), to illustrate the complex associations exist between SNPs and traits under investigation. This model is capable of distinguishing pleiotropic and single-trait SNPs having direct as well as indirect effects. Further, it appropriately deals with the variables which are not normally distributed. The mtmlSEM cannot work with artificial phenotypes represented as linear combinations of traits like PCA-based methods, but the phenotypes are regressed on the latent constructs to a certain extent (Igolkina et al., 2020).

## 6 Multiple Hypothesis Testing in GWAS

Multiple hypothesis testing is a problem of decision-making based on results of a number of statistical tests. As GWAS involves a large number of statistical tests to be conducted concurrently, it faces the challenge of multiple hypothesis testing. A more stringent criteria often rejects true associations, whereas a less stringent criteria introduces large number of false positives. Therefore, *p*-value threshold for significance is adjusted appropriately with the aim to control overall false-positive rate. The *p*-value correction methods such as Bonferroni correction, permutation test, and false discovery rate (FDR) control are employed to achieve this adjustment. The Bonferroni correction (Sidák, 1967) divides the level of significance at each locus by the total number of tests. It assumes independence among the tests of association, which is practically not true due to existence of LD among markers (Reich et al., 2001). Thus, the correction is slightly stringent if tests are independent, whereas in case of correlated tests, it becomes highly stringent (Gao et al., 2010). Sequential Bonferroni correction (Holm, 1979) is an extension to standard Bonferroni correction that allows possible dependencies between association tests to some extent. However, the Bonferroni corrections are suitable, if a small number of null hypotheses among many are expected to be false.

The permutation test (Westfall & Young, 1993; Browning, 2008) generates the empirical distribution of test statistics under null hypothesis without disturbing the original correlation structure. It randomly permutes the phenotypes and computes the association statistics under each permutation. The test effectively breaks the genotype-phenotype relationship without affecting the existing LD structure of the dataset. The permutation test is regarded as the gold standard, as it reliably and correctly describes the genomic correlation structure. The *p*-value correction involving FDR was proposed by Benjamini and Hochberg (1995). FDR correction effectively minimizes the expected number of false discoveries and estimates the number of significant associations. An improvement over FDR by enforcing monotonicity was introduced by Benjamini and Yekutieli (2001), which is compatible with initial FDR definition.

## 7 Databases and Tools for GWAS

There exists many databases containing GWAS information on human, but for agricultural crops, few information resources are available. Among them, some databases are briefed in the subsequent subsections. However, plenty of tools for analyzing GWAs data are available in the public domain, including both online and offline tools.

## *7.1 Databases for GWAS*

### 7.1.1 GWAS Atlas

GWAS atlas is a curated compilation of genome-wide associations between variants and different traits for two animals and seven plants. It provides information on high-quality curated collection of 75,467 associations between the variants and 614 traits of 2 domesticated animals (goat and pig) and 7 crops (rice, maize, soybean, sorghum, rapeseed, Japanese apricot, and cotton) collected from 254 research publications (Tian et al., 2020). The GWAS atlas is accessible at https://bigd.big.ac.cn/gwas/.

### 7.1.2 GrainGenes

GrainGenes is a database of genes, alleles, QTLs, genetic maps, mapping probes, and primers for crops like wheat, barley, rye, and oat, accessible at http://www.graingenes.org (Matthews et al., 2003). It includes information on polymorphisms, genotypes, traits, disease symptoms, and mutant phenotypes. In addition, it provides information on mapping and QTL studies.

### 7.1.3 Triticeae Toolbox

The Triticeae Toolbox (T3; Blake et al., 2015) is the database for the phenotype and genotype data produced by the Triticeae Coordinated Agricultural Project. It enables the download of specific datasets in GWAS compatible formats for GWA and genomic prediction studies. Additionally, it includes various tools for data analysis.

### 7.1.4 Pea Marker Database (PMD)

The PMD is supplemented with information on 2484 genic markers with their corresponding locations in linkage groups, gene names, and transcript sequences. PMD Version 2 contains 15944 pea markers in a similar fashion with additional features (Kulaeva et al., 2017). The PMD is available at http://www.peamarker.arriam.ru/ for public use.

### 7.1.5 NABIC Marker Database

This online molecular marker database has been developed at National Agricultural Biotechnology Information Center (NABIC), Korea. An individual molecular marker entry contains the information on gene definition, expressed sequence tag number, marker name, and general marker information (Kim et al., 2013). The database is hosted at http://nabic.rda.go.kr/gere/rice/molecularMarkers/.

## 7.2 *Tools for GWAS Data Analysis*

### 7.2.1 PLINK

PLINK is a GWAS toolset designed to analyze genotype/phenotype data. Visualization, annotation, and storage of PLINK results are made feasible through its integration with gPLINK and Haploview. PLINK can handle large datasets in their entirety having numerous markers genotyped for a large number of individuals. The broad functions of PLINK include summary statistics, population stratification, identity-by-descent estimation, association analysis, and data management (Purcell et al., 2007). It determines population stratification based on identity-by-state as well as identity-by-descent information.

### 7.2.2 GAPIT

Genome Association and Prediction Integrated Tool (GAPIT) is an R-package that implements advanced statistical methods including the CMLM and CMLM-based genomic selection and prediction. This tool can efficiently handle large datasets (~10,000 individuals and ~ 1 million SNPs) in terms of computational time as well as can offer concise tables and graphs for result interpretations (Lipka et al., 2012). It is updated frequently to integrate the state-of-the-art methods for GWAS and Genomic Selection. GAPIT is available at http://www.maizegenetics.net/GAPIT.

### 7.2.3 STRUCTURE

The STRUCTURE software package is used for mainly analyzing the multi-locus genotype data to determine the population genetic structure by (i) deducing the existence of distinct populations, (ii) allotting individuals to populations, (iii) investigating hybrid zones, (iv) classifying migrants and admixed individuals, and (v) approximating allele frequencies in the population having several migrant or admixed individuals. The STRUCTURE package is compatible with most of the widely used genetic markers such as SNPS, microsatellites, RFLPs, and AFLPs (Pritchard et al., 2000; Kaeuffer et al., 2007).

### 7.2.4 TASSEL

Trait Analysis by aSSociation, Evolution and Linkage (TASSEL) is a widely used graphical user interface for GWAS data analysis. It implements GLM and MLM approaches for controlling the population and family structure (Bradbury et al., 2007). The software computes LD statistics, diversity statistics, and principal components as well as supports graphical vitalizations. TASSEL also facilitates the analysis of indels, integration of phenotypic and genotypic data, and imputation of missing values.

### 7.2.5 GWASpro

GWASpro is a web server for large-scale GWAS based on high-performance computing. It supports the analyses of large-scale GWAS data with intricate replicated experimental designs and optimized to handle up to 10 million markers and 10,000 samples (Kim et al., 2019). The web server is freely accessible at https://bioinfo.noble.org/GWASPRO.

### 7.2.6 METAL

METAL is a proficient tool for GWAS meta-analysis for improving the power of analysis involving complex traits (Willer et al., 2010). METAL includes a user-friendly scripting interface with memory management efficiency that supports analyses of large datasets from a broad range of input file formats.

### 7.2.7 GWAMA: Genome-Wide Association Meta-analysis

The GWAMA supports the meta-analysis of summary statistics obtained from GWA studies on dichotomous phenotypes or quantitative traits (Mägi & Morris, 2010). The software is freely available online with documentations and examples at http://www.well.ox.ac.uk/GWAMA.

## *7.3 R-Packages for GWAS*

R is being used as a user-friendly scripting interface for almost all statistical analysis. It involves many modules for GWAS data analysis. GAPIT (described earlier) is also an R-based GWAS tool that is being used reliably by the researchers across the globe. A list of R-packages for GWAS analysis along with brief description and availability is provided in Table 3.

# 8 Prioritization of Associated Genomic Regions/Prediction of Candidate Genes

Generally, GWAS provides a large number of significantly associated loci/associated regions for the target trait. Therefore, it is extremely important to narrow down to a few reliable SNPs/major true variants that might be actually controlling target trait that can be utilized in marker-assisted selection (MAS)-based crop improvement

**Table 3** Widely used R-packages for GWAS data analysis

| Packages | Description | Availability | Reference |
|---|---|---|---|
| GAPIT | Implements CMLM-based genomic prediction and selection | http://www.maizegenetics.net/GAPIT | Lipka et al. (2012) |
| statgenGWAS | Fast single trait GWAS that supports analysis of typical plant breeding experiments | https://cran.r-project.org/web/packages/statgenGWAS/index.html | Rossum et al. (2021) |
| GWASTools | Includes classes for storing very large GWAS datasets, annotations, and functions for GWAS data cleaning and analysis | https://www.bioconductor.org/packages/release/bioc/html/GWASTools.html | Gogarten et al. (2012) |
| rrBLUP | Best linear unbiased predictor (BLUP) can be calculated based on an additive relationship matrix or a Gaussian kernel. It is used to estimate marker effects by ridge regression | https://cran.r-project.org/web/packages/rrBLUP/rrBLUP.pdf | Endelman (2019) |
| R/fGWAS2 | The R/fGWAS2 (functional genome-wide association studies) is based on a single SNP analysis and provides three separate methods for data analysis | https://www.dept.psu.edu/statgen/software/fgwas-r2.html | Li et al. (2011) |
| GWAtoolbox | Standardizes and accelerates the data handling from GWAS for large-scale GWAS meta-analyses | http://www.eurac.edu/GWAtoolbox | Fuchsberger et al. (2012) |

program. The important and consistent QTLs/genomic regions for the target traits can be filtered out based on the strength of associations, which can be determined using various statistical methods, such as multiple corrections (FDR, Bonferroni correction) and *P*-value. Moreover, the physical position of the SNPs can be localized on the genome provided the whole-genome sequence of species is available, and the genomic region flanking the associated SNPs up to LD decay distance can be screened for finding candidate genes responsible for the target trait. However, sometimes there could be many genes present in LD decay distance region (significantly associated SNP ± LD decay distance region). In such cases, to pinpoint the exact causal variant responsible for a trait, firstly sequence polymorphisms in all the genes present in the LD decay distance can be discovered by sequencing a diverse panel of accessions. Then, the polymorphism effect of identified SNPs on the target trait can be estimated to find out the exact variant responsible for variation in trait. For the estimation of SNPs effect on phenotype, first of all, haplotypes for various genes falling in the genomic regions associated with the trait are identified; then effects of each haplotype on respective trait are determined by statistical analyses. Besides above-described analyses, expression pattern of genes located within the LD-decay distance of associated SNP can also help in narrowing down to causal variant responsible for the trait. The gene expression levels can be analyzed for correlation with the studied traits.

## 9 Limitations of GWAS

The GWAS has been proved as a successful approach to map complex QTLs in crop plants. However, few limitations have been identified in the case of GWAS, which may be attributed to complex genetic architectures, genetic heterogeneity, unexpected LD, size of small effects, lower allelic frequency, or missing genotypes. The major limitations of GWAS have been reviewed (Korte & Farlow, 2013; Tam et al., 2019), and few of these are presented as follows:

### *9.1 False-Negative/False-Positive Results During Multiple Testing*

The interpretation of GWAS data involves false-positive and false-negative concepts. Chances of false-negative results arise as a result of issues related to population structure and low-frequency causal alleles. This limitation may be addressed by increasing the size of sample or reducing the number of tests.

### *9.2 Missing Heritability*

In GWAS, all the phenotypic variations are sometimes not answered by the identified SNPs associated with phenotypes, leading to missing heritability (Manolio et al., 2009). These variations could be because of incomplete linkage between the causative variants and those genotyped or due to rare variants (Dickson et al., 2010). Epigenetic variation where sophisticated genotyping is required may also lead to missing heritability (Johannes et al., 2009). The gene expression may be affected due to loss or gain of DNA methylation being inherited across the generations. Such epigenetic variations may be considered as a source of heritable phenotypic variation when there is no change in the sequence of DNA. In a special case of missing heritability, pseudo-heritability may exceed the heritability estimated from the replicates for some of the traits, such as flowering time in *Arabidopsis* (Korte & Farlow, 2013). Missing inheritability is a major limitation of GWA studies in case of small heterogeneous sample, which may be overcome by including full sequence data and increasing the sample size.

### *9.3 Heritability of Complex Traits*

All the components of complex traits cannot be identified by GWAS or any other method, which may be due to difficulty in detecting complex interactions, rare variants with small effects, common variants with very small effects, and genes possessing ultrarare variants (Tam et al., 2019).

### *9.4 Genetic Heterogeneity*

Genetic heterogeneity refers to existence of same phenotypic effect in different individuals caused due to different loci, either within a single gene (allelic heterogeneity) or in different genes (genic/locus heterogeneity). Single-marker approaches generally undergo genetic heterogeneity in case of multiple major loci and LD with each other.

### *9.5 Synthetic Associations/Misleading Associations*

This situation occurs when GWAS identifies noncausal SNPs as more significant than truly causal variants (Dickson et al., 2010). SNPs at a different location, which are associated with the presence or absence of all the alleles responsible, may be detected as synthetic associations (Tibbs-Cortes et al., 2021).

## 10 GWAS Studies in Plants

Genome-wide association study has emerged as a promising technology for studying the complex traits in plants (Tibbs-Cortes et al., 2021). GWAS has been carried out for studying various agriculturally important traits including yield traits, morphological characters, resistance to biotic and abiotic stresses, etc. in different crops including cereals, pulses, and horticultural crops besides the model plant *Arabidopsis* (Table 4). It has also been employed to investigate other types of phenotypes, such as identification of genes associated with geographical divergence and adaptation during domestication (Chen et al., 2019) and study of biochemical and molecular phenotypes including amino acid, fatty acid, flavonoid, and nucleic acid metabolites (Chen et al., 2016b). GWAS is also used to identify novel associations with the important traits as well as to explore target genes for genetic engineering and genome editing for crop improvement (Owens et al., 2014; Wang et al. 2016a, b; Zhang et al., 2018).

## 11 Integration of GWAS and QTL Mapping for Fine Mapping and Candidate Gene Discovery of Complex Trait

The widely used QTL mapping approach has very low resolution, i.e., large intervals between causal variant and the candidate gene controlling target trait, which limits utilization of the genomic regions/QTLs identified by this approach, in the

**Table 4** Major GWA studies carried out in plants

| Crop | Trait | Genotyping method | Reference |
|---|---|---|---|
| *Arabidopsis* | A total of 170 diverse phenotypes | SNP genotyping (250 K SNP array) | Atwell et al. (2010) |
| | Defense metabolites (43 glucosinolates) | SNP genotyping (250 K SNP array) | Chan et al. (2010a) |
| | Dark-induced senescence | SNP genotyping (250 K SNP array) | Zhu et al. (2021) |
| Barley | Resistance to Spot blotch | SNP genotyping (9 K iSelect Illumina SNP array) | Gyawali et al. (2018) |
| | Flowering traits, barley yellow mosaic virus, barley mild mosaic virus | GBS | Milner et al. (2019) |
| | Yield, quality, and disease resistance | SNP genotyping (9 K iSelect Illumina SNP array) | Tsai et al. (2020) |
| *Citrus* varieties | Fruit quality traits | SNP array genotyping | Minamikawa et al. (2017) |
| Maize | Resistance to *Fusarium* ear rot | SNP genotyping (Illumina MaizeSNP50 BeadChip) | Chen et al. (2016a) and Wu et al. (2020) |
| | | GBS | |
| | Thermo-tolerance of seed-set | GBS | Gao et al. (2019) |
| | Cold tolerance | SNP genotyping (Illumina Maize SNP50 BeadChip) | Zhang et al. (2020a, b) |
| | Nitrogen use efficiency | GBS | Ertiro et al. (2020) |
| Peanut | Yield-related traits | GBS | Wang et al. (2019) |
| | Resistance to leaf spots (early leaf spot, late leaf spot) | SNP genotyping (48 K SNP array; Axiom Arachis2) | Zhang et al. (2020a, b) |
| | Seed composition traits | SNP genotyping (48 K SNP array; Axiom Arachis2) | Zhang et al. (2021a) |
| Pear (*Pyrus pyrifolia*) | Fruit quality and phenological traits | WGRS | Zhang et al. (2021b) |
| Rice | Agronomic traits | WGRS | Huang et al. (2010) |
| | Agronomic traits | WGRS | Yano et al. (2016) |
| | Panicle architecture | WGRS | Bai et al. (2016) |
| | Mesocotyl elongation | WGRS | Wu et al. (2015) and Liu et al. (2019) |
| | Flowering date, resistance to rice yellow mottle virus and panicle architecture | WGRS | Cubry et al. (2020) |
| | Fertility restoration | WGRS | Li et al. (2020) |

(continued)

**Table 4** (continued)

| Crop | Trait | Genotyping method | Reference |
|---|---|---|---|
| Sesame | Resistance to *Phytophthora* blight | WGRS | Asekova et al. (2021) |
| | Seed coat color | Specific-locus amplified fragment sequencing | Cui et al. (2021) |
| Soybean | Seed protein and oil content | SNP genotyping using Illumina Infinium and GoldenGate assays | Hwang et al. (2014) |
| | Agronomic traits (hundred-seed weight, plant height, seed yield) | SNP genotyping (Illumina BARCSoySNP6K BeadChip) | Contreras-Soto et al. (2017) |
| | Photosynthesis-related traits (intercellular carbon dioxide concentration, net photosynthetic rate, stomatal conductance, transpiration rate) | SNP genotyping (NJAU 355K Soy SNP array) | Wang et al. (2020) |
| | Grain yield and related traits (seed maturity, plant height, seed weight) | GBS | Ravelombola et al. (2021) |
| Tomato | Fruit traits | SNP genotyping (51 K Axiom® tomato array) | Kim et al. (2021) |
| | Genetic architecture of 27 agronomic traits | WGRS | Ye et al. (2021) |
| Wheat | Grain yield and related traits | SNP genotyping (90 K and 660 K SNP array) | Li et al. (2019) |
| | Resistance to leaf rust, stem rust and stripe rust | SNP genotyping (Illumina iSelect 90 K SNP bead chip genotyping) | Joukhadar et al. (2020) |
| | Resistance to powdery mildew, leaf rust, yellow rust, and cold tolerance | GBS | Pang et al. (2021) |
| | Vegetative stage salinity stress tolerance | 35 K Axiom array | Chaurasia et al. (2020) |

MAS program. The low mapping resolution makes it very difficult to identify the candidate gene controlling the target trait. In such cases, integration of GWAS with QTL mapping can be helpful in improving mapping resolution of any locus and accelerate identification of associated candidate gene. While integration of the two approaches, it should be kept in mind that GWAS association panel possesses sufficient variability for trait under consideration. Further, association panel should be densely genotyped and must have sufficient markers on the chromosome that carry the QTL with large marker intervals. Higher the marker density, greater would be the chance to find SNPs very close to causal variant, or sometimes the trait-associated SNP may even fall within the candidate gene itself. Recently, a number of studies have integrated GWAS with QTL mapping in order to fine map genomic regions for various traits (Basu et al., 2019; Guo et al., 2019; Asekova et al., 2021). A total of 16 reliable SNPs linked with photosynthesis efficiency traits have been

identified in chickpea, using an integrated approach combining GWAS and QTL mapping, candidate gene-based association mapping, and expression profiling of candidate genes falling within the associated regions (Basu et al., 2019). Guo et al. (2019) identified candidate genes for seed vigor in rice by integrating GWAS with QTL mapping and expression profiling. In another study, Asekova et al. (2021) reported the fine mapping of one of the major *Phytopthora* blight (PB) resistance by applying GWAS. In this study, firstly a major genomic region for PB resistance was identified on chromosome 7 of *Sesamum* using a RIL population derived from the cross of Goenbaek × Milsung cross. Then, GWAS was conducted on a diverse panel of *Sesamum* genotypes for mapping of this trait, which revealed 10 SNPs associated with PB resistance. The identified SNPs were located within a 0.79 Mb region and co-located with the QTL intervals identified in RIL populations. The above study clearly suggests that integration of GWAS approach with QTL mapping can accelerate discovery of candidate genes for the target trait.

## 12 Integration of "Omics" Data in GWAS

The past decade saw revolution in the field "Omics," as during this period many new approaches for high-throughput analyses of transcriptome as well as other biochemical phenotypes were developed. It is now possible to generate transcriptome, proteome, ionome and metabolome data from large number of genotypes in a very short period of time with a low cost. These parameters represent important phenotypes and can be targeted for association analysis. Marker-trait association analysis that exploits transcriptomics or proteomics data has been accordingly redesignated as transcriptome wide association study (TWAS) or proteome-wide association analysis (PWAS), respectively. These approaches may help identification of eQTLs that can be directly linked to genes/pathways which may be important for variation in targeted phenotypes (Lee & Lee, 2021). Further integration of transcriptome, proteome, and metabolome data with conventional GWAS may represent a network-based augmentation for the identification of candidate genes/variants associated with complex traits. Therefore, currently in GWAS analysis, a greater emphasis is given on integrating multiple data associated with a target phenotype because this will not only minimize spurious associations but also would accelerate candidate gene discovery for complex traits.

## 13 Conclusion and Prospects

In summary, GWAS is a powerful approach for genetic mapping in plants that has a great potential to accelerate the utilization of genetic diversity in crop improvement. Over the past two decades, there has been refinement of association analysis methodology, software tools, and approaches towards interpretation of results which has

catalyzed wide-scale adoption of GWAS in plants. GWAS has been reported in different crops and has led to identification of hundreds of genomic regions for various traits. However, there has been less follow-up work toward identification of candidate gene governing important traits from the associated genomic regions, limiting utilization of trait-associated genomic regions in the breeding programs. Therefore, future studies on GWAS should focus on integrating data generated using other omics tools such as transcriptomics, proteomics, ionomics, metabolomics, as well as gene editing to accelerate the discovery of candidate genes for various complex traits in plants.

**Acknowledgments** Authors acknowledge the financial support received from SERB project grant EMR/2017/005133 and DBT project grant BT/Ag/Network/Wheat/2019-20.

## References

Abecasis, G. R., Altshuler, D., Auton, A., Brooks, L. D., Durbin, R. M., Gibbs, R. A., et al. (2010). A map of human genome variation from population-scale sequencing. *Nature, 467*(7319), 1061–1073.

Alseekh, S., Kostova, D., Bulut, M., & Fernie, A. R. (2021). Genome-wide association studies: Assessing trait characteristics in model and crop plants. *Cellular and Molecular Life Sciences, 78*(15), 5743–5754.

Alvarez-Prado, S., Sanchez, I., Cabrera-Bosquet, L., et al. (2019). To clean or not to clean phenotypic datasets for outlier plants in genetic analyses? *Journal of Experimental Botany, 70*(15), 3693–3698. https://doi.org/10.1093/jxb/erz191

Asekova, S., Oh, E., Kulkarni, K. P., Siddique, M. I., Lee, M. H., Kim, J. I., Lee, J. D., Kim, M., Oh, K. W., Ha, T. J., Kim, S. U., & Cho, K. S. (2021). An integrated approach of QTL mapping and genome-wide association analysis identifies candidate genes for *Phytophthora* blight resistance in sesame (*Sesamum indicum* L.). *Frontiers in Plant Science, 12*, 604709.

Atwell, S., Huang, Y. S., Vilhjálmsson, B. J., Willems, G., Horton, M., Li, Y., Meng, D., Platt, A., Tarone, A. M., Hu, T. T., Jiang, R., Muliyati, N. W., Zhang, X., Amer, M. A., Baxter, I., Brachi, B., Chory, J., Dean, C., Debieu, M., de Meaux, J., Ecker, J. R., Faure, N., Kniskern, J. M., Jones, J. D., Michael, T., Nemri, A., Roux, F., Salt, D. E., Tang, C., Todesco, M., Traw, M. B., Weigel, D., Marjoram, P., Borevitz, J. O., Bergelson, J., & Nordborg, M. (2010). Genome-wide association study of 107 phenotypes in *Arabidopsis thaliana* inbred lines. *Nature, 465*(7298), 627–631.

Bai, X., Zhao, H., Huang, Y., Xie, W., Han, Z., Zhang, B., Guo, Z., Yang, L., Dong, H., Xue, W., Li, G., Hu, G., Hu, Y., & Xing, Y. (2016). Genome-wide association analysis reveals different genetic control in panicle architecture between *Indica* and *Japonica* rice. *The Plant Genome, 9*(2), 1–10.

Barnett, V., & Lewis, T. (1994). *Outliers in statistical data* (3rd ed.). Wiley. ISBN:978-0-471-93094-5.

Basu, U., Bajaj, D., Sharma, A., et al. (2019). Genetic dissection of photosynthetic efficiency traits for enhancing seed yield in chickpea. *Plant, Cell & Environment, 42*, 158–173. https://doi.org/10.1111/pce.13319

Benjamini, Y., & Hochberg, Y. (1995). Controlling the false discovery rate: A practical and powerful approach to multiple testing. *JRSS(B), 57*, 289–300.

Benjamini, Y., & Yekutieli, D. (2001). The control of the false discovery rate under dependence. *The Annals of Statistics, 29*, 1165–1188.

Blake, V., Birkett, C. L., Matthews, D. E., Hane, D., Bradbury, P., & Jannink, J. (2015). The triticeae toolbox: Combining phenotype and genotype data to advance small-grains breeding. *The Plant Genome*. https://doi.org/10.3835/PlantGenome2014.12.0099

Borevitz, J. O., Hazen, S. P., Michael, T. P., Morris, G. P., Baxter, I. R., Hu, T. T., Chen, H., Werner, J. D., Nordborg, M., Salt, D. E., Kay, S. A., Chory, J., Weigel, D., Jones, J. D., & Ecker, J. R. (2007). Genome-wide patterns of single-feature polymorphism in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences of the United States of America, 104*(29), 12057–12062.

Bradbury, P. J., Zhang, Z., Kroon, D. E., Casstevens, T. M., Ramdoss, Y., & Buckler, E. S. (2007). TASSEL: Software for association mapping of complex traits in diverse samples. *Bioinformatics, 23*(19), 2633–2635.

Browning. (2008). PRESTO: Rapid calculation of order statistic distributions and multiple-testing adjusted P-values via permutation for one and two-stage genetic association studies. *BMC Bioinformatics, 9*, 309.

Camargo, A., Papadopoulou, D., Spyropoulou, Z., Vlachonasios, K., Doonan, J. H., & Gay, A. P. (2014). Objective definition of rosette shape variation using a combined computer vision and data mining approach. *PLoS One, 9*, e96889. https://doi.org/10.1371/journal.pone.0096889

Chan, E. K., Rowe, H. C., Hansen, B. G., & Kliebenstein, D. J. (2010a). The complex genetic architecture of the metabolome. *PLoS Genetics, 6*(11), e1001198.

Chan, E. K., Rowe, H. C., & Kliebenstein, D. J. (2010b). Understanding the evolution of defense metabolites in *Arabidopsis thaliana* using genome-wide association mapping. *Genetics, 185*(3), 991–1007.

Chaurasia, S., Singh, A. K., Songachan, L. S., Sharma, A. D., Bhardwaj, R., & Singh, K. (2020). Multi-locus genome-wide association studies reveal novel genomic regions associated with vegetative stage salt tolerance in bread wheat (*Triticum aestivum* L.). *Genomics, 112*(6), 4608–4621.

Chen, J., Shrestha, R., Ding, J., Zheng, H., Mu, C., Wu, J., & Mahuku, G. (2016a). Genome-wide association study and QTL mapping reveal genomic loci associated with *Fusarium* ear rot resistance in tropical maize germplasm. *G3 (Bethesda), 6*(12), 3803–3815.

Chen, W., Wang, W., Peng, M., Gong, L., Gao, Y., Wan, J., Wang, S., Shi, L., Zhou, B., Li, Z., Peng, X., Yang, C., Qu, L., Liu, X., & Luo, J. (2016b). Comparative and parallel genome-wide association studies for metabolic and agronomic traits in cereals. *Nature Communications, 7*, 12767.

Chen, E., Huang, X., Tian, Z., Wing, R. A., & Han, B. (2019). The genomics of *Oryza* species provides insights into rice domestication and heterosis. *Annual Review of Plant Biology, 70*, 639–665.

Contreras-Soto, R. I., Mora, F., de Oliveira, M. A. R., Higashi, W., Scapim, C. A., & Schuster, I. (2017). A genome-wide association study for agronomic traits in soybean using SNP markers and SNP based haplotype analysis. *PLoS One, 12*(2), e0171105.

Costa, C., Schurr, U., Loreto, F., Menesatti, P., & Carpentier, S. (2019). Plant phenotyping research trends, a science mapping approach. *Frontiers in Plant Science, 9*, 1933. https://doi.org/10.3389/fpls.2018.01933

Cubry, P., Pidon, H., Ta, K. N., Tranchant-Dubreuil, C., Thuillet, A. C., Holzinger, M., Adam, H., Kam, H., Chrestin, H., Ghesquière, A., François, O., Sabot, F., Vigouroux, Y., Albar, L., & Jouannic, S. (2020). Genome wide association study pinpoints key agronomic QTLs in African rice *Oryza glaberrima*. *Rice (NY), 13*(1), 66.

Cui, C., Liu, Y., Liu, Y., Cui, X., Sun, Z., Du, Z., Wu, K., Jiang, X., Mei, H., & Zheng, Y. (2021). Genome-wide association study of seed coat color in sesame (*Sesamum indicum* L.). *PLoS One, 16*(5), e0251526.

Dickson, S. P., Wang, K., Krantz, I., Hakonarson, H., & Goldstein, D. B. (2010). Rare variants create synthetic genome-wide associations. *PLoS Biology, 8*(1), e1000294.

Endelman, J. (2019). Ridge regression and other kernels for genomic selection, rrBLUP, Version 4.6.1. https://CRAN.R-project.org/package=rrBLUP

Ertiro, B. T., Labuschagne, M., Olsen, M., Das, B., Prasanna, B. M., & Gowda, M. (2020). Genetic dissection of nitrogen use efficiency in tropical maize through genome-wide association and genomic prediction. *Frontiers in Plant Science, 11*, 474.

Evanno, G., Regnaut, S., & Goudet, J. (2005). Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. *Molecular Ecology, 14*(8), 2611–2620. https://doi.org/10.1111/j.1365-294X.2005.02553.x

Exposito-Alonso, M., 500 Genomes Field Experiment Team, Burbano, H. A., Bossdorf, O., Nielsen, R., & Weigel, D. (2019). Natural selection on the Arabidopsis thaliana genome in present and future climates. *Nature, 573*(7772), 126–129.

Fiorani, F., & Schurr, U. (2013). Future scenarios for plant phenotyping. *Annual Review of Plant Biology, 64*, 267–291. https://doi.org/10.1146/annurev-arplant-050312-120137

Fuchsberger, C., Taliun, D., Pramstaller, P. P., et al. (2012). GWAtoolbox: An R package for fast quality control and handling of genome-wide association studies meta-analysis data. *Bioinformatics, 28*(3), 444–445. https://doi.org/10.1093/bioinformatics/btr679

Furlotte, N. A., & Eskin, E. (2015). Efficient multiple-trait association and estimation of genetic correlation using the matrix-variate linear mixed model. *Genetics, 200*(1), 59–68. https://doi.org/10.1534/genetics.114.171447

Fusari, C. M., Kooke, R., Lauxmann, M. A., Annunziata, M. G., Enke, B., Hoehne, M., Krohn, N., Becker, F. F. M., Schlereth, A., Sulpice, R., Stitt, M., & Keurentjes, J. J. B. (2017). Genome-wide association mapping reveals that specific and pleiotropic regulatory mechanisms fine-tune central metabolism and growth in *Arabidopsis*. *Plant Cell, 29*(10), 2349–2373.

Gao, X., Becker, L. C., Becker, D. M., Starmer, J. D., & Province, M. A. (2010). Avoiding the high Bonferroni penalty in genome-wide association studies. *Genetic Epidemiology, 34*(1), 100–105.

Gao, J., Wang, S., Zhou, Z., Wang, S., Dong, C., Mu, C., Song, Y., Ma, P., Li, C., Wang, Z., He, K., Han, C., Chen, J., Yu, H., & Wu, J. (2019). Linkage mapping and genome-wide association reveal candidate genes conferring thermotolerance of seed-set in maize. *Journal of Experimental Botany, 70*(18), 4849–4864.

Glaubitz, J. C., Casstevens, T. M., Lu, F., Harriman, J., Elshire, R. J., Sun, Q., et al. (2014). TASSEL-GBS: A high capacity genotyping by sequencing analysis pipeline. *PLoS One, 9*, e90346. https://doi.org/10.1371/journal.pone.0090346

Gogarten, S. M., Bhangale, T., Conomos, M. P., Laurie, C. A., McHugh, C. P., Painter, I., Zheng, X., Crosslin, D. R., Levine, D., Lumley, T., Nelson, S. C., Rice, K., Shen, J., Swarnkar, R., Weir, B. S., & Laurie, C. C. (2012). GWASTools: An R/Bioconductor package for quality control and analysis of genome-wide association studies. *Bioinformatics, 28*(24), 3329–3331. https://doi.org/10.1093/bioinformatics/bts610

Grubbs, F. E. (1950). Sample criteria for testing outlying observations. *Annals of Mathematical Statistics, 21*, 27–58.

Guo, T., Yang, J., Li, D., Sun, K., Luo, L., Xiao, W., Wang, J., Liu, Y., Wang, S., Wang, H., & Chen, Z. (2019). Integrating GWAS, QTL, mapping and RNA-seq to identify candidate genes for seed vigor in rice (Oryza sativaL.). *Molecular Breeding, 39*(6), 1–16. https://doi.org/10.1007/s11032-019-0993-4

Gupta, P. K., Kulwalb, P. L., & Jaiswal, V. (2019). Association mapping in plants in the post-GWAS genomics era. *Advances in Genetics*. https://doi.org/10.1016/bs.adgen.2018.12.00

Gyawali, S., Chao, S., Vaish, S. S., Singh, S. P., Rehman, S., Vishwakarma, S. R., & Verma, R. P. S. (2018). Genome wide association studies (GWAS) of spot blotch resistance at the seedling and the adult plant stages in a collection of spring barley. *Molecular Breeding, 38*, 62.

Hellwege, J. N., Keaton, J. M., Giri, A., Gao, X., Velez Edwards, D. R., & Edwards, T. L. (2017). Population stratification in genetic association studies. *Current Protocols in Human Genetics, 95*, 1.22.1–1.22.23. https://doi.org/10.1002/cphg.48

Holland, J. B. (2007). Genetic architecture of complex traits in plants. *Current Opinion in Plant Biology, 10*(2), 156–161. https://doi.org/10.1016/j.pbi.2007.01.003

Holm, S. (1979). A simple sequentially rejective multiple test procedure. *Scandinavian Journal of Statistics, 6*(2), 65–70. JSTOR 4615733. MR 0538597.

Huang, X., Wei, X., Sang, T., Zhao, Q., Feng, Q., Zhao, Y., Li, C., Zhu, C., Lu, T., Zhang, Z., Li, M., Fan, D., Guo, Y., Wang, A., Wang, L., Deng, L., Li, W., Lu, Y., Weng, Q., Liu, K., Huang, T., Zhou, T., Jing, Y., Li, W., Lin, Z., Buckler, E. S., Qian, Q., Zhang, Q. F., Li, J., & Han,

B. (2010). Genome-wide association studies of 14 agronomic traits in rice landraces. *Nature Genetics, 42*(11), 961–967.

Hwang, E. Y., Song, Q., Jia, G., Specht, J. E., Hyten, D. L., Costa, J., & Cregan, P. B. (2014). A genome-wide association study of seed protein and oil content in soybean. *BMC Genomics, 15*, 1.

Igolkina, A. A., Meshcheryakov, G., Gretsova, M. V., et al. (2020). Multi-trait multi-locus SEM model discriminates SNPs of different effects. *BMC Genomics, 21*, 490. https://doi.org/10.1186/s12864-020-06833-2

Ingvarsson, P. K., & Street, N. R. (2011). Association genetics of complex traits in plants. *New Phytologist, 189*, 909–922. https://doi.org/10.1111/j.1469-8137.2010.03593.x

Jaiswal, V., Gahlaut, V., Meher, P. K., Mir, R. R., Jaiswal, J. P., Rao, A. R., et al. (2016). Genome wide single locus single trait, multi-locus and multi-trait association mapping for some important agronomic traits in common wheat (T. aestivum L.). *PLoS One, 11*(7), e0159343. https://doi.org/10.1371/journal.pone.0159343

James, R. A., Davenport, R. J., Munns, R. (2006). Physiological characterization of two genes for Na+ exclusion in durum wheat, Nax1 and Nax2. *Plant Physiology 142*(4), 1537–1547. https://doi.org/10.1104/pp.106.086538

Jennings, H. S. (1917). The numerical results of diverse systems of breeding, with respect to two pairs of characters, linked or independent, with special relation to the effects of linkage. *Genetics, 2*, 97–154.

Johannes, F., Porcher, E., Teixeira, F. K., Saliba-Colombani, V., Simon, M., Agier, N., Bulski, A., Albuisson, J., Heredia, F., Audigier, P., Bouchez, D., Dillmann, C., Guerche, P., Hospital, F., & Colot, V. (2009). Assessing the impact of transgenerational epigenetic variation on complex traits. *PLoS Genetics, 5*, e10000530.

Joiret, M., Mahachie John, J. M., Gusareva, E. S., et al. (2019). Confounding of linkage disequilibrium patterns in large scale DNA based gene-gene interaction studies. *BioData Mining, 12*, 11. https://doi.org/10.1186/s13040-019-0199-7

Joukhadar, R., Hollaway, G., Shi, F., Kant, S., Forrest, K., Wong, D., Petkowski, J., Pasam, R., Tibbits, J., Bariana, H., Bansal, U., Spangenberg, G., Daetwyler, H., Gendall, T., & Hayden, M. (2020). Genome-wide association reveals a complex architecture for rust resistance in 2300 worldwide bread wheat accessions screened under various Australian conditions. *Theoretical and Applied Genetics, 133*(9), 2695–2712.

Kaeuffer, R., Réale, D., Coltman, D. W., & Pontier, D. (2007). Detecting population structure using STRUCTURE software: Effect of background linkage disequilibrium. *Heredity (Edinb), 99*(4), 374–380. https://doi.org/10.1038/sj.hdy.6801010

Kang, H. M., Zaitlen, N. A., Wade, C. M., et al. (2008). Efficient control of population structure in model organism association mapping. *Genetics, 178*(3), 1709–1723. https://doi.org/10.1534/genetics.107.080101

Kim, S., Zhao, K., Jiang, R., Molitor, J., Borevitz, J. O., Nordborg, M., & Marjoram, P. (2006). Association mapping with single-feature polymorphisms. *Genetics, 173*, 1125–1133.

Kim, C. K., Seol, Y. J., Lee, D. J., Jeong, I. S., Yoon, U. H., Lee, G. S., Hahn, J. H., & Park, D. S. (2013). NABIC marker database: A molecular markers information network of agricultural crops. *Bioinformation, 9*(17), 887–888. https://doi.org/10.6026/97320630009887

Kim, B., Dai, X., Zhang, W., Zhuang, Z., Sanchez, D. L., Lübberstedt, T., Kang, Y., Udvardi, M. K., Beavis, W. D., Xu, S., & Zhao, P. X. (2019). GWASpro: A high-performance genome-wide association analysis server. *Bioinformatics, 35*(14), 2512–2514. https://doi.org/10.1093/bioinformatics/bty989

Kim, M., Nguyen, T. T. P., Ahn, J. H., Kim, G. J., & Sim, S. C. (2021). Genome-wide association study identifies QTL for eight fruit traits in cultivated tomato (*Solanum lycopersicum* L.). *Horticulture Research, 8*, 203.

Klasen, J., Barbez, E., Meier, L., et al. (2016). A multi-marker association method for genome-wide association studies without the need for population structure correction. *Nature Communications, 7*, 13299. https://doi.org/10.1038/ncomms13299

Klein, R. J., Zeiss, C., Chew, E. Y., Tsai, J. Y., Sackler, R. S., Haynes, C., Henning, A. K., SanGiovanni, J. P., Mane, S. M., Mayne, S. T., Bracken, M. B., Ferris, F. L., Ott, J., Barnstable,

C., & Hoh, J. (2005). Complement factor H polymorphism in age-related macular degeneration. *Science, 308*(5720), 385–389.

Korte, A., & Farlow, A. (2013). The advantages and limitations of trait analysis with GWAS: A review. *Plant Methods, 9*, 29. https://doi.org/10.1186/1746-4811-9-29

Korte, A., Vilhjálmsson, B. J., Segura, V., Platt, A., Long, Q., & Nordborg, M. (2012). A mixed-model approach for genome-wide association studies of correlated traits in structured populations. *Nature Genetics, 44*, 1066–1071.

Köttgen, A., Pattaro, C., Böger, C. A., Fuchsberger, C., Olden, M., et al. (2010). New loci associated with kidney function and chronic kidney disease. *Nature Genetics, 42*, 376–384.

Kulaeva, O. A., Zhernakov, A. I., Afonin, A. M., Boikov, S. S., Sulima, A. S., Tikhonovich, I. A., et al. (2017). Pea Marker Database (PMD) – A new online database combining known pea (Pisum sativum L.) gene-based markers. *PLoS One, 12*(10), e0186713. https://doi.org/10.1371/journal.pone.0186713

Lasky-Su, J. (2017). Chapter 19: Statistical techniques for genetic analysis. *Clinical and Translational Science*, 347–362.

Lee, T., & Lee, I. (2021). Genome-wide association studies in Arabidopsis thaliana: Statistical analysis and network-based augmentation of signals. *Methods in Molecular Biology, 2200*, 187–210. https://doi.org/10.1007/978-1-0716-0880-7_9

Lewontin, R. C., & Kojima, K. (1960). The evolutionary dynamics of complex polymorphisms. *Evolution, 14*, 458–472. https://doi.org/10.1111/j.1558-5646.1960.tb03113.x

Li, J., Das, K., Fu, G., Li, R., & Wu, R. (2011). The bayesian lasso for genome-wide associations studies. *Bioinformatics, 27*(4), 516–523.

Li, L., Zhang, Q., & Huang, D. (2014a). A review of imaging techniques for plant phenotyping. *Sensors, 14*, 20078–20111. https://doi.org/10.3390/s141120078

Li, M., Liu, X., Bradbury, P., Yu, J., Zhang, Y. M., Todhunter, R. J., Buckler, E. S., & Zhang, Z. (2014b). Enrichment of statistical power for genome-wide association studies. *BMC Biology, 17*(12), 73. https://doi.org/10.1186/s12915-014-0073-5

Li, F., Wen, W., Liu, J., Zhang, Y., Cao, S., He, Z., Rasheed, A., Jin, H., Zhang, C., Yan, J., Zhang, P., Wan, Y., & Xia, X. (2019). Genetic architecture of grain yield in bread wheat based on genome-wide association studies. *BMC Plant Biology, 19*, 168.

Li, P., Zhou, H., Yang, H., Xia, D., Liu, R., Sun, P., Wang, Q., Gao, G., Zhang, Q., Wang, G., & He, Y. (2020). Genome-wide association studies reveal the genetic basis of fertility restoration of CMS-WA and CMS-HL in xian/indica and aus accessions of rice (*Oryza sativa* L.). *Rice (NY), 13*(1), 11.

Lin, D. Y., Hu, Y., & Huang, B. E. (2008). Simple and efficient analysis of disease association with missing genotype data. *American Journal of Human Genetics, 82*(2), 444–452. https://doi.org/10.1016/j.ajhg.2007.11.004

Lipka, A. E., Tian, F., Wang, Q., Peiffer, J., Li, M., Bradbury, P. J., Gore, M. A., Buckler, E. S., & Zhang, Z. (2012). GAPIT: Genome association and prediction integrated tool. *Bioinformatics, 28*(18), 2397–2399.

Lipka, A. E., Kandianis, C. B., Hudson, M. E., Yu, J., Drnevich, J., Bradbury, P. J., & Gore, M. A. (2015). From association to prediction: Statistical methods for the dissection and selection of complex traits in plants. *Current Opinion in Plant Biology, 24*, 110–118. https://doi.org/10.1016/j.pbi.2015.02.010

Lippert, C., Listgarten, J., Liu, Y., Kadie, C. M., Davidson, R. I., & Heckerman, D. (2011). FaST linear mixed models for genome-wide association studies. *Nature Methods, 8*(10), 833–835. https://doi.org/10.1038/nmeth.1681

Lippert, C., Casale, F. P., Rakitsch, B., & Stegle, O. (2014). LIMIX: Genetic analysis of multiple traits. *bioRxiv*. https://doi.org/10.1101/003905

Liu, X., Huang, M., Fan, B., Buckler, E. S., & Zhang, Z. (2016). Iterative usage of fixed and random effect models for powerful and efficient genome-wide association studies. *PLoS Genetics, 12*(2), e1005767. https://doi.org/10.1371/journal.pgen.1005767

Liu, H., Zhan, J., Li, J., Lu, X., Liu, J., Wang, Y., Zhao, Q., & Ye, G. (2019). Genome-wide association study (GWAS) for mesocotyl elongation in rice (*Oryza sativa* L.) under multiple culture conditions. *Genes (Basel), 11*(1), 49.

Mackay, I., & Powell, W. (2007). Methods for linkage disequilibrium mapping in crops. *Trends in Plant Science, 12*(2), 57–63. https://doi.org/10.1016/j.tplants.2006.12.001

Mackay, T. F., Stone, E. A., & Ayroles, J. F. (2009). The genetics of quantitative traits: Challenges and prospects. *Nature Reviews. Genetics, 10*(8), 565–577.

Mägi, R., & Morris, A. P. (2010). GWAMA: Software for genome-wide association meta-analysis. *BMC Bioinformatics, 11*, 288. https://doi.org/10.1186/1471-2105-11-288

Manolio, T. A., Collins, F. S., Cox, N. J., Goldstein, D. B., Hindorff, L. A., Hunter, D. J., McCarthy, M. I., Ramos, E. M., Cardon, L. R., & Chakravarti, A. (2009). Finding the missing heritability of complex diseases. *Nature, 461*(7265), 747–753.

Marees, A. T., de Kluiver, H., Stringer, S., Vorspan, F., Curis, E., Marie-Claire, C., & Derks, E. M. (2019). A tutorial on conducting genome-wide association studies: Quality control and statistical analysis. *Molecular Ecology Resources, 19*(3), 639–647. https://doi.org/10.1111/1755-0998.12995

Matthews, D. E., Carollo, V. L., Lazo, G. R., & Anderson, O. D. (2003). GrainGenes, the genome database for small-grain crops. *Nucleic Acids Research, 31*(1), 183–186. https://doi.org/10.1093/nar/gkg058

Milner, S. G., Jost, M., Taketa, S., et al. (2019). Genebank genomics highlights the diversity of a global barley collection. *Nature Genetics, 51*, 319–326. https://doi.org/10.1038/s41588-018-0266-x

Minamikawa, M. F., Nonaka, K., Kaminuma, E., Kajiya-Kanegae, H., Onogi, A., Goto, S., Yoshika, T., Imai, A., Hamada, H., Hayashi, T., Matsumoto, S., Katayose, Y., Toyoda, A., Fujiyama, A., Nakamura, Y., Shimizu, T., & Iwata, H. (2017). Genome-wide association study and genomic prediction in citrus: Potential of genomics-assisted breeding for fruit quality traits. *Scientific Reports, 7*, 4721.

Otyama, P. I., Wilkey, A., Kulkarni, R., et al. (2019). Evaluation of linkage disequilibrium, population structure, and genetic diversity in the U.S. peanut mini core collection. *BMC Genomics, 20*(1), 481. https://doi.org/10.1186/s12864-019-5824-9

Owens, B. F., Lipka, A. E., Magallanes-Lundback, M., Tiede, T., Diepenbrock, C. H., Kandianis, C. B., Kim, E., Cepela, J., Mateos-Hernandez, M., Buell, C. R., Buckler, E. S., DellaPenna, D., Gore, M. A., & Rocheford, T. (2014). A foundation for provitamin A biofortification of maize: Genome-wide association and genomic prediction models of carotenoid levels. *Genetics, 198*(4), 1699–1716.

Pagano, M., & Gauvreau, K. (2000). *Principles of biostatistics*. Duxbury Press.

Pang, Y., Wu, Y., Liu, C., Li, W., St. Amand, P., Bernardo, A., Wang, D., Dong, L., Yuan, X., Zhang, H., Zhao, M., Li, L., Wang, L., He, F., Liang, Y., Yan, Q., Lu, Y., Su, Y., Jiang, H., Wu, J., Li, A., Kong, L., Bai, G., & Liu, S. (2021). High-resolution genome-wide association study and genomic prediction for disease resistance and cold tolerance in wheat. *Theoretical and Applied Genetics, 134*, 2857–2873.

Pavan, S., Delvento, C., Ricciardi, L., Lotti, C., Ciani, E., & D'Agostino, N. (2020). Recommendations for choosing the genotyping method and best practices for quality control in crop genome-wide association studies. *Frontiers in Genetics, 5*(11), 447. https://doi.org/10.3389/fgene.2020.00447

Price, A., Patterson, N., Plenge, R., Weinblatt, M., Shadick, N., et al. (2006). Principal components analysis corrects for stratification in genome-wide association studies. *Nature Genetics, 38*, 904–909.

Pritchard, J. K., Stephens, M., & Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics, 155*(2), 945–959. https://doi.org/10.1093/genetics/155.2.945

Purcell, S., Neale, B., Todd-Brown, K., Thomas, L., Ferreira, M. A. R., Bender, D., Maller, J., Sklar, P., de Bakker, P. I. W., Daly, M. J., & Sham, P. C. (2007). PLINK: A toolset for whole-genome association and population-based linkage analysis. *American Journal of Human Genetics, 81*.

Ravelombola, W., Qin, J., Shi, A., Song, Q., Yuan, J., Wang, F., Chen, P., Yan, L., Feng, Y., Zhao, T., Meng, Y., Guan, K., Yang, C., & Zhang, M. (2021). Genome-wide association study and genomic selection for yield and related traits in soybean. *PLoS One, 16*(8), e0255761.

Reich, D. E., Cargill, M., Bolk, S., Ireland, J., Sabeti, P. C., Richter, D. J., et al. (2001). Linkage disequilibrium in the human genome. *Nature, 411*(6834), 199–204. https://doi.org/10.1038/35075590

Reimann, C., Filzmoser, P., Garrett, R. G., & Dutter, R. (2008). Multivariate outlier detection. In *Statistical data analysis explained: Applied environmental statistics with R* (pp. 201–210). Wiley.

Rossum, B., Kruijer, W., Eeuwijk, F., Boer, M., Malosetti, M., Bustos-Korts, D., Millet, E., Paulo, J., Verouden, M., Wehrens, R., & Zheng, C. (2021). statgenGWAS: Genome wide association studies. Version 1.0.7. https://cran.r-project.org/web/packages/statgenGWAS

Rousseeuw, P. J., & Hubert, M. (2011). Robust statistics for outlier detection. *WIREs Data Mining and Knowledge Discovery, 1*, 73–79.

Segura, V., Vilhjálmsson, B., Platt, A., et al. (2012). An efficient multi-locus mixed-model approach for genome-wide association studies in structured populations. *Nature Genetics, 44*, 825–830.

Shi, S., Yuan, N., Yang, M., Du, Z., Wang, J., Sheng, X., Wu, J., & Xiao, J. (2018). Comprehensive assessment of genotype imputation performance. *Human Heredity, 83*, 107–116. https://doi.org/10.1159/000489758

Sidák, Z. (1967). Rectangular confidence regions for the means of multivariate normal distributions. *Journal of the American Statistical Association, 62*(318), 626–633.

Tam, V., Patel, N., Turcotte, M., Bossé, Y., Paré, G., & Meyre, D. (2019). Benefits and limitations of genome-wide association studies. *Nature Reviews. Genetics, 20*, 467–484.

Tamba, C. L., & Zhang, Y. M. (2018). A fast mrMLM algorithm for multi-locus genome-wide association studies. *bioRxiv*. https://doi.org/10.1101/341784

Teslovich, T. M., Musunuru, K., Smith, A. V., Edmondson, A. C., Stylianou, I. M., et al. (2010). Biological, clinical and population relevance of 95 loci for blood lipids. *Nature, 466*, 707–713.

The International HapMap Consortium. (2003). The international HapMap project. *Nature, 426*, 789–796. https://doi.org/10.1038/nature02168

The Wellcome Trust Case Control Consortium. (2007). Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature, 447*, 661–678.

Tian, D., Wang, P., Tang, B., Teng, X., Li, C., Liu, X., Zou, D., Song, S., & Zhang, Z. (2020). GWAS Atlas: A curated resource of genome-wide variant-trait associations in plants and animals. *Nucleic Acids Research, 48*(D1), D927–D932. https://doi.org/10.1093/nar/gkz828

Tibbs-Cortes, L., Zhang, Z., & Yu, J. (2021). Status and prospects of genome-wide association studies in plants. *The Plant Genome, 14*(1), e20077.

Tsai, H. Y., Janss, L. L., Andersen, J. R., et al. (2020). Genomic prediction and GWAS of yield, quality and disease-related traits in spring barley and winter wheat. *Scientific Reports, 10*, 3347. https://doi.org/10.1038/s41598-020-60203-2

Turley, P., Walters, R. K., Maghzian, O., et al. (2018). Multi-trait analysis of genome-wide association summary statistics using MTAG. *Nature Genetics, 50*, 229–237. https://doi.org/10.1038/s41588-017-0009-4

Utz, H. F. (2003). *'PLABSTAT' a computer program for statistical analysis of plant breeding experiments*. Institute for Plant Breeding, Seed Science and Population Genetics, University of Hohenheim.

Varshney, R. K., Bohra, A., Yu, J., Graner, A., Zhang, Q., & Sorrells, M. E. (2021). Designing future crops: Genomics-assisted breeding comes of age. *Trends in Plant Science, 26*(6), 631–649. https://doi.org/10.1016/j.tplants.2021.03.010

Wang, Q., Tian, F., Pan, Y., Buckler, E. S., & Zhang, Z. (2014). A SUPER powerful method for genome wide association study. *PLoS One, 9*(9), e107684. https://doi.org/10.1371/journal.pone.0107684

Wang, X., Wang, H., Liu, S., Ferjani, A., Li, J., Yan, J., Yang, X., & Qin, F. (2016a). Genetic variation in ZmVPP1 contributes to drought tolerance in maize seedlings. *Nature Genetics, 48*, 1233–1241.

Wang, S. B., Feng, J. Y., Ren, W. L., et al. (2016b). Improving power and accuracy of genome-wide association studies via a multi-locus mixed linear model methodology. *Scientific Reports, 6*, 19444. https://doi.org/10.1038/srep19444

Wang, J., Yan, C., Li, Y., Li, C., Zhao, X., Yuan, C., Sun, Q., & Shan, S. (2019). GWAS discovery of candidate genes for yield-related traits in peanut and support from earlier QTL mapping studies. *Genes (Basel), 10*(10), 803.

Wang, L., Yang, Y., Zhang, S., Che, Z., Yuan, W., & Yu, D. (2020). GWAS reveals two novel loci for photosynthesis-related traits in soybean. *Molecular Genetics and Genomics, 295*(3), 705–716.

Wen, Y. J., Zhang, H., Ni, Y. L., Huang, B., Zhang, J., Feng, J. Y., Wang, S. B., Dunwell, J. M., Zhang, Y. M., & Wu, R. (2018). Methodological implementation of mixed linear models in multi-locus genome-wide association studies. *Briefings in Bioinformatics, 19*(4), 700–712. https://doi.org/10.1093/bib/bbw145

Westfall, P. H., & Young, S. S. (1993). *Resampling-based multiple testing: Examples and methods for P-value adjustment* (p. 340). Wiley. ISSN:0271-6356.

Wigginton, J. E., Cutler, D. J., & Abecasis, G. R. (2005). A note on exact tests of Hardy-Weinberg equilibrium. *American Journal of Human Genetics, 76*(5), 887–893. https://doi.org/10.1086/429864

Willer, C. J., Li, Y., & Abecasis, G. R. (2010). METAL: Fast and efficient meta-analysis of genomewide association scans. *Bioinformatics, 26*(17), 2190–2191. https://doi.org/10.1093/bioinformatics/btq340

Wright, S. (1918). On the nature of size factors. *Genetics, 3*, 367–374.

Wright, S. (1921). Correlation and causation. *Journal of Agricultural Research, 20*, 557–585.

Wu, J., Feng, F., Lian, X., Teng, X., Wei, H., Yu, H., Xie, W., Yan, M., Fan, P., Li, Y., Ma, X., Liu, H., Yu, S., Wang, G., Zhou, F., Luo, L., & Mei, H. (2015). Genome-wide Association Study (GWAS) of mesocotyl elongation based on re-sequencing approach in rice. *BMC Plant Biology, 15*, 218.

Wu, S., Alseekh, S., Cuadros-Inostroza, Á., Fusari, C. M., Mutwil, M., Kooke, R., Keurentjes, J. B., Fernie, A. R., Willmitzer, L., & Brotman, Y. (2016). Combined use of genome-wide association data and correlation networks unravels key regulators of primary metabolism in *Arabidopsis thaliana*. *PLoS Genetics, 12*(10), e1006363.

Wu, S., Tohge, T., Cuadros-Inostroza, Á., Tong, H., Tenenboim, H., Kooke, R., Méret, M., Keurentjes, J. B., Nikoloski, Z., Fernie, A. R., Willmitzer, L., & Brotman, Y. (2018). Mapping the *Arabidopsis* metabolic landscape by untargeted metabolomics at different environmental conditions. *Molecular Plant, 11*(1), 118–134.

Wu, Y., Zhou, Z., Dong, C., Chen, J., Ding, J., Zhang, X., Mu, C., Chen, Y., Li, X., Li, H., Han, Y., Wang, R., Sun, X., Li, J., Dai, X., Song, W., Chen, W., & Wu, J. (2020). Linkage mapping and genome-wide association study reveals conservative QTL and candidate genes for *Fusarium* rot resistance in maize. *BMC Genomics, 21*(1), 357.

Yang, X., Li, P., Yang, Z., & Chenwu, X. (2017). Genetic mapping of quantitative trait loci in crops. *The Crop Journal, 5*(2), 175–184. https://doi.org/10.1016/j.cj.2016.06.003

Yano, K., Yamamoto, E., Aya, K., Takeuchi, H., Lo, P. C., Hu, L., Yamasaki, M., Yoshida, S., Kitano, H., Hirano, K., & Matsuoka, M. (2016). Genome-wide association study using whole-genome sequencing rapidly identifies new genes influencing agronomic traits in rice. *Nature Genetics, 48*(8), 927–934.

Ye, J., Wang, X., Wang, W., Yu, H., Guo, A., Li, C., Sun, P., Wang, X., Li, H., Ouyang, B., Zhang, J., Zhang, Y., Han, H., Giovannoni, J. J., Fei, Z., & Ye, Z. (2021). Genome-wide association study reveals the genetic architecture of 27 agronomic traits in tomato. *Plant Physiology, 186*(4), 2078–2092.

Yu, J., & Buckler, E. S. (2006). Genetic association mapping and genome organization of maize. *Current Opinion in Biotechnology, 17*(2), 155–160.

Yu, J., Pressoir, G., Briggs, W. H., Bi, I. V., Yamasaki, M., & Doebley, J. F. (2006). A unified mixed-model method for association mapping that accounts for multiple levels of relatedness. *Nature Genetics, 38*, 203–208. https://doi.org/10.1038/ng1702

Zhan, X., Zhao, N., Plantinga, A., Thornton. T. A., Conneely, K. N., Epstein, M. P., & Wu, M.C. (2017). Powerful Genetic Association Analysis for Common or Rare Variants with High-Dimensional Structured Traits. *Genetics, 206*(4), 1779–1790. https://doi.org/10.1534/genetics.116.199646

Zhang, Y. M., Mao, Y., Xie, C., Smith, H., Luo, L., & Xu, S. (2005). Mapping quantitative trait loci using naturally occurring genetic variance among commercial inbred lines of maize. *Genetics, 169*, 2267–2275. https://doi.org/10.1534/genetics.104.033217

Zhang, X., Zou, F., & Wang, W. (2008). FastANOVA: An efficient algorithm for genome-wide association study. *KDD*, 821–829.

Zhang, Z., Ersoz, E., Lai, C. Q., et al. (2010). Mixed linear model approach adapted for genome-wide association studies. *Nature Genetics, 42*(4), 355–360. https://doi.org/10.1038/ng.546

Zhang, Y., Massel, K., Godwin, I. D., & Gao, C. (2018). Applications and potential of genome editing in crop improvement. *Genome Biology, 19*, 210.

Zhang, H., Chu, Y., Dang, P., Tang, Y., Jiang, T., Clevenger, J. P., Ozias-Akins, P., Holbrook, C., Wang, M. L., Campbell, H., Hagan, A., & Chen, C. (2020a). Identification of QTLs for resistance to leaf spots in cultivated peanut (*Arachis hypogaea* L.) through GWAS analysis. *Theoretical and Applied Genetics, 133*(7), 2051–2061.

Zhang, H., Zhang, J., Xu, Q., et al. (2020b). Identification of candidate tolerance genes to low-temperature during maize germination by GWAS and RNA-seq approaches. *BMC Plant Biology, 20*, 333. https://doi.org/10.1186/s12870-020-02543-9

Zhang, H., Wang, M. L., Dang, P., Jiang, T., Zhao, S., Lamb, M., & Chen, C. (2021a). Identification of potential QTLs and genes associated with seed composition traits in peanut (*Arachis hypogaea* L.) using GWAS and RNA-Seq analysis. *Gene, 769*, 145215.

Zhang, M. Y., Xue, C., Hu, H., Li, J., Xue, Y., Wang, R., Fan, J., Zou, C., Tao, S., Qin, M., Bai, B., Li, X., Gu, C., Wu, S., Chen, X., Yang, G., Liu, Y., Sun, M., Fei, Z., Zhang, S., & Wu, J. (2021b). Genome-wide association studies provide insights into the genetic determination of fruit traits of pear. *Nature Communications, 12*, 1144.

Zhou, X., & Stephens, M. (2012). Genome-wide efficient mixed-model analysis for association studies. *Nature Genetics, 44*(7), 821–824. https://doi.org/10.1038/ng.2310

Zhu, C., Gore, M., Buckler, E. S., & Yu, J. (2008). Status and prospects of association mapping in plants. *The Plant Genome, 1*(1), 5–20.

Zhu, F., Alseekh, S., Koper, K., Tong, H., Nikoloski, Z., Naake, T., Liu, H., Yan, J., Brotman, Y., Wen, W., Maeda, H., Cheng, Y., & Fernie, A. R. (2021). Genome-wide association of the metabolic shifts underpinning dark-induced senescence in *Arabidopsis*. *Plant Cell, 34*, 557–578.

# Tweaking CRISPR/Cas for Developing Salt and Drought Tolerant Crop Plants

**Mahrukh, Khazin Hussain, Jafar K. Lone, Ragini Bhardwaj, and Muntazir Mushtaq**

**Abstract** Drought and soil salinity stresses are major consequences of climate change and the main source of yield loss and diminishing crop productivity. Abiotic stress-tolerant crops are expected to ensure global food security under conditions of anthropogenic climate change. Various studies demonstrated the application of CRISPR/Cas system in developing crops with enhanced drought and salinity tolerance; those studies clearly show the potential and effective role of the CRISPR/Cas system for future applications to enhance abiotic stress tolerance. Here in this chapter, we have tried to highlight all the recent work carried out regarding the applications of CRISPR-Cas-mediated gene editing in crop plants to combat drought and salinity stress.

**Keywords** CRISPR · Genome editing · Molecular breeding · Salinity · Drought · Abiotic stress · Crop improvement

## 1 Introduction

Climate change during the last three decades caused a serious yield loss and decrease production of many important agricultural crops globally. Among the major abiotic factors, drought and salinity hamper global agricultural productivity in most valuable crops (Kaushal & Wani, 2016; Singh et al., 2018). About 45% of agricultural

Mahrukh · K. Hussain
Division of Genetics and Plant Breeding, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Srinagar, India

J. K. Lone · R. Bhardwaj · M. Mushtaq (✉)
Division of Genomic Evaluation, ICAR-National Bureau of Plant Genetic Resources, New Delhi, India



A. Raina et al. (eds.), *Advanced Crop Improvement, Volume 2*,
https://doi.org/10.1007/978-3-031-26669-0_18

lands are estimated to be subjected to continuous or frequent drought conditions. In addition, about 52% of the world's population is seriously affected by soil salinity, which represents around 4.03 billion people living in 13 countries. Soil salinization is a severe form of soil degradation that can be caused by both natural and human-mediated activities like irrigation in mostly arid and semiarid regions (Rengasamy et al., 2010). Physiological reactions in plant are very similar to drought and salinity stress. Both cause cellular dehydration, which ultimately causes osmotic stress, and water loss from the cytoplasm into the intercellular space results in stomatal closure and affects $CO_2$ fixation (Flexas et al., 2004, 2007).

Salinity affect plant functions by means of two main mechanisms (i) by external osmotic pressure nearby the roots, which decreases assimilation of water, which leads to changes which are similar as caused by drought, and (ii) by negative effects of salt ions, especially $Na^+$ and $Cl^-$, which later accumulate in plant cell, mainly in the leaves so the consequence is seen at very low level of salinity and at the time of the first stages of salt exposure, while the ionic effect is observed during long-term exposure and increasing level of salinity (Arzani & Ashraf, 2016). One of the most common abiotic conditions is drought stress, which irreversibly affects plant growth and development, and it remains a serious concern for agricultural researchers, especially plant breeders, and ultimately causes water shortage in the root, resulting in osmotic unbalance and serious yield loss (Salekdeh et al., 2009). Adaptive morphophysiological variations in plants' biochemical processes enable them to withstand drought. Drought stress frequently causes stomatal closure, which inhibits $CO_2$ diffusion into the leaf, or non-stomatal limitations, which result in a decrease in carbon absorption and other photosynthetic activities. Because salinity reduces water intake through the roots, a combination of water scarcity and saline conditions could cause considerable stress.

Drought and salinity stress tolerance, as well as resource usage efficiency, are required to meet the goal of breaching yield limitations. Conventional breeding methods are time-consuming, tedious, and expensive but for so many years have been the most practical and lucidest strategy for improvement of crops and have aided their growth in abiotic stresses like drought and salinity. Apart from considering the contribution of traditional and molecular breeding approaches for improved crop production against abiotic stresses, inclusion of genome editing technologies is need of the hour. Genome editing is a technique which enables the scientists to make highly specific mutations for functional genomics and crop improvement. Because of its ease, variety, flexibility, and broad use, the clustered and regularly interspaced short palindromic repeat-Cas (CRISPR/Cas) technologies have emerged as a progressive genome editing approach. CRISPR-Cas technology is emerging at a rapid pace, and new molecular tools are readily accessible. The CRISPR-Cas genome editing is a flexible technology that allows precise genetic manipulation of crop species and has been used for the improvement of various abiotic and biotic stresses providing an opportunity to develop genotypes with desirable traits to achieve sustainable agricultural systems. The CRISPR/Cas genome editing method incorporates a single-guide RNA (sgRNA) with an endonuclease (Cas9) to induce a double-stranded break (DSB) at a specified place on the DNA. These DSBs are

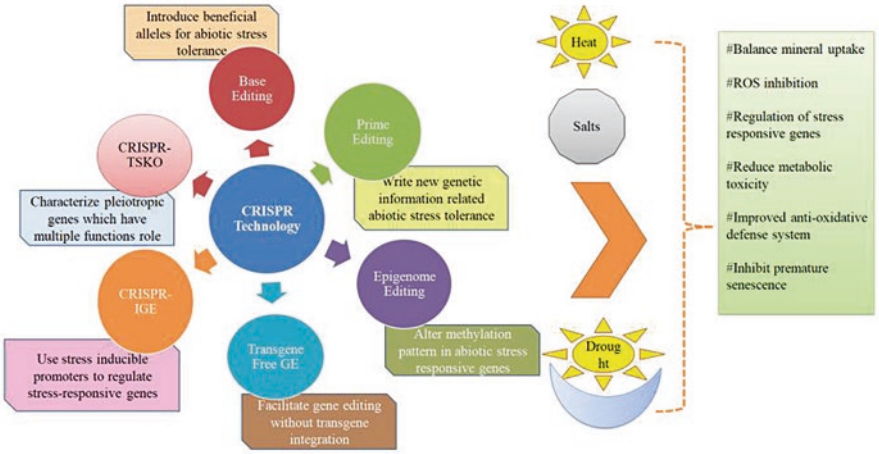



**Fig. 1** Mechanistic insights into CRISPR/Cas-mediated genome editing toward enhancing drought and salinity tolerance in plants

amended by endogenous repair mechanisms of the cell giving rise to unique new mutants (Voytas & Gao, 2014). CRISPR/Cas technology has been proficiently utilized at present times for the cognizance of improved resilience against stresses particularly abiotic such as drought and salinity in plants (Shi et al., 2017; Zhang et al., 2019a, b). This chapter will focus on the utilization of CRISPR/Cas9 genome editing technology toward drought and salinity tolerance in crops and foresight of this system toward the production of drought- and salinity-tolerant crops, which is also summarized in Fig. 1.

## 2 Molecular Breeding Approaches for Drought- and Salt-Tolerant Crop Plants

Quantitative trait locus (QTL) is a fragment of DNA linked with a specific trait in the genome of an organism. QTLs linked to salt and drought tolerance are complex playing an important part in acknowledging reaction against stress and producing tolerance in plants. Technological advancements has been made to analyze genes associated with QTLs by introducing novel methods such as microarray-based profiling of differential gene expression or combo of genetic mapping, and transcriptional profiling is utilized for recognizing genes linked with QTLs. Recently, numerous QTLs are reported pertaining to salinity and drought tolerance. Success achieved in genetic marker analysis made it possible to examine simply inherited along with the quantitative characters and identification of sole gene controlling the particular trait. Genetic markers can be utilized to speed up genetic advance by tagging QTL and by evaluating their share toward the phenotype by selection of

favorable alleles at these loci. To investigate mapping donor introgression in the genetic background of an elite recurrent parent, advanced backcross QTL analysis is used. Conventional breeding approaches for drought and salt resilience are ineffective owing to the reproductive barrier and possibility of transfer of unwanted characters. To evade this issue, genome editing approaches are desirable and preferred over conventional approaches as the former is involved in transfer of specific genes only.

## 3 Genome Editing Strategies Crop Improvement

Genome editing technologies have facilitated researchers to perform precise genetic manipulations in target organisms. Golden Gate system like innovative cloning approaches made the construction of those tools effortless (Cermak et al., 2011); nevertheless, protocols for altering genome are still comparatively tedious and laborious. Most researchers now have access to genome editing, thanks to the development of CRISPR-associated protein 9 (CRISPR/Cas9) technologies for planned mutagenesis (Gasiunas et al., 2012). This technology for editing genome has particularity for the specific sequence conferred by an editable small fragment of RNA (guide RNA); hence, the structure of Cas9 doesn't need any tempering to alter target recognition same as in case of ZFNs and TALENs (Cong et al., 2013). CRISPR/Cas9 technology originated from the adaptive immune system of bacteria in response to invaders like phages observed first in *E. coli* where repeats of DNA were set apart by sequences known as spacers (Ishino et al., 1987). The spacers are sequences of DNA from viruses which the bacteria save as a kind of immune memory.

These sequences are transcribed and processed into CRISPR RNAs (crRNAs), which are then coupled to a transactivating CRISPR RNA (tracrRNA). The Cas9, crRNA, and tracrRNA ribonucleoprotein complex is designed to invade the DNA adjacent to the spacer (Jansen et al., 2002; Bolotin et al., 2005; Pourcel et al., 2005). The guide RNA is made by combining crRNA and tracrRNA into a unique fragment (gRNA). The spacer can quickly be changed to lead Cas9 to a certain sequence (Cong et al., 2013). CRISPR's application in agriculture has created new possibilities for agricultural scientists, especially plant breeders (Shan et al., 2013). The most basic application of CRISPR/Cas9 is the development of out-of-frame loss-of-function mutants. During the cultivation of crops, loss-of-function mutations are a common genetic alteration. From a genetic standpoint, crop domestication was achieved by stacking variants with loss of function for critical genes regulating features including blooming time, seed breaking, seed size, and color (Meyer & Purugganan, 2013). By focusing on these key genes, researchers may follow crop improvement back to many years using a method called de novo domestication (Zsogon et al., 2017). Conceptually, this process could aid in speedy enhancement of highly tolerant, indigenously acclimatized species to attain new economically suitable crops that contain the unchanged stress tolerance traits of their wild ancestors. De novo domestication can be more effective than breeding for present-day

economic varieties as stress-tolerant characters controlled by various genes might be lost during crop domestication. Genome editing aids in acceleration of molecular breeding and improvement of crops which are essential for local food security but not important globally (orphan crops), e.g., chickpea, sorghum, and sweet potato (Lemmon et al., 2018). Genome editing is close to de novo domestication approach like improved resilience, locally suited, and extremely particularized crop may give satisfactory outcomes rather than aiming to bring back stress resistance in currently established varieties, during which complex polygenic characters are lost over crop domestication (Khan et al., 2019). Plant transformation efficiency is a drawback of genome editing in crops hampering the transfer of genome editing material into the specific cells. Genetic transformation is unattempted for many plant species, and existing protocols are structured for a little portion of the lab responsive varieties. In spite of that, genome editing has promising role in plant sciences which compels to develop more efficient crop transformation methods.

## 4 CRISPR for Developing Abiotic Stress Tolerance in Crop Plants

Breeders attempt to regulate a gene for analyzing its role as well as enhancing quantity, quality, and tolerance of crops toward abiotic stresses. CRISPR/Cas genome editing occurs naturally in bacteria and is used as a tool that helps in understanding role of gene and precision crop breeding by choosing a particular DNA and RNA sequence. It can be applied to select a sequence for gene knocking, knockout, and replacement. It can also be used to monitor and regulate gene expression at genomic and epigenomic levels by binding to a particular sequence.

CRISPR/Cas system combines endonuclease (Cas9) procured from bacteria *Streptococcus pyogenes* and a single-guide RNA (sgRNA) conferring precision for the target. This complex of Cas9-sgRNA binds to a specific location present on the DNA and induces breaks in the target genomic region depending on the sequence in the downstream known as protospacer adjacent motif (PAM). Hence, the use of different protospacer adjacent motif (PAM) sequence makes it an exceptionally basic, quick, cost-effective, and highly precise genome editing tool that has been incredibly useful for improvement of crops (Zhang et al., 2018a, b). Almost all important crops, including maize and rice, have benefited from CRISPR/Cas technology. Barley, wheat, sorghum, watermelon, potato, cucumber, lettuce, grapes, and soybeans are some of the most common crops. Abiotic stresses like drought and salinity are notably conforming to be critical for crop growth and development due to global warming.

Multiple structural and regulatory genes, as well as noncoding RNAs, play a role in plant responses to varying environmental conditions (Zhang, 2015). These genes can be altered using traditional transgenic technologies and advanced genome editing tools to improve crop tolerance to abiotic stressors. As a sole abiotic stress may be controlled by many genes rather than an individual gene having a dominant effect

**Table 1** Applications of CRISPR to develop crop plants tolerant to drought and salinity

| Crop | Delivery mode | Target gene | Function | Reference(s) |
|---|---|---|---|---|
| *Drought stress* | | | | |
| *Brassica napus* | *A. tumefaciens* | *BnaA6.RGA* | Transcription factor | Wu et al. (2020) |
| Rice (*O. sativa*) | *A. tumefaciens* | *OsNAC006*<br>*OsmiR535*<br>*OsDST*<br>*OsPYL9*<br>*OsERA1*<br>*SRL (1 and 2)*<br>*NAC14*<br>*SAPK2*<br>*MYB5,DERF1, PMS3, SPPEPSPS, MSH1* | Drought<br>Drought and salinity tolerance<br>Drought and salt tolerance<br>Drought tolerance<br>Drought tolerance<br>Leaf rolling<br>Transcription factor<br>Signalling (ABA)<br>Amino acid synthesis | Wang et al. (2020), Yue et al. (2020), Kumar et al. (2020), Usman et al. (2020), Ogata et al. (2020), Liao et al. (2019), Shim et al. (2018), Lou et al. (2017), and Zhang et al. (2014) |
| Tomato (*S. lycopersicum* L.) | *A. tumifaciens*<br>*A. tumifaciensA. tumifaciens*<br>*A. tumefaciens* | *SlNPR1*<br>*NPR1*<br>*SIMAPK3*<br>*NPR1* | Drought tolerance<br>Drought tolerance<br>Plant growth and development | Li et al. (2019) and Wang et al. (2017) |
| Wheat (*Triticum aestivum*) | PEG-mediated transformation | *DREB2*, *DREB3*, *ERF3* | Dehydration responsive element binding protein | Kim et al. (2018) |
| Maize (*Zea mays*) | Particle bombardment | *ARGOS8* | Gene family regulator of ethylene | Shi et al. (2017) |
| *A. thaliana* | *A. tumefaciens*<br>*A. tumefaciens*<br>*A. tumefaciens*<br>*A. tumefaciens*<br>*A. tumefaciens* | Trehalase<br>*AREB1*<br>*Vacuolar $H^+$-pyrophosphatase* (AVP1)<br>*OST2*<br>*miR169a* | Drought tolerance<br>ABA signaling<br>As transcription factor<br>Stomatal movement<br>Acts in drought tolerance as negative factor | Nunez-Munoz et al. (2021), Roca-Paixao et al. (2019), and Park et al. (2017) |
| *Glycine max* | *A. tumefaciens* | *GmMYB118* | Drought and salt tolerance | Du et al. (2018) |
| Populus clone NE-19 | *A. tumefaciens* | *PdNF-YB21* | Drought tolerance | Zhou et al. (2020) |

(continued)

**Table 1** (continued)

| Crop | Delivery mode | Target gene | Function | Reference(s) |
|---|---|---|---|---|
| *Salinity stress* | | | | |
| Rice (*O. sativa*) | *A. tumefaciens* | *GTγ-2* | Transcription factor | Liu et al. (2020) |
| | | *PQT3* | Ubiquitin ligase | Alfatih et al. (2020) |
| | | *PIL14* | Transcription factor | Mo et al. (2020) |
| | | *BGE3* | Cytokinin transport | Yin et al. (2020) |
| | | *DST* | Transcription factor (zinc finger) | Kumar et al. (2020) |
| | | *FLN2* | Sucrose metabolism | Chen et al. (2019) |
| | | *RR9*, *RR10* | Cytokinin signaling | Wang et al. (2019) |
| | | *DOF15* | Transcription factor | Qin et al. (2019) |
| | | *NCA1a, NCA1b* | Catalase activity regulated by chaperones | Liu et al. (2019) |
| | | *OsRR22* | Transcription factor | Zhang et al. (2019a, b) |
| | | *NAC041* | Transcription factor | Bo et al. (2019) |
| | | *OTS1* | Response regulator for salt stress | Zhang et al. (2019a, b) |
| | | *SAPK1*, *SAPK2* | Pathway regulator for BA | Lou et al. (2018) |
| | | *OsBBS1* | Signaling mediated by chaperones | Zeng et al. (2018) |
| | | *MIR528* | Response regulator for salt stress | Zhou et al. (2017) |
| | | *SAPK2* | Signaling (ABA) | Lou et al. (2017) |
| | | *RAV2* | Transcription factor | Duan et al. (2016) |

(continued)

**Table 1** (continued)

| Crop | Delivery mode | Target gene | Function | Reference(s) |
|---|---|---|---|---|
| *Solanum lycopersicum* | *A. tumefaciens*<br>*A. tumefaciens*<br>*A. tumefaciens*<br>*A. tumefaciens* | *SlARF4*<br>*CLV3*<br>*SP5G*, *SP*<br>*GGP1*<br>*WUS* | Osmotic and salt stresses<br>Regulates floral meristem development<br>Regulates sensitivity for daylength<br>Regulates gene transcription process in shoot apical meristem | Bouzroud et al. (2020), Li et al. (2018), Van Eck et al. (2019), Li et al. (2018), and Zhang et al. (2018a, b) |
| *Arabidopsis thaliana* | *A. tumefaciens* | *SAUR41* | Salinity stress | Qiu et al. (2020) |

as in case of biotic stress, a mild advancement is scored to generate genome editing mutants for enhancing plant tolerance to abiotic stresses by employing CRISPR/Cas system (Table 1). CRISPR/Cas9-mutated AGROS8 gene (negative regulator of ethylene response) improved maize tolerance to drought recently (Shi, 2017). Recently, CRISPR/Cas-edited *Arabidopsis* mutants of *dpa4-sod7-aitr256* improved tolerance to drought stress (Chen, 2019). In tomato, water usage efficiency was enhanced by knocking down and knocking out of ARF4 transcription factor gene thereby improving salinity and osmotic stress in mutated plants (Bouzroud, 2020). Mutants of G protein genes *gs3* and *dep1* in rice obtained from CRISPR/Cas genome editing improved tolerance to salinity stress (Cui, 2020). Removal of *ppa6* gene improved tolerance to alkaline stress in rice (Wang, 2019). Expression of several genes can be mediated using CRISPR/Cas system. This implies the prospects of CRISPR/Cas genome editing technology on enhancing plant resilience to abiotic stresses (Mushtaq et al., 2018).

## 5 CRISPR/Cas Genome Editing for Plant Tolerance to Drought Stress

Drought tolerance is a complicated trait governed by multiple genes; therefore, understanding the underlying molecular and physiological mechanisms is crucial. Reactive oxygen species, abscisic acid, allied phytohormones, calcium, cross talk between various factors, and many other signaling molecules are known to be involved in signal transmission under drought condition (Hu & Xiong, 2014). The synthesis of plant hormones and their signaling regulates ion transporters, MAPKs, CDPKs, protein kinase (CIPK), CIPKs, and calcineur in Blike interacting and sucrose non-fermenting protein (SNF1)-related kinase 2 (SnF2) (Fang & Xiong, 2015). Drought stress in rice is modulated by increased expression of OsCIPK23 and OsCDPK7 (Yang et al., 2008). In rice, OsMPK5 and MAP kinase kinase kinase

(M3K) gene *DSM1* has been recognized as an essential molecule linked in improving tolerance toward drought (Sinha et al., 2011). SnRK2C in *Arabidopsis* enables drought stress tolerance by regulating stress-related genes (Umezawa et al., 2004). Plants with transcription factors, such ZF-TFs, AP2/EREBF, MYB, NAC, and AREB/ABFs, should be able to withstand drought (Joshi et al., 2016). Master transcription factors AREB1, AREB2, and AREB3 in *Arabidopsis* showed a linked ABA-mediated direct regulation for drought tolerance (Yoshida et al., 2010). Similarly, by starting the biosynthesis of wax in *Arabidopsis*, AP2/EREBF TF SHN have shown improved drought tolerance (Aharoni et al., 2004). ABA is closely linked to drought stress among the phytohormones linked with signaling cues. The synchronized interaction within three groups of proteins, the pyrabactin resistance 1 (PYR1) and/or regulatory component of the ABA receptor (RCAR), protein phosphatase 2C (PP2C), and SnRK2s, provides ABA-mediated drought tolerance in plants.

CRISPR/Cas9 system was utilized in *Arabidopsis* to introduce a plasma membrane $H^+$ ATPase, OPEN STOMATA 2 (OST2) encoded by novel alleles accountable for stomatal response (Osakabe et al., 2016). CRISPR/Cas9 system has improved drought tolerance by using truncated sgRNA (tru-sgRNA) and Cas9 combination which induced mutation at the *OST2* locus by improving stomatal response. CRISPR/Cas9 system was also utilized to ensure the role of non-expresser of pathogenesis-related gene 1 (NPR1 gene) in tomato drought tolerance by generating mutant lines for NPR1 gene (Li et al., 2019). Loss-of-function s1npr1 mutants induced by CRISPR/Cas9 system exhibited deteriorated drought characters in contrast to tomatoes of wild origin such as lowered tolerance to drought, broad higher electrolytic leakage, stomatal aperture, increased levels of hydrogen peroxide, and malondialdehyde and lower levels of antioxidant enzymes. Decrease in expression of the genes responsible for water stress tolerance such as SIGST, SIDHN, and SIDREB validated the vulnerability of *s1npr1* variants toward drought. Hence, *S1NPR1* could be committed to perform a vital function in regulating tolerance to drought, and various mutants of *SlNPR1* can be achieved using genome editing tools for extended drought tolerance in tomato and various crops. CRISPR/Cas9 system can also be modified to enhance drought tolerance in plants; for example, in *Arabidopsis*, drought tolerance was enhanced by using a defunct Cas9 including the catalytic domain of histone acetyltransferase and single-guide RNA (sgRNA) for targeting *AREB1* gene promotor region (Roca-Paixaao et al., 2019). CRISPR/Cas9 system has been utilized for developing mutants with loss of function of *OsSAPK2* to validate its function in the development of ABA-mediated tolerance against drought in rice (Lou et al., 2017). In recent times, CRISPR/Cas9 technology has been utilized to generate novel *ARGOS8* mutants to incorporate valuable traits, including drought tolerance in maize (Shi et al., 2017). This genome editing tool is recently employed to generate variants with rolled leaves in rice genotypes by altering the genes (*SRL1* and *SRL2*) (Liao et al., 2019). Lower stomatal number, vascular bundles, chlorophyll content, stomatal conductance, transpiration rate, and other related characters have been reported for homozygous mutant lines for *SRL1* and *SRL2*. Overall, CRISPR/Cas system has been widely accepted for altering genes with precision and corresponding mechanisms for drought tolerance as needed for improvement of crops.

## 6 CRISPR/Cas Genome Editing for Plant Tolerance to Salinity

Salinity affects productivity of economically important crops due to ion toxicity and osmotic stress as it reduces plant vigor. Plants tolerant to salinity balance the accretion of organic solutes and inorganic ions to decrease osmotic stress. The presence of salinity in growth medium leads to increase in concentration of $Na^+$, which takes part with $K^+$ causing efflux of inorganic ions, leading to potassium ion shortage in the cytoplasm. As a result, increased proportion of $Na^+$ in plant cells causes unbalance in ion equilibrium. Photosynthesis is obstructed by both osmotic stress and ion toxicity in plant cells producing reactive oxygen species (ROS) in abundance which lead to reduction in growth and ultimately cell death. Plants counter salinity stress by morphophysiological adjustments, which are the consequences of changes in the regulation of genes and signaling pathways (Julkowska & Testerink, 2015). The initial changes at cellular level are quite recognizable including development of intracellular signal messengers like reactive oxygen species, instantaneous changes in $Ca^{2+}$ levels, differential activity of $Ca^{2+}$/calmodulin-dependent kinase activation, ABA synthesis, and activation of SOS homeostatic signaling pathways (Zeng et al., 2015).

CRISPR/Cas genome editing has confirmed a number of genes that improve salt tolerance. In rice, for example, the *OsBBS1* gene has been linked to salinity susceptibility and early leaf maturation. In addition, the gene *OsMIR528* works as a positive salinity regulator (Sun et al., 2019; Ganie et al., 2021). The GT-1 element is required for stimulating the expression of the *OsRAV2* gene, which confers salinity tolerance, according to the CRISPR/Cas directed variation (Duan et al., 2016). Salinity tolerance was conferred by loss-of-function mutations of the SnRK2 and SAPK-1 and 2 genes in rice, which were mediated by CRISPR/Cas (Lou et al., 2017). Aside from that, rice's *OsRR22* and *OsNAC041* genes have been shown to improve salinity tolerance (Sun et al., 2019; Ganie et al., 2021). In tomato plants, knocking down the SlMAP3 gene resulted in a considerable reduction in the expression of *SlLOX*, *SlGST*, and *SlDREB*, resulting in salt tolerance (Wang et al., 2017). With the use of targeted genome editing utilizing the CRISPR/Cas system, such case studies can be exploited to increase salinity tolerance in crops.

## 7 Conclusion and Future Directions

We still lack efficient ways to comprehend the potential applications of the different advanced gene editing tools such as base editing, prime editing, and chromosome engineering regarding tolerance to drought, temperature, and salinity stress, though existing reports mostly used CRISPR/Cas9 gene knockout technology. Very recently, the introduction of CRISPR-interference (CRISPRi) and CRISPR-mediated activation (CRISPRa) in the CRISPR toolkit, based on dCas9, as already

is utilized in maize (Gentzel et al., 2020), has tremendous potential for additional development of field plant genome editing. Targeting the core promoter of a gene by gene editing technology could be an unprecedented approach for modification of any desired trait, unraveling newer opportunities for breeding improved crop varieties tolerant to drought and salinity stress. Furthermore, novel delivery methods utilized in the recent approaches for genome editing will develop transgene-free products, therefore, surmounting the constraints of ethical, regulatory, and commercialization concerns. Application gene editing tools in functional genomics together with other strategies could possibly help in combating global food crisis and will facilitate in accomplishing the zero-hunger goal, one of the sustainable development goals set by the United Nations, by 2030.

## References

Aharoni, A., Dixit, S., Jetter, R., Thoenes, E., van Arkel, G., & Pereira, A. (2004). The SHINE clade of AP2 domain transcription factors activates wax biosynthesis, alters cuticle properties, and confers drought tolerance when overexpressed in Arabidopsis. *The Plant Cell, 16*, 2463–2480. https://doi.org/10.1105/tpc.104.022897

Alfatih, A., Wu, J., Jan, S. U., Zhang, Z.-S., Xia, J.-Q., & Xiang, C.-B. (2020). Loss of rice PARAQUAT TOLERANCE 3 confers enhanced resistance to abiotic stresses and increases grain yield in field. *Plant, Cell and Environment, 43*, 2743–2754.

Arzani, A., & Ashraf, M. (2016). Smart engineering of genetic resources for enhanced salinity tolerance in crop plants. *Critical Reviews in Plant Sciences, 35*, 146–189. https://doi.org/10.1080/07352689.2016.1245056

Bo, W., Zhaohui, Z., Huanhuan, Z., Xia, W., Binglin, L., Lijia, Y., et al. (2019). Targeted mutagenesis of NAC transcription factor gene, OsNAC041, leading to salt sensitivity in rice. *Rice Science, 26*(2), 98–108.

Bolotin, A., Quinquis, B., Sorokin, A., & Ehrlich, S. D. (2005). Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. *Microbiology, 151(8), 2551–2561.*

Bouzroud, S. (2020). Down regulation and loss of auxin response factor 4 function using CRISPR/Cas9 alters plant growth, stomatal function and improves tomato tolerance to salinity and osmotic stress. *Genes, 11*, 272.

Bouzroud, S., Gasparini, K., Hu, G., et al. (2020). Down regulation and loss of Auxin Response Factor 4 function using CRISPR/Cas9 alters plant growth, stomatal function and improves tomato tolerance to salinity and osmotic stress. *Genes (Basel), 11*(3), 272. https://doi.org/10.3390/genes11030272

Cermak, T., Doyle, E. L., Christian, M., Wang, L., Zhang, Y., Schmidt, C., & Voytas, D. F. (2011). Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. *Nucleic Acids Research, 39*(12), e82–e82.

Chen, S. (2019). Genome editing to integrate seed size and abiotic stress tolerance traits in *Arabidopsis* reveals a role for DPA4 and SOD7 in the regulation of inflorescence architecture. *International Journal of Molecular Sciences, 20*, 2695.

Chen, Z. Q., Liu, Y., Yin, Y. J., et al. (2019). Expression of AtGA2ox1 enhances drought tolerance in maize. *Plant Growth Regulation, 89*, 203–215. https://doi.org/10.1007/s10725-019-00526-x

Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., & Zhang, F. (2013). Multiplex genome engineering using CRISPR/Cas systems. *Science, 339*(6121), 819–823.

Cui, Y. (2020). Heterotrimeric G proteins are involved in the regulation of multiple agronomic traits and stress tolerance in rice. *BMC Plant Biology, 20*, 90.

Du, Y. T., Zhao, M. J., Wang, C. T., et al. (2018). Identification and characterization of GmMYB118 responses to drought and salt stress. *BMC Plant Biology, 18*, 320. https://doi.org/10.1186/s12870-018-1551-7

Duan, Y. B., Li, J., Qin, R. Y., Xu, R. F., Li, H., Yang, Y. C., et al. (2016). Identification of a regulatory element responsible for salt induction of riceOsRAV2 through ex situ and *in situ* promoter analysis. *Plant Molecular Biology, 90*(1–2), 49–62.

Fang, Y., & Xiong, L. (2015). General mechanisms of drought response and their application in drought resistance improvement in plants. *Cellular and Molecular Life Sciences, 72*, 673–689. https://doi.org/10.1007/s00018-014-1767-0

Flexas, J., Bota, J., Loreto, F., Cornic, G., & Sharkey, T. D. (2004). Diffusive and metabolic limitations to photosynthesis under drought and salinity in C3 plants. *Plant Biology, 6*, 1–11. https://doi.org/10.1055/s-2004-820867

Flexas, J., Diaz-Espejo, A., Galmes, J., Kaldenhoff, R., Medrano, H., & Ribas-Carbo, M. (2007). Rapid variations of mesophyll conductance in response to changes in $CO_2$ concentration around leaves. *Plant, Cell & Environment, 30*, 1284–1298. https://doi.org/10.1111/j.1365-3040.2007.01700.x

Ganie, S. A., Wani, S. H., Henry, R., & Hensel, G. (2021). Improving rice salt tolerance by precision breeding in a new era. *Current Opinion in Plant Biology, 60*, 101996.

Gasiunas, G., Barrangou, R., Horvath, P., & Siksnys, V. (2012). Cas9–crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proceedings of the National Academy of Sciences, 109*(39), E2579–E2586.

Gentzel, I. N., Park, C. H., Bellizzi, M., Xiao, G., Gadhave, K. R., Murphree, C., ... & Wang, G. L. (2020). A CRISPR/dCas9 toolkit for functional analysis of maize genes. *Plant Methods, 16, 1–9.*

Hu, H., & Xiong, L. (2014). Genetic engineering and breeding of drought-resistant crops. *Annual Review of Plant Biology, 65*, 715–741. https://doi.org/10.1146/annurev-arplant-050213-040000

Ishino, Y., Shinagawa, H., Makino, K., Amemura, M., & Nakata, A. (1987). Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in *Escherichia coli*, and identification of the gene product. *Journal of Bacteriology, 169*(12), 5429–5433.

Jansen, R., Embden, J. D. V., Gaastra, W., & Schouls, L. M. (2002). Identification of genes that are associated with DNA repeats in prokaryotes. *Molecular Microbiology, 43(6), 1565–1575.*

Joshi, R., Wani, S. H., Singh, B., Bohra, A., Dar, Z. A., Lone, A. A., Pareek, A., & Singla-Pareek, S. L. (2016). Transcription factors and plants response to drought stress: Current understanding and future directions. *Frontiers in Plant Science, 7*, 1029. https://doi.org/10.3389/fpls.2016.01029

Julkowska, M. M., & Testerink, C. (2015). Tuning plant signaling and growth to survive salt, *Trends in Plant Science, 20*(9), 586–594.

Kaushal, M., & Wani, S. P. (2016). Rhizobacterial-plant interactions: Strategies ensuring plant growth promotion under drought and salinity stress. *Agriculture, Ecosystems and Environment, 231*, 68–78. https://doi.org/10.1016/j.agee.2016.06.031

Khan, M. Z., Zaidi, S. S. E. A., Amin, I., & Mansoor, S. (2019). A CRISPR way for fast-forward crop domestication. *Trends in Plant Science, 24*(4), 293–296.

Kim, D., Alptekin, B., & Budak, H. (2018). CRISPR/Cas9 genome editing in wheat. *Functional and Integrative Genomics, 18*(1), 31–41.

Kumar, V. V. S., Verma, R. K., Yadav, S. K., Yadav, P., Watts, A., Rao, M. V., & Chinnusamy, V. (2020). CRISPR-Cas9 mediated genome editing of drought and salt tolerance (OsDST) gene in indica mega rice cultivar MTU1010. *Physiology and Molecular Biology of Plants, 26*, 1099–1110. https://doi.org/10.1007/s12298-020-00819-w

Lemmon, Z. H., Reem, N. T., Dalrymple, J., Soyk, S., Swartwood, K. E., Rodriguez-Leal, D., & Lippman, Z. B. (2018). Rapid improvement of domestication traits in an orphan crop by genome editing. *Nature Plants, 4*(10), 766–770.

Li, T., Yang, X., Yu, Y., Si, X., Zhai, X., Zhang, H., et al. (2018). Domestication of wild tomato is accelerated by genome editing. *Nature Biotechnology, 36*, 1160–1163.

Li, R., Liu, C., Zhao, R., Wang, L., Chen, L., Yu, W., ... & Shen, L. (2019). CRISPR/Cas9-Mediated SlNPR1 mutagenesis reduces tomato plant drought tolerance. *BMC Plant Biology, 19(1), 1–13.*

Liao, S., Qin, X., Luo, L., Han, Y., Wang, X., Usman, B., Nawaz, G., Zhao, N., Liu, Y., & Li, R. (2019). CRISPR/Cas9-induced mutagenesis of semi-rolled leaf 1,2 confers curled leaf phenotype and drought tolerance by influencing protein expression patterns and ROS scavenging in rice (*Oryza sativa* L.). *Agronomy, 9*, 728.

Liu, J., Cui, L., Xie, Z., Zhang, Z., Liu, E., & Peng, X. (2019). Two NCA1 isoforms interact with catalase in a mutually exclusive manner to redundantly regulate its activity in rice. *BMC Plant Biology, 19*, 105.

Liu, X., Wu, D., Shan, T., Xu, S., Qin, R., Li, H., et al. (2020). The trihelix transcription factor OsGTg-2 is involved adaption to salt stress in rice. *Plant Molecular Biology, 103*, 545–560.

Lou, D., Wang, H., Liang, G., & Yu, D. (2017). OsSAPK2 confers abscisic acid sensitivity andtolerance to drought stress in rice. *Frontiers in Plant Science, 8*, 993.

Lou, D., Wang, H., & Yu, D. (2018). The sucrose non-fermenting-1-related protein kinases SAPK1 and SAPK2 function collaboratively as positive regulators of salt stress tolerance in rice. *BMC Plant Biology, 18*(1), 203.

Meyer, R. S., & Purugganan, M. D. (2013). Evolution of crop species: Genetics of domestication and diversification. *Nature Reviews Genetics, 14*(12), 840–852.

Mo, W., Tang, W., Du, Y., Jing, Y., Bu, Q., & Lin, R. (2020). PHYTOCHROMEINTERACTING FACTOR-LIKE14 and SLENDER RICE1 interaction controls seedling growth under salt stress. *Plant Physiology, 184*, 506–517.

Mushtaq, M., Bhat, J. A., Mir, Z. A., Sakina, A., Ali, S., Singh, A. K., et al. (2018). CRISPR/Cas approach: A new way of looking at plant-abiotic interactions. *Journal of Plant Physiology, 224–225*, 156–162.

Nuñez-Muñoz, L., Vargas-Hernández, B., Hinojosa-Moya, J., Ruiz-Medrano, R., & Xoconostle-Cázares, B. (2021). Plant drought tolerance provided through genome editing of the trehalase gene. *Plant Signaling & Behavior, 16*(4), 1877005. https://doi.org/10.1080/15592324.2021.1877005

Ogata, T., Ishizaki, T., Fujita, M., & Fujita, Y. (2020). CRISPR/Cas9-targeted mutagenesis of OsERA1 confers enhanced responses to abscisic acid and drought stress and increased primary root growth under nonstressed conditions in rice. *PLoS ONE, 15*, e0243376.

Osakabe, Y., Watanabe, T., Sugano, S. S., Ueta, R., Ishihara, R., Shinozaki, K., & Osakabe, K. (2016). Optimization of CRISPR/Cas9 genome editing to modify abiotic stress responses in plants. *Scientific Reports, 6*, 26685.

Park, J. J., Dempewolf, E., Zhang, W., & Wang, Z. Y. (2017). RNA-guided transcriptional activation via CRISPR/dCas9 mimics overexpression phenotypes in Arabidopsis. *PLoS One, 12*, e0179410.

Pourcel, C., Salvignol, G., & Vergnaud, G. (2005). CRISPR elements in Yersinia pestis acquire new repeats by preferential uptake of bacteriophage DNA, and provide additional tools for evolutionary studies. *Microbiology, 151(3), 653–663.*

Qin, H., Wang, J., Chen, X., Wang, F., Peng, P., Zhou, Y., et al. (2019). Rice OsDOF15 contributes to ethylene inhibited primary root elongation under salt stress. *The New Phytologist, 223*, 798–813.

Qiu, T., Qi, M., Ding, X., et al. (2020). The SAUR41 subfamily of SMALL AUXIN UP RNA genes is abscisic acid inducible to modulate cell expansion and salt tolerance in Arabidopsis thaliana seedlings. *Annals of Botany, 125*, 805–819. https://doi.org/10.1093/aob/mcz160

Rengasamy, P., North, S., & Smith, A. (2010). *Diagnosis and management of sodicity and salinity in soil and water in the Murray irrigation region*. The University of Adelaide.

Roca-Paixao, J. F., Gillet, F. X., Ribeiro, T. P., Bournaud, C., Lourenco Tessutti, T., Noriega, D. D., et al. (2019). Improved drought stress tolerance in Arabidopsis by CRISPR/dCas9 fusion with a histone acetyl transferase. *Scientific Reports, 9*, 8080.

Salekdeh, G. H., Reynolds, M., Bennett, J., & Boyer, J. (2009). Conceptual framework for drought phenotyping during molecular breeding. *Trends in Plant Science, 14*, 488–496. https://doi.org/10.1016/j.tplants.2009.07.007

Shan, Q., Wang, Y., Chen, K., Liang, Z., Li, J., Zhang, Y., & Gao, C. (2013). Rapid and efficient gene modification in rice and Brachypodium using TALENs. *Molecular Plant, 6*(4), 1365–1368.

Shi, J. (2017). ARGOS8 variants generated by CRISPR-Cas9 improve maize grain yield under field drought stress conditions. *Plant Biotechnology Journal, 15*, 207–216.

Shi, J., Gao, H., Wang, H., Lafitte, H. R., Archibald, R. L., Yang, M., et al. (2017). ARGOS8 variants generated by CRISPR-Cas9 improve maize grain yield under field drought stress conditions. *Plant Biotechnology Journal, 15*, 207–216. https://doi.org/10.1111/pbi.12603

Shim, J. S., Oh, N., Chung, P. J., Kim, Y. S., Choi, Y. D., & Kim, J. K. (2018). Overexpression of OsNAC14 improves drought tolerance in rice. *Frontiers in Plant Science, 9*, 310.

Singh, V. K., Singh, A. K., Singh, P. P., & Kumar, A. (2018). Interaction of plant growth promoting bacteria with tomato under abiotic stress: A review. *Agriculture, Ecosystems and Environment, 267*, 129–140. https://doi.org/10.1016/j.agee.2018.08.020

Sinha, A. K., Jaggi, M., Raghuram, B., & Tuteja, N. (2011). Mitogen-activated protein kinase signaling in plants under abiotic stress. *Plant Signaling & Behavior, 6*, 196–203. https://doi.org/10.4161/psb.6.2.14701

Sun, B. R., Fu, C. Y., Fan, Z. L., Chen, Y., Chen, W. F., Zhang, J., et al. (2019). Genomic and transcriptomic analysis reveal molecular basis of salinity tolerance in a novel strong salt-tolerant rice landrace Changmaogu. *Rice, 12*(1), 99.

Umezawa, T., Yoshida, R., Maruyama, K., Yamaguchi-Shinozaki, K., & Shinozaki, K. (2004). SRK2C, a SNF1-related protein kinase 2, improves drought tolerance by controlling stress-responsive gene expression in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences of the United States of America, 101*, 17306–17311.

Usman, B., Nawaz, G., Zhao, N., Liao, S., Liu, Y., & Li, R. (2020). Precise editing of the OsPYL9 gene by RNA-guided Cas9 nuclease confers enhanced drought tolerance and grain yield in rice (*Oryza sativa* L.) by regulating circadian rhythm and abiotic stress responsive proteins. *International Journal of Molecular Sciences, 21*, 7854.

Van Eck, J., Keen, P., & Tjahjadi, M. (2019). *Agrobacterium tumefaciens* mediated transformation of tomato. *Methods in Molecular Biology, 1864*, 225–234.

Voytas, D. F., & Gao, C. (2014). Precision genome engineering and agriculture: opportunities and regulatory challenges. *PLoS Biology, 12*, e1001877. https://doi.org/10.1371/journal.pbio.1001877

Wang, B. (2019). Mutagenesis reveals that the OsPPa6 gene is required for enhancing the alkaline tolerance in rice. *Frontiers in Plant Science, 10*, 759.

Wang, L., Chen, L., Li, R., Zhao, R. R., Yang, M. J., Sheng, J. P., & Shen, L. (2017). Reduced drought tolerance by CRISPR/Cas9-mediated SlMAPK3 mutagenesis in tomato plants. *Journal of Agricultural and Food Chemistry, 65*(39), 8674–8682. https://doi.org/10.1021/acs.jafc.7b02745

Wang, W. C., Lin, T. C., Kieber, J., & Tsai, Y. C. (2019). Response regulators 9 and 10 negatively regulate salinity tolerance in rice. *Plant and Cell Physiology, 60*, 2549–2563.

Wang, B., Zhong, Z., Wang, X., Han, X., Yu, D., Wang, C., et al. (2020). Knockout of the OsNAC006 transcription factor causes drought and heat sensitivity in rice. *International Journal of Molecular Sciences, 21*(7), 2288.

Wu, J., Yan, G., Duan, Z., Wang, Z., Kang, C., Guo, L., et al. (2020). Roles of the *Brassica napus* DELLA protein n BnaA6.RGA, in modulating drought tolerance by interacting with the ABA signaling component BnaA10. ABF2. *Frontiers in Plant Science, 11*, 577.

Yang, W., Kong, Z., Omo-Ikerodah, E., Xu, W., Li, Q., & Xue, Y. (2008). Calcineurin B-like interacting protein kinase OsCIPK23 functions in pollination and drought stress responses in rice (*Oryza sativa* L.). *Journal of Genetics and Genomics, 35*, S531–S532. https://doi.org/10.1016/S1673-8527(08)60073-9

Yin, W., Xiao, Y., Niu, M., Meng, W., Li, L., Zhang, X., et al. (2020). ARGONAUTE2 enhances grain length and salt tolerance by activating BIG GRAIN3 to modulate cytokinin distribution in rice. *The Plant Cell, 32*(7), 2292–2306.

Yoshida, T., Fujita, Y., Sayama, H., Kidokoro, S., Maruyama, K., Mizoi, J., Shinozaki, K., & Yamaguchi-Shinozaki, K. (2010). AREB1, AREB2, and ABF3 are master transcription factors that cooperatively regulate ABRE-dependent ABA signaling involved in drought stress tolerance and require ABA for full activation. *The Plant Journal, 61*, 672–685. https://doi.org/10.1111/j.1365-313X.2009.04092.x

Yue, E., Cao, H., & Liu, B. (2020). OsmiR535, a potential genetic editing target for drought and salinity stress tolerance in *Oryza sativa*. *Plants, 9*, 1337.

Zeng, H., Xu, L., Singh, A., Wang, H., Du, L., & Poovaiah, B. W. (2015). Involvement of calmodulin and calmodulin-like proteins in plant responses to abiotic stresses. *Frontiers in Plant Science, 6*, 600.

Zeng, D. D., Yang, C. C., Qin, R., Alamin, M., Yue, E. K., Jin, X. L., & Shi, C. H. (2018). A guanine insert in OsBBS1 leads to early leaf senescence and salt stress sensitivity in rice (*Oryza sativa* L.). *Plant Cell Reports, 37*(6), 933–946.

Zhang, B. (2015). MicroRNA: A new target for improving plant tolerance to abiotic stress. *Journal of Experimental Botany, 66*, 1749–1761.

Zhang, H., Zhang, J., Wei, P., Zhang, B., Gou, F., Feng, Z., et al. (2014). The CRISPR/Cas9 system produces specific and homozygous targeted gene editing in rice in one generation. *Plant Biotechnology Journal, 12*(6), 797–807.

Zhang, S., Jiao, Z., Liu, L., Wang, K., Zhong, D., Li, S., et al. (2018a). Enhancerpromoter interaction of SELF PRUNING 5G shapes photoperiod adaptation. *Plant Physiology, 178*(4), 1631–1642.

Zhang, Y., Massel, K., Godwin, I. D., & Gao, C. (2018b). Applications and potential of genome editing in crop improvement. *Genome Biology, 19*, 210. https://doi.org/10.1186/s13059-018-1586-y

Zhang, A., Liu, Y., Wang, F., Li, T., Chen, Z., Kong, D., Bi, J., Zhang, F., Luo, X., Wang, J., Tang, J., Yu, X., Liu, G., & Luo, L. (2019a). Enhanced rice salinity tolerance via CRISPR/Cas9-targeted mutagenesis of the OsRR22 gene. *Molecular Breeding, 39*(3), 47. https://doi.org/10.1007/s11032-019-0954-y

Zhang, C., Srivastava, A. K., & Sadanandom, A. (2019b). Targeted mutagenesis of the SUMO protease, overly tolerant to Salt1 in rice through CRISPR/Cas9-mediated genome editing reveals a major role of this SUMO protease in salt tolerance. *bioRxiv*, 555706.

Zhou, J., Deng, K., Cheng, Y., Zhong, Z., Tian, L., Tang, X., et al. (2017). CRISPR-Cas9 based genome editing reveals new insights into MicroRNA function and regulation in rice. *Frontiers in Plant Science, 8*, 1598.

Zhou, Y., Zhang, Y., Wang, X., et al. (2020). Root-specifc NF-Y family transcription factor, PdNF-YB21, positively regulates root growth and drought resistance by abscisic acid-mediated indoylacetic acid transport in Populus. *The New Phytologist, 227*, 407–426. https://doi.org/10.1111/nph.16524

Zsögön, A., Cermak, T., Voytas, D., & Peres, L. E. P. (2017). Genome editing as a tool to achieve the crop ideotype and de novo domestication of wild relatives: case study in tomato. *Plant Science, 256*, 120–130.

# CRISPR/Cas in Improvement of Food Crops for Feeding the World into the Future


**Suraiya Akhtar, Raja Ahmed, Khaleda Begum, Ankur Das, and Sofia Banu**

**Abstract** In the past few decades, hunger and undernourishment at national and global levels are the truths we deny owing to many variables involved, and among the variables, abrupt rise in global population and limited availability of quality food crops are the prominent ones. On top of that, climate change, urbanization, uprising sea level and COVID-19 pandemic have added more adverse and lasting effects on global food security. Therefore, steps should be taken to raise climate resilient, biotic and abiotic stress-resistant, high-yielding and quality food crops to meet the ongoing and upcoming food security and scarcity. On the other hand, conventional breeding and genetic modification techniques are not only limited to few cultivable crops but also raise questions on food safety and security. This has initiated the shift to new nuclease-based tools like CRISPR/Cas, which has recently gained popularity by virtue of its specificity and versatility. CRISPR/Cas technology has been bestowed with precise and remarkable results in manipulating plant traits for sustainable agricultural production. In this chapter, we intended to summarize the necessity for food crop improvement, its prevailing challenges and technologies adopted to overcome the same. The applicability of CRISPR/Cas in achieving zero hunger goals, its reliability to feed the world in the future and its current progress in food crops improvement are also discussed. CRISPR/Cas limitations, its challenges and future perspectives were addressed as well.

**Keywords** Food security · Zero hunger · Crop improvement · Genome-editing technology · CRISPR/Cas

S. Akhtar · R. Ahmed · K. Begum · A. Das · S. Banu (✉)
Department of Bioengineering and Technology, Gauhati University, Guwahati, Assam, India



A. Raina et al. (eds.), *Advanced Crop Improvement, Volume 2*,
https://doi.org/10.1007/978-3-031-26669-0_19

## 1 Introduction

The increase in global population and climate change brings a great threat to global food security (FAO, 2015). It was observed that due to climate change and urbanization, both loss in biodiversity and decline in agricultural production occurred (UN Report, 2019). Recent reports reveal that approximately 811 million people around the globe are going to bed hungry each night, and 25,000 people, including 10,000 children, die daily due to hunger (FAO, 2021). Besides, 99.1 million people in 23 countries faced hunger in 2020, and an additional 78 and 30 million people would be projected to undernourishment and hunger in 2030 due to dispute, climate change and the outbreak of COVID-19 pandemic (Grebmer et al., 2021). The Food and Agriculture Organization of the United Nations anticipated that the global population will be going to reach 9.1 billion by 2050 that requires raise in food production by 70% (FAO, 2009). To solve the above-mentioned problems, several researchers nowadays are trying to develop different improved varieties using different techniques to fight against hunger.

Presently, conventional breeding is the most frequently used technique for the improvement of different crop plants, but it is a very time-consuming technique which may require many years to develop improved plant variety (Ahmar et al., 2020). Therefore, to cut the time and labour during the process of crop improvement, scientists shifted from conventional techniques to gene editing approach (e.g. clustered regularly interspaced short palindromic repeats/associated protein 9 (CRISPR/Cas9), transcription activator-like effector nucleases (TALENs), zinc-finger nucleases (ZFNs), etc.) (Abdallah et al., 2015; Rashid et al., 2017; Wanga et al., 2021). CRISPR/Cas9 is less expensive with more efficient and user-friendly compared to other gene editing tools (Li et al., 2020). Due to this, the use of CRISPR/Cas9 in the field of plant science has increased rapidly in recent years (Haque et al., 2018; El-Mounadi et al., 2020; Wada et al., 2020). Using CRISPR, scientists not only tried to increase the yield of crop plants but also raise varieties resistant against different stresses (Jaganathan et al., 2018). Scientists and food producers are increasingly optimistic that CRISPR will be the instrument that may eliminate hunger around the globe in the near future (Ahmad et al., 2021).

## 2 Need for Crop Improvement

The growing global population may require up to 59–98% more food by 2050. Many agricultural development aspects must be addressed in order to meet the increasing food crop demand (Fig. 1). To feed the global population, farmers need to expand agricultural land or adopt new farming techniques on the existing agricultural land (Islam & Karim, 2019). Rapid transitions of food system to meet the global food demands have many challenges. The nature of the produce from agriculture and food demand is affected by the increase in population, urbanization and

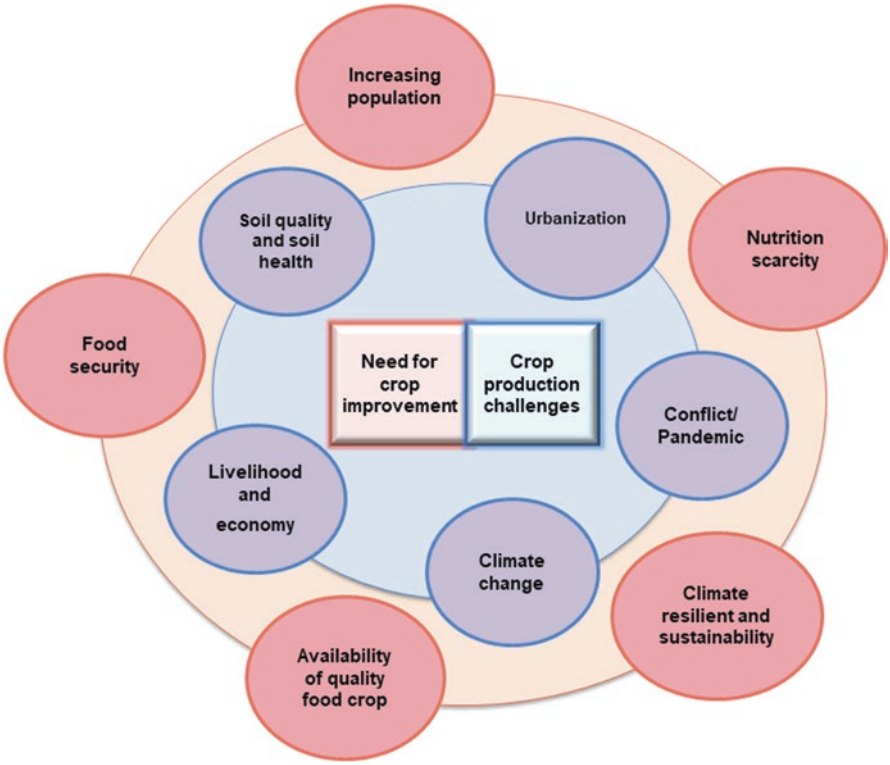



**Fig. 1** Factors relating to crop improvement

individual income. Increasing food demand and stagnant improvement in crop yield encourage farmers to rigorously use chemicals, which are harmful to the environment. In order to address the aforementioned issues, it is vital to promote the implementation of a sustainable food system which might face a great challenge. In low-income countries, the agricultural land demand will increase, and hence, availability of land for agriculture in a confined area of the country may cause environmental and social problems. Therefore, ensuring that natural resources like forests, water biodiversity and land suitable for agriculture will be another difficulty in food production and security on a long-term basis. Moreover, climate change has impacts on agricultural production, soil, forests, water and other natural resources and ultimately affects the ecosystems. Crop yield and food stock are also affected by climate change, thereby jeopardizing the stability and availability of food supplies. Food and agricultural systems are under threat because of the rise in intensity and number of transboundary outbreaks of plant and animal pests and diseases, possibly resulting in food safety issues and radiation contamination. Agriculture can be made more productive by integrating management of pests because it prevents pests and diseases from spreading, minimizes yield loss and reduces pest infestations. Production of sustainable food entails eradicating severe poverty and eliminating

inequality in order to boost agricultural profitability and production, as well as linking farmers to markets. Food that's both sustainable and nutritious strengthens resilience in the face of extended crises, wars and catastrophes; improves resilience, efficiency and the accessibility of the food system; expands income possibilities in rural areas; addresses the root reasons of migration; etc. (Calicioglu et al., 2019). With recent advancements in genome-editing technologies, particularly the CRISPR-Cas system, targeted and precise genetic alteration of crops can be accomplished with greater viability and an accelerated shift toward precise crop improvement to increase food supply and highlight nutritious food sustainability and future agricultural growth potential (Ahmad et al., 2021).

## 3 Genome-Editing Technology Over Conventional Genetic Modification in Crop Improvement

Conventional crop breeding techniques involve marker-assisted selection (MAS), MAS-assisted gene pyramiding and marker-assisted recurrent selection. The ultimate goal for the evolution of these technologies was to identify and propagate crop plants with desirable characters and produce high-quality food with minimum inputs (Fig. 2a) (Tester & Langridge, 2010). However, due to poor breeding capabilities and lack of proper trained staff, communication from developers to users, especially in the developing countries, led to the clampdown of these ideas and emergence of an era of genetically modified (GM) crop. GM crops are generated by incorporating a gene (trait) of interest into a vector and delivering the same into the target plant to obtain a plant with a trait of interest (disease or pest resistance, high yield, abiotic and biotic tolerant, etc.) (Fig. 2b). The upstream or downstream promoters of the transferred genes are identified and characterized for proper regulation in the target plant (Moller et al., 2009). Undoubtedly, GM crops can be considered as sustainable agriculture considering a few of its aspects, including less application of pesticides, less harmful gas emission, less utilization of fossil fuels and potential to solve hunger problem in the developing countries (Conner et al., 2003; James, 2011). But certain aspects of it are still arguable, such as human health and environmental impact due to utilization of agrochemicals, engineered genes, gene transfer into wild and non-targeted population, expensive end products and difficulties in varieties production in consideration to the current needs (Buiatti et al., 2013). Additionally, due to limited public acknowledgement and persisting questions on food security and safety, GM crops were not accepted globally and strict global regulations were established (Turnbull et al., 2021), which have not only resulted in restriction of GM crops to a minimum number of cultivable crops (Prado et al., 2014) but also have started a shift in the interest of the researchers and scientists towards new breeding and genome editing techniques for development and cultivation of novel verities of crops.

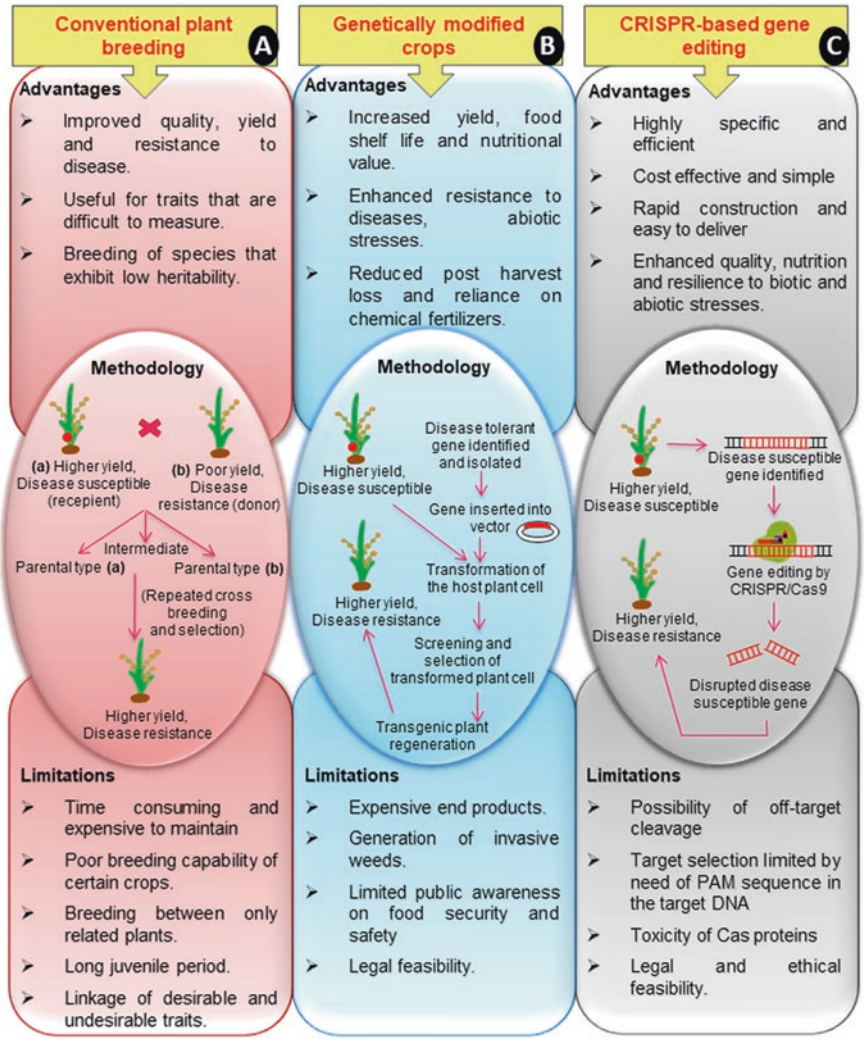



**Fig. 2** Comparison of advantages, schematic working pathway and limitations of prominent approaches for crop improvement. (**a**) Conventional plant breeding, (**b**) genetically modified crops and (**c**) CRISPR-based gene editing

The new breeding or genome editing involves utilization of modern and optimized nuclease-based biological techniques for changes in the genome precisely at particular location (Gao, 2015). Nuclease-based editing tools specifically recognized DNA sequences and introduce breaks, which were fixed by plant repair systems by nucleotide insertion or deletion resulting in gene replacements or insertions or knockouts (Symington & Gautier, 2011). For example, zinc-finger nucleases (ZFNs) (Kim et al., 1996) are developed by fusion of a DNA recognition domain

which recognizes a specific region in DNA, and cleavage domain (Fok1), where the latter acts as the restriction enzyme (Urnov et al., 2010). In crop plants such as maize (trait stacking and herbicide resistance) (Shukla et al., 2009) and rice (trait stacking), ZFNs have been successfully employed (Cantos et al., 2014). In rice, ZFNs have been reported in identification of specific loci in the DNA for future gene insertion (Cantos et al., 2014). Secondly, transcription activator-like effector nucleases (TALENs) (Christian et al., 2010) are another example, which allows more flexible and efficient site detection where each endonuclease works on one nucleotide basis. Also, TALENs have been successfully employed in rice (bacterial resistance, fragrant rice) (Li et al., 2012; Shan et al., 2015), wheat (mildew resistance) (Wang et al., 2014), maize (reduced wax in leaves, haploid induction) (Char et al., 2015; Kelliher et al., 2017), sugarcane (cell wall composition) (Jung & Altpeter, 2016), soybean (high oleic and low linoleic contents) (Haun et al., 2014; Demorest et al., 2016) and tomato (high anthocyanin content) (Cermak et al., 2015). Nevertheless, ZFNs and TALENs are not simple and often challenging due to off-target effects and low efficacy (Szczepek et al., 2007). Thirdly, the Cas proteins integrated clustered regularly interspaced short palindromic repeats (CRISPR/Cas system) have advanced as an innovative tool for genome or gene editing and manipulation (Fig. 2c) (Li et al., 2013). Compared to other nucleases, CRISPR/Cas9, also called type II CRISPR/SpCas9, has gained popularity due to its efficiency, low cost, simplicity and, most importantly, its contribution to many industrially valuable food crops, including maize, rice, tomato, wheat, cotton, potato and soybean (Chen et al., 2019). Upgradation of Cas9 variants into type V CRISPR/Cpf1 has overcome certain limitations on detection of potential sites and restrictive protospacer adjacent motifs (PAM). Cpf1 and its orthologue have been in notice due to their capability to induce targeted mutations (Endo et al., 2016; Xu et al., 2017). Similar to earlier Cas9 variants, Cpf1 may also be coupled with base editing (Li et al. 2018a, b, c, d) and employed in the improvement of crop plants.

## 4 CRISPR/Cas as a Tool to Feed the World into the Future

CRISPR/Cas tool has gained popularity as a promising second-generation editing tool (Jaganathan et al., 2018). Naturally, CRISPR/Cas9 systems act as prokaryotic acquired immune system against the invading phages, and the system was first discovered in *E. coli* (Mutezo et al., 2021). There exist 2 classes of CRISPR/Cas system with 5 types and 16 subtypes (Makarova et al., 2015) in diverse bacteria and archaea which differ in their components and working action, and the data of types and subtypes expanded rapidly to 6 types and 33 subtypes (Makarova et al., 2020). In the updated classification of CRISPR/Cas system, four subtypes – III-E, III-F, IV-B and IV-C – were added in Class 1 (total of 3 types and 16 subtypes) and Class 2 included 3 types and 17 subtypes (Table 1). Additionally, former subtype 'I-U' was reclassified as subtype 'I-G'; former variant 'V-U3' was reclassified as 'V-F1' variant; and a former variant 'V-U5' was upgraded to subtype 'V-K' in 2020 classification

**Table 1** Updated classification of CRISPR/Cas system

| Type | Subtype and variant | | Association of Cas protein variant with type and its function | Organism, corresponding gene range |
|---|---|---|---|---|
| *Class 1* | | | | |
| I | I-A | | **Cas1 (**DNA nuclease) | *Archaeoglobus fulgidus*, AF1859, AF1870–AF1879 |
| | I-B | | **Cas2 (**RNA nuclease) | *Clostridium kluyveri*, CKL_2758–CKL_2751 |
| | I-C | | **Cas3** (DNA nuclease and helicase) | *Bacillus halodurans*, BH0336–BH0342 |
| | I-D | | **Cas7** (RNA recognition, crRNA binding) | *Cyanothece* sp. *8802*, Cyan8802_0527–Cyan8802_0520 |
| | I-E | | | *Escherichia coli K12*, ygcB–ygbF |
| | I-F | I-F1 | | *Yersinia pseudo-tuberculosis*, YPK_1644–YPK-1649 |
| | | I-F2 | | *Shewanella putrefaciens CN-32*, Sputcn32_1819–Sputcn32_1823 |
| | | I-F3 | | *Vibrio crassostreae J5 20*, VCR20J5_310088–VCR20J5_310108 |
| | I-G (I-U)* | | | *Geobacter sulfurreducens*, GSU0051–GSU0054, GSU0057–GSU0058 |
| III | III-A | | **Cas7** (RNA recognition, crRNA binding) | *Staphylococcus epidermidis*, SERP2463–SERP2455 |
| | III-B | | **Cas11** (small subunit of effector complexes) | *Pyrococcus furiosus*, PF1131–PF1124 |
| | III-C | | | *Methanothermobacter thermautotrophicus*, MTH328–MTH323 |
| | III-D | | | *Synechocystis* sp. *6803*, sll7067–sll7063 |
| | III-E | | | *Candidatus Scalindua brodae*, SCABRO_02601, SCABRO_02597 SCABRO_02593, SCABRO_02595 |
| | III-F | | | *Thermotoga lettingae TMO*, Tlet_0097–Tlet_0100 |
| IV | IV-A | | **Cas1** (DNA nuclease) | *Thioalkalivibrio* sp. *K90mix*, (TK90_2699–TK90_2703) |
| | IV-B | | **Cas5** (pre-crRNA processing) | *Rhodococcus jostii RHA1*, RHA1_ro10069–RHA_ro10072 |
| | IV-C | | **Cas7** (RNA recognition, crRNA binding) | *Thermoflexia bacterium*, D6793_05715–D6793_05700 |
| *Class 2* | | | | |
| II | II-A | | **Cas1** (DNA nuclease) | *Streptococcus thermophilus*, str0657–str0660 |
| | II-B | | **Cas2** (RNA nuclease) | *Legionella pneumophila str. Paris*, lpp0160–lpp0163 |
| | II-C | II-C1 | **Cas4** (DNA nuclease) | *Neisseria lactamica 020-06*, NLA_17660–NLA_17680 |
| | | II-C2 | **Cas9** (DNA nuclease) | *Micrarchaeum acidiphilum ARMAN-1*, BK997_03320–BK997_03335 |

(continued)

**Table 1** (continued)

| Type | Subtype and variant | | Association of Cas protein variant with type and its function | Organism, corresponding gene range |
|---|---|---|---|---|
| V | V-A | | **Cas2** (RNA nuclease) | *Francisella cf. Novicida Fx1*, FNFX1_1431–FNFX1_1428 |
| | V-B | V-B1 | **Cas4** (DNA nuclease) | *Alicyclobacillus acidoterrestris*, N007_06525–N007_06535 |
| | | V-B2 | **Cas12** (crRNA processing, DNA nuclease) | *Planctomycetes bacterium RGB_13_46_10*, A2167_01675–A2167_01685 |
| | V-C | | | *Oleiphilus* sp., A3715_16885–A3715_16890 |
| | V-D | | | *Bacterium CG09_39_24*, BK003_02070–BK003_02075 |
| | V-E | | | *Deltaproteobacteria bacterium*, A2Z89_08250–A2Z89_08265 |
| | V-F | V-F1 | | *Uncultured archaeon*, NDOCEIEL_00008–NDOCEIEL_00011 |
| | | V-F1 (V-U3)** | | *Bacillus thuringiensis HD-771*, BTG_31928 |
| | | V-F2 | | *Uncultured archaeon*, ICDLJNLD_00049–ICDLJNLD_00052 |
| | | V-F3 | | *Candidatus Micrarchaeota archaeon*, COU37_03050–COU37_03065 |
| | | V-U1 | | *Gordonia otitidis*, GOOTI_RS19525 |
| | | V-U2 | | *Cyanothece* sp. *PCC 8801*, PCC8801_4127 |
| | | V-U4 | | *Rothia dentocariosa M567*, HMPREF0734_01291 |
| | V-G | | | *Hot springs metagenome*, FLYL01000025.1 (182949–185252) |
| | V-H | | | *Hypersaline lake sediment metagenome (JGI)*, Ga0180438_100006283 |
| | V-I | | | *Freshwater metagenome (JGI)*, Ga0208225_100001036 |
| | V-K (V-U5)*** | | | *Cyanothece* sp. *PCC 8801*, PCC8801_2993–PCC8801_2997 |
| VI | VI-A | | **Cas13** (crRNA processing, RNA nuclease) | *Leptotrichia shahii*, B031_RS0110445 |
| | VI-B | VI-B1 | | *Prevotella buccae*, HMPREF6485_RS00335–HMPREF6483_RS00340 |
| | | VI-B2 | | *Bergeyella zoohelcum*, HMPREF9699_02005–HMPREF9699_02006 |
| | VI-C | | | *Fusobacterium perfoetens*, T364_RS0105110 |
| | VI-D | | | *Ruminococcus bicirculans*, RBI_RS12820 |

An '*' and '**' represent a former subtype (I-U) and variant (V-U3), reclassified as I-G subtype and V-F1 variant, respectively; '***' represents a former variant (V-U5), upgraded to subtype V-K (Makarova et al., 2015, 2020)

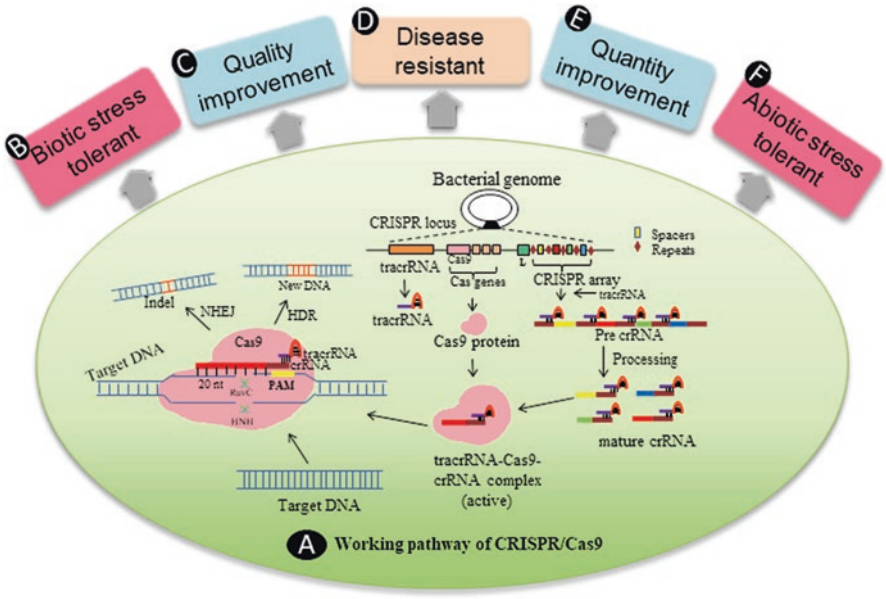



**Fig. 3** (**a**) CRISPR/Cas: Mechanism of action, (**b–f**) its application in crop improvement

(Makarova et al., 2020). The principle of working mechanism of all CRISPR/Cas systems lies on the induction of site-specific double-strand breaks (DSBs) in the DNA of the invading virus or target DNA (Fig. 3a). In consequence to the generated DSBs, a cellular DNA repair mechanism is induced that includes either non-homologous end-joining (NHEJ) or homology-directed repair (HDR). Non-homologous end-joining mechanism leads to imprecise repair, whereas homology-directed repair leads to precise repair. However, repair mechanism leads to dysfunctional viruses by creating insertions or deletions (indels) in the invading DNA of the virus, thus providing with a natural defence against the viruses (Zaidi et al., 2020).

The site-specific DSBs are facilitated by the CRISPR RNA (crRNA) in nature or the guide RNA (gRNA) in the experimental CRISPR/Cas system, which is used for guidance and site-specificity. The spacer region of the crRNA hybridizes to the complementary sequence within the target genome present near to protospacer adjacent motif (PAM) or protospacer flanking sequence (PFS) in type VI systems. Following hybridization, the Cas nuclease cleaves the targeted DNA sequence (Pickar-Oliver & Gersbach, 2019).

With Cas9 protein and tailored crRNA with proper spacer sequences, site-specific cleavage can be performed at any locus harbouring PAM or PFS in an engineered system (Pickar-Oliver & Gersbach, 2019). Cas9 protein, namely, Cas9 of *Streptococcus pyogenes* (spCas9), was the first protein to be reprogrammed and employed outside of prokaryotic cells for genome editing (Jinek et al., 2012). The PAM – a trinucleotide sequence of 5′-NGG (N representing any nucleotide) and the

20 nucleotide long spacer – is used by the spCas9 for selective recognition and binding to the DNA of the target (Wright et al., 2016) and usually creates a blunt DSBs (Garneau et al., 2010). In experimental systems, a short crRNA is transcribed from a CRISPR locus which hybridizes with a target sequence near the PAM site followed by the binding of a trans-activating RNA (tracr RNA) to the crRNA. The interaction of tracrRNA with crRNA aids in the processing of mature guide RNA (gRNA) which then forms the Cas9 complex with Cas9 and RNase III. The gRNA directs the Cas9 nuclease to create a DSB after the Cas9 complex binds to the target site. The DSB is generated three nucleotides upstream of the target DNA's PAM site (O'Connell et al., 2014). The functionalities of crRNA and tracrRNA can be replicated using a designed single gRNA. CRISPR/Cas9 can thereby produce precise, site-specific genome editing by causing Cas9-mediated DSBs at the target sites through the guidance of designed single gRNAs (Hanna & Doench, 2020).

The CRISPR/Cas system, in particular, has established itself as the dominant, ground-breaking site-specific nuclease (SSN), and despite the fact that its efficacy for plant genome editing was first shown in 2013 (Li et al., 2013; Nekrasov et al., 2013; Shan et al., 2013), its plant applications have grown fast in recent years (Zaidi et al., 2020). With continuous upgradation and the development of newer variants of CRISPR/Cas timely and precisely with growing needs and obstacles, this novel gene-editing tool has become more utilizable and dependable than the conventional ones. Later, introduction of non-native DNA might affect the final product (He & Zhao, 2019), whereas the nucleases target precisely into the plant genome which also can be regulated (Liang et al. 2018a, b). Advances in technologies and procedures have resulted in efficient product development and easy market availability of crops for human consumption at reasonable prices. This makes CRISPR/Cas system an excellent tool for addressing questions associated with plants that have been genetically modified and produce food crops to meet hunger in the near future (Fig. 3b–f).

## 5 Progress of CRISPR in Crop Improvement

Many studies have concentrated on the usage of CRISPR/Cas9-based gene editing in enhancing resilience to stress, quality and agricultural output in crop plants (Table 2). CRISPR/Cas9 system has demonstrated gene knock-in and knockout, expression regulation and gene knock-down-like genome-editing capabilities in a variety of food crop plants. The following sections outline the progress of CRISPR/Cas9 for gene editing in several food crop species based on literature retrieval.

### *5.1 Vegetables*

Application of CRISPR/Cas system for improvement of vegetable crops has been well demonstrated. Susceptibility to various stresses, low yield, nutritional value and shelf life were some major concerns for vegetable crops, especially for

**Table 2** List of CRISPR/Cas-mediated gene editing in food crops

| Food crop type | Food crop | Gene targeted | Character | Transfer method | Reference |
|---|---|---|---|---|---|
| Vegetables | *Solanum tuberosum* | 16DOX | Reduction of steroidal glycoalkaloid content | CRISPR/Cas9, *Agrobacterium* (shoot transformation) | Nakayasu et al. (2018) |
| GBSS | Increase quality of starch | CRISPR/Cas9, PEG (protoplast transfection) | Andersson et al. (2018) | SBE | Increase quality of starch |
| | | CRISPR/Cas9, PEG (protoplast transfection) | Tuncel et al. (2019) | StPPO2 | Reduce browning |
| | | CRISPR/Cas9, PEG (protoplast transfection) | Gonzalez et al. (2019) | ALS1 | Resistance to herbicide |
| | | CRISPR/Cas9, *Agrobacterium* (shoot transformation) | Butler et al. (2015) | IAA2 | Shoot morphogenesis |
| | | CRISPR/Cas9, *Agrobacterium* | Wang et al. (2015) | *Cucumis sativus* | RBOHD |
| | | Tolerance to salinity | CRISPR/Cas9, *Agrobacterium* (hairy root transformation) | Huang et al. (2019a, b) | elF4E |
| | Resistance to virus | CRISPR/Cas9, *Agrobacterium* | Chandrasekaran et al. (2016) | WIP1 | Generation of flower with carpel only |
| | | CRISPR/Cas9, *Agrobacterium* | Hu et al. (2017) | *Daucus carota* | PDS |
| | | Albino phenotype | CRISPR/Cas9, *Agrobacterium* | Xu et al. (2019) | *Brassica oleracea var. capitata* |
| | PDS | Albino phenotype | CRISPR/Cas9, *Agrobacterium* | Ma et al. (2019) | Cereals |
| | *Triticum aestivum* | TaGW2 | Increase seed size | CRISPR/Cas9 | Wang et al. (2018a, b, c) |

(continued)

**Table 2** (continued)

| Food crop type | Food crop | Gene targeted | Character | Transfer method | Reference |
|---|---|---|---|---|---|
| TaMLO | Resistance against powdery mildew | CRISPR/Cas9 | Wang et al. (2016a, b) | TaLpx-1 | Enhance resistance against *Fusarium graminearum* |
| | | CRISPR/Cas9 | Zhang et al. (2017) | TaLOX2 | Resistance against powdery mildew |
| | | CRISPR/Cas9 | Bhowmik et al. (2018) | TaEDR1 | Resistance against powdery mildew |
| | | CRISPR/Cas9 | Zhang et al. (2017) | TaCer9 | Improve drought resistance |
| | | CRISPR/Cas9, Particle bombardment | Liang et al. (2018a, b) | TaALS | Enhance herbicide resistance |
| | | CRISPR/Cas9 | Zong et al. (2017) | *Oryza sativa* | GW2, GW5 and TGW6 |
| | | Increase weight of grain | CRISPR/Cas9 | Xu et al. (2016a, b) | Hd2, Hd4, and Hd5 |
| | Early maturity | CRISPR/cas9 | Li et al. (2017) | OsVP1 | Bypass seed dormancy |
| | | CRISPR/Cas9 | Jung et al. (2019) | OsMPK5 | Enhance disease resistance |
| | | CRISPR/Cas9 | Jun et al. (2019) | Os8N3 | Resistance against *Xanthomonas oryzae* pv. *oryzae* strains |
| | | CRISPR/Cas9 | Jun et al. (2019) | OsSWEET | Resistance against *Xanthomonas oryzae* pv. *oryzae* strains |
| | | CRISPR/Cas9 | Zafar et al. (2020) | OsERF922 | Enhance blast resistance in rice |
| | | CRISPR/Cas9 | Wang et al. (2016a, b) | AAC | Osmotic stress tolerance |
| | | CRISPR/Cas9 | Romero and Gatica-Arias (2019) | ALS | Herbicide resistance |
| | | CRISPR/Cas9 | Romero and Gatica-Arias (2019) | | |
| | | DST | Drought and salt tolerance | CRISPR/Cas9 | Kumar et al. (2020) |

| | | | | | |
|---|---|---|---|---|---|
| *Zea mays* | CLE | Boost grain yields | CRISPR/Cas9 | Lei et al. (2021) | *Hordeum vulgare* |
| | β-1,3-Glucanase | Increase cellulose formation | CRISPR/Cas9 | Kim et al. (2020) | Nud |
| | Production of naked grains | CRISPR/Cas9 | Gasparis et al. (2018) | *Sorghum bicolor* | k1C |
| | | Reduce kafirin quantity | CRISPR/Cas9 | Li et al. (2018a, b, c, d) | Fruits |
| | *Solanum lycopersicum* | phytoene synthase 1 (PSY1 | Yellow-coloured tomato | CRISPR/Cas9, *Agrobacterium* (Cotyledon transformation) | Wang et al. (2019a, b) |
| MYB transcription factor 12 (MYB12) | Pink-coloured tomato | CRISPR/Cas9, *Agrobacterium* | Wang et al. (2019a, b) | Anthocyanin 2 (ANT2) | Purple-coloured tomato |
| | | CRISPR/Cas9, *Agrobacterium* | Wang et al. (2019a, b) | CLV-WUS | Fruits locule number increase |
| | | CRISPR/Cas9, *Agrobacterium* | Ma et al. (2015) | PL | Increase shelf life |
| | | CRISPR/Cas9 | Uluisik et al. (2016) | ALC | Increase shelf life |
| | | CRISPR/Cas9, *Agrobacterium* | Yu et al. (2017) | MPK20 | Sugar metabolism controlling gene repression |
| | | CRISPR/Cas9, *Agrobacterium* | Chen et al. (2018) | ALMT9 | Decrease malate content |
| | | CRISPR/Cas9, *Agrobacterium* (cotyledon transformation) | Ye et al. (2017) | AGL6 | Production seedless parthenocarpic fruit |
| | | CRISPR/Cas9, *Agrobacterium* | Klap et al. (2017) | | |
| | | IAA9 | Production seedless parthenocarpic fruit | CRISPR/Cas9, *Agrobacterium* (cotyledon transformation) | Ueta et al. (2017) |

(continued)

**Table 2** (continued)

| Food crop type | Food crop | Gene targeted | Character | Transfer method | Reference |
|---|---|---|---|---|---|
| ARF7 | Production seedless parthenocarpic fruit | CRISPR/Cas9, *Agrobacterium* | Hu et al. (2018) | MBP21 | Generation of 'jointless' fruit stem |
| | | CRISPR/Cas9, *Agrobacterium* | Soyk et al. (2017) | GAI | Generation of dwarf plant |
| | | CRISPR/Cas9, *Agrobacterium* | Tomlinson et al. (2019) | BOP1, BOP2, BOP3 | Simplified inflorescences and early flowering |
| | | CRISPR/Cas9, *Agrobacterium* | Xu et al. (2016a, b) | SP5G | Generation of resistant tomato against duration of day length |
| | | CRISPR/Cas9, *Agrobacterium* | Soyk et al. (2017) | CBF1 | Decrease in chilling stress tolerance |
| | | CRISPR/Cas9, *Agrobacterium* | Li et al. (2018a, b, c, d) | CP and Rep of virus | Resistance against tomato yellow leaf curl virus |
| | | CRISPR/Cas9, *Agrobacterium* | Tashkandi et al. (2018) | DCL | Resistance against potato virus X, tobacco mosaic virus and tomatomosaic virus |
| | | CRISPR/Cas9, *Agrobacterium* | Wang et al. (2018a, b, c) | DMR6 | Resistance against downy mildew |
| | | CRISPR/Cas9, *Agrobacterium* | Thomazella et al. (2016) | MLO1 | Resistance against powdery mildew |
| | | CRISPR/Cas9, *Agrobacterium* | Nekrasov et al. (2017) | Solyc08g075770 | resistant against *Fusarium* wilting disease caused by *Fusarium oxysporum* |
| | | CRISPR/Cas9, *Agrobacterium* | Prihatna et al. (2018) | | |
| | | MAPK3 | Susceptibility to grey mould disease | *B. cinerea* spore suspension | Zhang et al. (2018) |

| | | | | | |
|---|---|---|---|---|---|
| JAZ2 | Resistance against bacterial speck disease | CRISPR/Cas9, *Agrobacterium* | Ortigosa et al. (2019) | MAPK3 | Decrease in drought stress tolerance |
| | | CRISPR/Cas9, *Agrobacterium* | Wang et al. (2017) | BZR1 | Decrease in heat stress tolerance |
| | | CRISPR/Cas9, *Agrobacterium* | Yin et al. (2018) | *Musa paradisiaca* | LCYε |
| | | Flux for β-carotene biosynthesis | CRISPR/Cas9, *Agrobacterium* | Kaur et al. (2020) | MaACO1 |
| | Increase shelf life | CRISPR/Cas9, embryogenic cell suspension | Hu et al. (2021) | *Fragaria ananassa* | MYB10 |
| | | Mutagenesis study | CRISPR/Cas9, *Agrobacterium* | Xing et al. (2018) | ARF8 |
| | Increase growth rate | PEG (protoplast transfection) | Zhou et al. (2018) | TM6 | Confirm role of this gene for stamen development |
| | | CRISPR/Cas9, *Agrobacterium* | Martín-Pizarro et al. (2019) | *Vitis vinifera* | MLO7 |
| | | Resistance against powdery mildew | Protoplast transfection | Malnoy et al. (2016) and Wang et al. (2019a, b) | WRKY52 |
| | Resistance against grey mould disease | CRISPR/Cas9, *Agrobacterium* | *Citrus paradisi* | LOB1 promoter | Resistance against citrus canker |
| | | CRISPR/Cas9, *Agrobacterium* | Peng et al. (2017) | *Malus pumila* | |
| | DIPM1, 2, 4 | Resistance against fire blight disease | Protoplast transfection | Malnoy et al. (2016) | |
| | *Citrullus lanatus* | SWEET3, AGA2, TST2 | Sugar accumulation | CRISPR/Cas9 | Ren et al. (2021) |

(continued)

**Table 2** (continued)

| Food crop type | Food crop | Gene targeted | Character | Transfer method | Reference |
|---|---|---|---|---|---|
| ALS | Resistance to herbicide | CRISPR/Cas9, Agrobacterium (shoot transformation) | Tian et al. (2018) | PSK1 | Resistance to *F. oxysporum* |
| | | CRISPR/Cas9, Agrobacterium (shoot transformation) | Zhang et al. (2020a, b) | PDS | Albino phenotype |
| | | CRISPR/Cas9, PEG (protoplast transfection) | Tian et al. (2017) | Pulses | *Medicago truncatula* |
| | | Enhancer1, phytoene desaturase and symbiosis receptor-like kinase | Mutagenesis study | CRISPR/Cas9, Agrobacterium (hairy root transformation) | Curtin et al. (2018) |
| *Vigna unguiculata* | VuSPO-11-1 | Development of hybrid of cowpea plant | CRISPR/Cas9, *Agrobacterium* | Che et al. (2021) | *Glycine max* |
| | GmFAD2–1A and GmFAD2–1B | Improve the profile of seed oil | CRISPR/Cas9, *Agrobacterium* (hairy root transformation) | Do et al. (2019) | GmF3H1, GmF3H2 GmFNSII-1 |
| | Increase leaf isoflavone content providing resistant against soybean mosaic virus (SMV) | CRISPR/Cas9, *Agrobacterium* (hairy root transformation) | Zhang et al. (2020a, b) | MtSUP | Confirm orthologue sequence of *Arabidopsis* present in soybean |
| | | CRISPR/Cas9, *Agrobacterium* | Rodas et al. (2021) | *Cicer arietinum* | 4CL and RVE7 |
| | | Editing of drought-tolerant gene | PEG (protoplast transfection) | Badhan et al. (2021) | Seeds and nuts |
| | *Brassica napus* | FAD2-2 and BNFAD2 | Reduce linoleic acid | CRISPR/cas9 | Okuzaki et al. (2018) and Al Amin et al. (2019) |

| | | | | | |
|---|---|---|---|---|---|
| GmFATB1 | Reduction in fatty acid concentration | CRISPR/cas9 | Ma et al. (2021) | BnWRKY70 | Resistance against *Sclerotinia sclerotiorum* |
| | | CRISPR/Cas9, *Agrobacterium* | Sun et al. (2018) | BnD14 | Develop variety with prolific branched dwarf phenotype, short internode length and increase of the total flower |
| | | CRISPR/cas9 | Stanic et al. (2021) | *Camelina sativa* | CsFAD2 |
| | | Enhance fatty acid content | CRISPR/cas9 | Jiang et al. (2017) | CsCRUC |
| | Enhance fatty acid content | CRISPR/cas9 | Lyzenga et al. (2019) | CsFAE1 | Enhance fatty acid content |
| | | CRISPR/cas9 | Ozseyhan et al. (2018) | CsDGAT1 | Enhance fatty acid content |
| | | CRISPR/cas9 | Aznar-Moreno and Durrett (2017) | *Arachis hypogaea* | AhNFR5 |
| | | Hairy root disease tolerance | CRISPR/cas9 | Shu et al. (2020) | Herbs and species |
| | *Ocimum basilicum* | DMR1 | Resistance to *Hyaloperonospora arabidopsis* | CRISPR/Cas9, *Agrobacterium* | Navet and Tian (2020) |
| *Capsicum annuum* | CaERF28 | Resistance to *Colletotrichum truncatum* | CRISPR/Cas9, *Agrobacterium* | Mishra et al. (2021) | |

*Cucurbitaceae*, *Brassica*, *Solanaceae*, *Apiaceae* and *Asteraceae* family species. CRISPR/Cas9 has bought a revolution through its precise, specific genome editing and mutation-inducing nature to overcome such issues. In this part, we summarized the progress of CRISPR/Cas9 towards vegetable crops improvement to date.

### 5.1.1 Quality Improvement

Overall quality improvement relating to nutrition, size, life, colour, flavour and taste has been accomplished in a few fleshy vegetable crops through CRISPR/Cas9 system. For instance, in cucumber, genes in carotenoid pathway have been edited to boost the lycopene content (5.1-fold) and also to inhibit α- and β-carotene formation from lycopene (Chandrasekaran et al., 2016). The technique of CRISPR/Cas has also been implemented to develop gynoecious lines of cucumber for fast development of hybrids and concentrated fruit sets and to enhance productivity rate by turning off the carpel development inhibitory genes *WIP domain containing protein 1 (WIP1)*, and the resultant mutant cucumber displayed female flower only (Hu et al., 2017). In potato, steroid 16α-hydroxylase mutant was generated to reduce the content of steroidal glycoalkaloids, thus, eliminating the bitter taste that potato exhibits due to high α-solanine and α-chaconine (Nakayasu et al., 2018). Potato starch quality was also induced by generating starch-branching enzyme (*SBE*) and granule-bound starch synthase (*GBSS)* mutant variety using CRISPR/Cas9 (Andersson et al., 2018; Tuncel et al., 2019). Further mutation in the *StPPO2* gene has resulted in downregulation of polyphenol oxidases and reduced browning (Gonzalez et al., 2019). Hence, CRISPR/Cas has been successfully employed in certain leafy vegetables to overcome the harvest and post-harvest costing and obtain comparatively high returns in the market (Xu et al. 2016a, b).

### 5.1.2 Biotic and Abiotic Stress

The CRISPR/Cas9 system has been employed in vegetable crops either to detect crucial gene functions or impart resistance to abiotic or biotic factors including microbes, pests, herbicide, disease, etc. *RBOHD* through CRISPR/Cas9-mediated knockout was functionally characterized to be connected to salt resistance in pumpkin (Huang et al. 2019a, b). A potato variant with herbicide resistance was also generated through transformation mediated by *Agrobacterium* using CRISPR/Cas system (Butler et al., 2015). Pathogenic fungi, *Sclerotina sclerotiorum*-resistant *Brassica nupus* and virus-resistant *Cucumis sativus* (cucumber), were generated by editing *WRKY70* and *elF4E* gene, respectively, using CRISPR/Cas9 (Chandrasekaran et al., 2016; Sun et al., 2018).

### 5.1.3 Others

High expression of phytoene desaturase (*PDS*) specifically in carrot (Xu et al., 2019) and *Brassica* crops (Ma et al., 2019) affects photosynthesis and leaf colouration. Therefore, using CRISPR/Cas9 the *PDS* gene of the plants was knocked out. Other aspects of CRISPR/Cas9 towards crop improvement include parthenocarpy and domestication of wild variety, which are successful mainly in tomato, and have not yet been applied in vegetable crops (Ueta et al., 2017; Li et al. 2018a, b, c, d), as these CRISPR/Cas9 system-induced mutations have no side effects, reliable and reproducible. Therefore, the same can be applied to vegetable crops as well in the future.

## *5.2 Cereals*

As a staple food, cereals under the family Gramineae are rich in fibre, carbohydrate and proteins (Sarwar et al., 2013). Because of its nutritional value, it is consumed as food by a large population of the world (Laskowski et al., 2019). Although it is rich in nutritional value, crop loss to a larger extent, due to stress caused by biotic and abiotic factors, affects the productivity of these crops, due to which farmers suffer (Jun et al., 2019; Jeyasri et al., 2021). In recent times, scientists not only tried to boost its resistance against biotic and abiotic stresses but also attempted to raise its quality and yield to resolve the food problem in today's world and the near future (Atkinson & Urwin, 2012). For this to accomplish, scientists use CRISPR technology, an important gene-editing tool which has emerged in the recent past (Jaganathan et al., 2018).

### 5.2.1 Quality Improvement

CRISPR has been employed widely in recent years by different researchers to increase the quality and production of grains. In wheat, *TaGW2* is a significant gene responsible for seed size which was mutated by Wang et al. to enhance the size of seeds (Wang et al. 2018a, b, c). Wheat, barley and rye are the source of gliadins and glutenins, which may lead to coeliac disease. To overcome this problem, researchers use CRISPR/Cas system mutated genes producing gliadins and glutenins, which could perhaps lower the exposure of individuals to coeliac disease epitopes (Jouanin et al., 2020). A huge percentage of global population depends upon rice directly or indirectly. Several researchers are now involved in methods to improve both qualitative and quantitative qualities using CRISPR technology. For this, genes like *Gnla*, *DEP1*, *GS3*, *CCD7*, *IPA1* and *SBE* are mutated using CRISPR to enhance the grain number, panicle architecture, productivity, grain size, starch biosynthesis, etc. (Li et al., 2016; Ricroch et al., 2017; Miao et al., 2018). In 2016, Xu et al. increased the weight of rice grain by targeting *GW2*, *GW5* and *TGW6* genes using CRISPR/Cas9

tool (Xu et al. 2016a, b). Genes like *Hd2*, *Hd4* and *Hd5*, responsible for maturity of rice targeted by Li et al. to obtain rice variety with early maturity (Li et al., 2017). Rice seed dormancy was bypassed via targeted mutagenesis of *OsVP1* gene using CRISPR/Cas9 tool (Jung et al., 2019). In maize, the *CLE* genes are edited using CRISPR/Cas9 tool to boost grain yields (Lei et al., 2021). The *β-1,3-Glucanase* gene of barley was mutated using CRISPR to increase cellulose formation (Kim et al., 2020). Using CRISPR technology, the *Nud* gene, which is responsible for the production of a layer of cementation between the pericarp and both the palea and lemma in barley as a result of an ethylene response, was modified to produce naked grains (Gasparis et al., 2018). Kafirins are the major storage protein of sorghum regulated by *k1C* gene. Since kafirins are devoid of essential amino acids and have poor digestibility, Li and his colleagues focused on the *k1C* gene to reduce the kafirin quantity with increase in digestibility and quality of protein (Li et al. 2018a, b, c, d).

### 5.2.2 Biotic and Abiotic Stress

CRISPR has been used extensively in rice, wheat and maize to boost tolerance to biotic and abiotic stress. In rice, *OsMPK5* gene (a counter-regulator of rice defence response) was mutated using CRISPR/Cas9 for the enhancement of disease-resistant quality (Jun et al., 2019). By the knock-down of *Os8N3* gene using CRISPR technology, researchers developed a mutant variety which is resistant against *Xanthomonas oryzae* pv. *oryzae* strains (Jun et al., 2019). In 2020, Zafar et al. developed traits, where effector binding elements of *AvrXa7* (transcription activator-like receptor) from *OsSWEET* gene family were deleted using CRISPR/Cas9 tool, which enhanceed the resistance against *Xanthomonas oryzae* pv. oryzae strains (Zafar et al., 2020). Also, to improve blast resistance rice variety, scientists mutated *OsERF922* gene using CRISPR/Cas9 tool (Wang et al. 2016a, b). In wheat, researchers mutated *TaMLO* gene by applying CRISPR/Cas9 tool in wheat protoplast to obtain a variety that is resistant to powdery mildew (Wang et al. 2016a, b). Additionally, by editing *TaLOX2* and *TaEDR1* genes, researchers obtained powdery mildew-resistant variety of wheat (Zhang et al., 2017; Bhowmik et al., 2018). In 2015, using CRISPR/Cas9 tool, Zhang et al. silenced *TaLpx-1* gene to boost the resistance of wheat against *Fusarium graminearum* by activating jasmonic acid-mediated defence response (Zhang et al., 2017). A wheat homolog *TaCer9* was edited by Liang et al. to create a variety with improved drought resistance and efficiency with water use (Liang et al. 2018a, b). In 2018, *TaALS* gene was edited by Zong and his coworkers using CRISPR/Cas9 for the generation of herbicide-resistant wheat (Zong et al., 2017). In rice, *AAC*, *ALS* and *DST* genes are edited to develop herbicide, drought, osmotic stress as well as salt stress-tolerant variety (Romero & Gatica-Arias, 2019; Kumar et al., 2020).

## *5.3 Fruits*

For the purpose of improving fruit harvests with reference to consumer-desired traits, CRISPR/Cas technology was employed to edit genome of different fruit crops to bring about the change in desired traits, and this has been carried out, namely, in apple, tomato, banana, citrus, coconut, date palm, grapefruits, grape, kiwifruits, watermelon, pear and orange. Tomato acts as a model plant system for fruit biology research because of its short life cycle, simple diploid species with small genome, simple reproductive biology, insensitivity to the photoperiod length and ease to culture in any environmental condition (Foolad, 2007). In 2014, CRISPR/Cas technology was applied for the first time on a tomato plant, with the gene *Argonaute 7* being targeted which resulted in wiry phenotype. Subsequently, CRISPR/Cas technology has been implemented to edit tomato plant genome to obtain virus, fungal and bacterial infection-resistant plants (Brooks et al., 2014). The utilization of the CRISPR/Cas technology for tomato and other fruits has been summarized in this part in terms of quality enhancement and tolerance to biotic and abiotic stress.

### 5.3.1 Quality Improvement

Researchers have successfully grown yellow, pink and purple colour tomato using CRISPR/Cas technology by editing the *phytoene synthase 1* (*PSY1*), *MYB transcription factor 12* (*MYB12*) and *Anthocyanin 2* (*ANT2*), respectively (Wang et al. 2019a, b). Stem cell circuit *CLAVATA-WUSCHEL* (*CLV-WUS*) associated with fruit size was inactivated by applying CRISPR/Cas technology that resulted in larger tomato fruits as compared to the wild type (WT) (Ma et al., 2015). Although CRISPR/Cas edited inactivation of *DNA demethylase 2* (*DML2*) and *ripening inhibitor* (*RIN*) confers prolong shelf life and incomplete fruit ripening, these edited crops showed inferior characters, for which researchers mutated *pectate lyase (PL)* and *alcobaca (ALC)* gene of tomato (Uluisik et al., 2016; Yu et al., 2017). In sugar the carotenoid biosynthetic gene of tomato, mitogen-activated protein kinase 20 (*MPK20*) functions were disrupted by CRISPR/Cas technology (Chen et al., 2018). Gene expressions related to biosynthesis pathway of compounds with bioactive properties of tomato such as anthocyanin, GABA and lycopene have been modulated to enhance their content employing CRISPR/Cas technology (Wang et al. 2019a, b). *Aluminium-activated malate transporter 9* (*ALMT9*) associated with malate content of tomato has been detected using CRISPR/Cas technology (Ye et al., 2017). Seedless tomato was produced by silencing the gene *Agamous-like 6* (*AGL6*) using CRISPR/Cas technology along with heat stress (Klap et al., 2017). The role of auxin signalling pathway genes – *indole-3-acetic acid inducible9* (*IAA9*) and *auxin response factor 7* (*ARF7*) in the parthenocarpic tomato development – was validated by inactivating these two genes using CRISPR/Cas technology (Ueta et al., 2017; Hu et al., 2018). 'Stem jointless' fruit has been produced by knockout *MADS-box protein 21* (*MBP21*) gene for easy fruit plucking with picking

manipulators by the farmers (Soyk et al., 2017). Dwarf tomato plant generated by silencing *gibberellic-acid insensitive* (*GAI*) gene showed great survival rate in windy environment (Tomlinson et al., 2019). Arabidopsis homolog gene 'blade-on-petiole' *(BOP)* associated with flower architecture in tomato, when knocked out, led to large plant productivity (Xu et al. 2016a, b). Inactivation of *self-pruning 5G* (*SP5G*) in tomato showed a fast rush of flower blossoming that shorten the mature fruit development period (Soyk et al., 2017).

β-carotene-enriched Cavendish banana cultivar (cv.) Grand Naine (AAA genome) has been developed by targeting *lycopene epsilon-cyclase (LCYε)* gene with CRISPR/Cas technology (Kaur et al., 2020). Hu et al. (2021) edited ethylene biosynthesis gene '*MaACO1*' (*aminocyclopropane-1-carboxylate oxidase 1*) to increase banana's shelf life. Functional study of *R2R3 MYB transcription factor 10* (*MYB10*) of strawberry, a model plant, produced loss-of-coloration fruits using CRISPR/Cas technology (Xing et al., 2018). Strawberry seedling growth was faster in *Auxin response factor 8* (*ARF8*) gene homozygous mutants than in WT plants (Zhou et al., 2018). The crucial role of *tomato MADS-box gene 6* (*TM6*) during stamen development was confirmed by editing with CRISPR/Cas technology as *TM6* knockout strawberry developed defects in anther development (Martín-Pizarro et al., 2019). Recently, CRISPR/Cas9 system has been employed as well to edit watermelon genome for better sugar accumulation (Ren et al., 2021). Knocking out of the *PDS* gene in watermelon whose high expression affects photosynthesis and leaf colouration was also achieved through CRISPR/Cas9 (Tian et al., 2017).

### 5.3.2 Biotic and Abiotic Stress

A virus-resistant tomato plant was developed by targeting the virus's genes *CP* and *Rep* to confer virus resistance (Tashkandi et al., 2018). The Tomato *Dicer-like 2 (DCL2)* gene was knocked out using CRISPR/Cas technology to confer virus resistance against potato virus X, tobacco mosaic virus and tomato mosaic virus (Wang et al. 2018a, b, c). In tomato, CRISPR/Cas technology was used to mute the *downy mildew resistant 6* (*DMR6*) orthologue gene which resulted in resistant tomato plant against *Pseudomonas syringae*, *Phytophthora capsici*, and *Xanthomonas* spp. (Thomazella et al., 2016). Also, *mildew-resistant locus O1* (*Mlo1*) coding for membrane-associated protein which provides susceptibility to powdery mildew disease causative agent *Oidium neolycopersici* was inactivated in tomato (Nekrasov et al., 2017). Knocking out of *Solyc08g075770* gene of tomato with CRISPR/Cas has demonstrated resistance against *Fusarium* wilting disease (caused by *Fusarium oxysporum*) (Prihatna et al., 2018). *Mitogen-activated protein kinase 3 (MAPK3)* has been shown to be resistant against grey mould disease-causing airborne pathogen *Botrytis cinerea* which causes economic losses during post-harvesting period of tomato was delineated with CRISPR/Cas technology (Zhang et al., 2018). Researchers developed tomato variant – *JAZ2* repressors lacking the C-terminal jasmonate-associated (Jas) domain (*JAZ2*) using CRISPR/Cas technology to impart

defence against bacterial speck disease caused by *Pseudomonas syringae* (Ortigosa et al., 2019).

Base editing of acetolactate synthase and knockout of *PSK1* of watermelon through CRISPR/Cas9 has resulted in herbicide-resistant variant and resistance to *F. oxysporum* (Tian et al., 2018; Zhang et al. 2020a, b). In grapevine and grape berry, resistance against *Erysiphe necator* and *Botrytis cinerea* was conferred by *mildew resistance locus O 7 (MLO7)* and *WRKY transcription factor 52 (WRKY52)* genes, respectively, and loss of functions of these two genes was validated with CRISPR/Cas technology (Malnoy et al., 2016; Wang et al. 2018a, b, c). Resistant papaya and cacao against fungal pathogens *Phytophthora palmivora* and *Phytophthora tropicalis* have been produced using CRISPR/Cas technology. *Lateral organ boundaries 1* (*LOB1*) genes promoter of citrus has been targeted for generation of resistant citrus plant against *Xanthomonas citri*, which causes serious loss of citrus crop (Peng et al., 2017). *Erwinia amylovora* resistance protein DspA/E--interacting proteins (*DIPM1*, *DIPM2* and *DIPM4*) were silenced using this technology (Malnoy et al., 2016). Improvement of desert agriculture crop date palm with CRISPR is challenging task due to its complex genome and high occurrence of single-nucleotide polymorphism introduced basic procedure of theoretical application of CRISPR/Cas technology for genetic improvement of date palm (Wang et al. 2019a, b).

CRISPR/Cas technology is also applied to improve crop plant against abiotic stresses like drought, flooding, chilling and heat. Tomatoes are easily harmed by cold temperatures because of their chill-sensitive nature. Cold-protecting genes *C-repeat binding factor 1* (*CBF1*) mutated tomato plant was developed using CRISPR/Cas technology, and it demonstrated more susceptibility to chilling injury (Li et al. 2018a, b, c, d). Grey mould-resistant gene *MAPK3* also associated with protecting plasma membrane from oxidative damage and provided drought stress resistance (Wang et al., 2017). *CRISPR-bzr1*- and *BZR1*-overexpressing lines confirmed that phytohormone genes *Brassinazole-resistant 1 (BZR1)* are engaged in thermotolerance through regulating *Feronia* genes *(FER)* as well as regulating BR-mediated developmental processes (Yin et al., 2018). Herbicide-resistant healthy growing watermelon was developed by editing high herbicide-resistant gene *Acetolactate synthase (ALS)* by employing CRISPR/Cas technology conferred resistant to herbicide tribenuron (Tian et al., 2018).

## *5.4 Pulses*

Legumes belong to third largest Angiosperm family after Asteraceae and Orchidaceae and in second place relating to its economic importance after Gramineae (grasses). They are a rich source of plant-derived proteinand essential amino acids for vegetarians. One important ecological role played by leguminous crops is maintaining soil health for sustainable farming with their symbiotic nitrogen fixation properties. Using the available annotated genome sequences and transformation

protocol, CRISPR/Cas technology has been implemented to edit *Medicago truncatula* and *Lotus japonicus*, and in soybean, cowpea genome to improve desired agronomic traits (Bhowmik et al., 2021). *M. truncatula* and *L. japonicus* are model species for physiological study of leguminous plant and are studied for features related to nitrogen fixation through root nodules owing to their short growth period, diploidy, self-fertility and ease of transformation method (Young & Udvardi, 2009). Development of CRSPPR/Cas technology allowed targeted mutagenesis in nodulation gene of these plants. Curtin and his colleagues used CRISPR/Cas for functional study of nodulation-specific genes of *M. truncatula* (Curtin et al., 2017). Function of *nodule-specific PLAT domain* (*NPD1–5*) and *nitrate peptide* family *(NPD)* genes has been confirmed by silencing the genes with CRSPPR/Cas technology (Trujillo et al., 2019; Wang et al. 2020a, b). *Hua enhancer1*, *phytoene desaturase* and *symbiosis receptor-like kinase* genes of *M. truncatula* mutated using CRISPR/Cas technology (Michno et al., 2015; Meng et al., 2017; Curtin et al., 2018; Wolabu et al., 2020). Rodas et al. (2021) confirmed true orthologue of *Arabidopsis* of SUPERMAN (*AtSUP*) in *M. truncatula* (*MtSUP*). Mutational analysis of three genes – *symbiosis receptor-like kinase (LjSYMRK)*, *leghaemoglobin (Ljlb1-3)* and *lotus histidine kinase 1 (LHK1)* interacting with the protein (*LjCZF1-2*) – has been done with *L. japonicus* genome using CRISPR/Cas technology (Wang et al. 2016a, b, 2019a, b; Cai et al., 2018). In *L. japonicus*, *CYP716A51-1* gene associated with C28 oxidation of triterpenes has been validated using this technology. Che et al. in 2021 overcame the cowpea recalcitrant properties to transformation, and *VuSPO-11-1* genes of meiosis were edited using CRSPPR/Cas tool for production of suitable hybrid of cowpea plant, which showed 68.8% of editing activity. In peas, CRISPR/Cas9-targeted *lipoxygenase (LOX)* mutations are likely to prevent the emission of VOC-related 'off-flavours'. In addition, other contributors for the 'off-flavour', namely, saponin B and DDMP saponin, can be removed or altered in pea seeds through CRISPR/Cas9. This technology can also be used to eliminate key allergen proteins (vicilin and convicillin) found in peas (Bhowmik et al., 2021). Soybean is the first oil and protein-rich crop to be genetically improved with CRISPR/Cas technology. Hairy root transformation mediated by *Agrobacterium rhizogenes* has been utilized to modify agronomic features such as soybean architecture, flowering times, seed oil and storage protein. *Arabidopsis PEAPOD* gene orthologues in soybean, namely, *GmPPD1* and *GmPPD2*, have been edited using this technology (Kanazashi et al., 2018). Homologue *GmFAD2–1A* and *GmFAD2–1B* genes of soybean have been edited with CRISPR/Cas technology to improve the profile of seed oil (Do et al., 2019). Mutant soybean was developed by silencing lipoxygenase gene responsible for beany flavour (Wang et al. 2020a, b), and *GmF3H1*, *GmF3H2* and *GmFNSII-1* genes of soybean were targeted simultaneously with CRISPR/Cas9-mediated multiplex gene-editing technology, which has shown increase in leaf isoflavone content providing resistant against soyabean mosaic virus (SMV) (Zhang et al. 2020a, b).

By targeting a *symbiosis receptor-like kinase gene* with CRISPR/Cas technology, Ji et al. (2019) were able to disrupt nitrogen fixation, and the use of this approach in the *Vigna* system suggests that this editing technology might be applied to additional *Vigna* species, such as the mung bean. CRISPR/Cas-mediated gene editing of the genome of the second most important food legume, chickpea, has

been done for the first time in 2021 in which the knock-out drought-resistant genes *4-coumarate ligase* (*4CL*) and *Reveille 7*(*RVE7*) genes (Badhan et al., 2021). Understanding the genes involved in the biosynthesis pathway of Faba bean anti-nutritional factors and increasing sulphur-containing amino acids by expressing Met- and Cys-rich seed storage proteins hold enormous promise, thanks to advances in genetic transformation technologies and CRISPR (Bhowmik et al., 2021).

## *5.5 Seeds and Nuts*

In recent times, the demand for edible seeds and nuts is increasing owing to their nutrition richness and ease to consume (Zion Market Research 2018). But it was observed that farmers from around the globe face several problems regarding the development of edible seeds and nuts (Mullen, 2020). As a result, scientists around the globe are involved in the endeavour to develop more advance varieties with high stress-tolerant and nutritional properties (Huang et al., 2020; Shu et al., 2020). Of all the gene-editing technologies employed, the most notable of them is CRISPR, which has seen amazing success in recent times (Jaganathan et al., 2018).

### 5.5.1 Quality Improvement

Popular crops known for oil production, like rapeseed's genome responsible for fatty acid metabolism, have been modified to generate better oil quality (Okuzaki et al., 2018; Al Amin et al., 2019). Also, the lowering of fatty acid concentration in rapeseed was obtained using CRISPR/Cas9 by targeting *GmFATB1* gene (Ma et al., 2021). In false flax *CsFAD2* (Jiang et al., 2017), *CsDGAT1* (Aznar-Moreno & Durrett, 2017), *CsFAE1* (Ozseyhan et al., 2018) and *CsCRUC* (Lyzenga et al., 2019) genes were knocked out using CRISPR/Cas9 to enhance fatty acid content of seeds.

### 5.5.2 Biotic and Abiotic Stress

CRISPR/Cas9 had been employed for the mutation or silencing different important genes which are responsible for different abiotic and biotic stresses. In peanut, *AhNFR5* genes were mutated using CRISPR/Cas9 for the production of variety tolerant to hairy root disease caused by *Rhizobium rhizogenes* (Shu et al., 2020). Sclerotinia, additionally known as white mould, is caused by the fungal pathogen *Sclerotinia sclerotiorum* in rapeseed, a severe problem faced by farmers nowadays. To solve this issue, Sun et al. targeted *BnWRKY70* gene using CRISPR/Cas9 tool to improve the resistance against *Sclerotinia sclerotiorum* (Sun et al., 2018) and also to develop a rapeseed variety with prolific branched dwarf phenotype along with shortened internode length and increase of the total flower, Stanic et al. edited *BnD14* gene using CRISPR/Cas9 (Stanic et al., 2021).

### 5.6 Herbs and Spices

Herbs associated with food and spices are comparatively less explored through CRISPR/Cas technology in contrast to other food plant species. CRISPR/Cas was utilized to modify *DMR1* gene (*downy mildew-resistant 1*) in the sweet basil genome to impart resistance against a mildew pathogen *Hyaloperonospora arabidopsis* (Navet & Tian, 2020). In spices, to confer resistance in *Capsicum annuum* against *Colletotrichum truncatum* was enhanced to prevent yield loss. Transcript-mediated CRISPR/Cas9 with alterations in the *CaERF28* locus was utilized to generate the resistant variant (Mishra et al., 2021). Moreover, a non-food traditional herb with medicinal value, *Salvia miltiorrhiza*, was edited by making knockout for terpene synthase gene (*SmCPS1*), proving the efficacy and simplicity of CRISPR/Cas9 in its genome editing (Li et al., 2017).

## 6 Limitations in CRISPR/Cas

### 6.1 Global Regulatory Bodies for CRISPR-Edited Crops

Given the growing use of the CRISPR/Cas9 technology for the production of better plant types, in different countries, concerns regarding its misuse and its impact in the present and future have been raised (Gonzalez-Avila et al., 2021). To address this concern, several countries have adopted different regulations for research and for the implementation of these regulations; several regulatory bodies are being framed in these countries (Entine et al., 2021). In 2018, the USDA announced that the genome-modified crops are safe similar to those crops, which are obtained through conventional breeding (Waltz, 2016). The Environmental Protection Agency, the Food and Drug Administration and the US Department of Agriculture (USDA) are the primary regulatory agencies in the USA which look after and evaluate the genetic engineering crops (FDA, 2020). In Canada, the plants developed with gene manipulation are required to be approved by the Canadian Food Inspection Agency (CFIA) (Ellens et al., 2019). In India, the genetically modified crops are regulated by the Food Safety and Standards Authority of India (Chimata & Bharti, 2019). Apart from these, several other countries like China, Japan, Australia, the European Union, the United Kingdom, etc. have their own regulatory bodies which regulate genetically engineered crop development and production (Entine et al., 2021).

### 6.2 Bioethics and Risk Assessment

CRISPR/Cas-mediated genome editing is most preferred among other genome editing tools due to its easy handling, cost-effectiveness and high accuracy for targeted gene alterations in a variety of organisms. Being the most important discovery of the

twenty-first century, CRISPR/Cas technology has many bioethical issues, apart from legal and social issues, regarding its application for modification of genetic background of plants, humans and animals (Ayanoglu et al., 2020). The editing of crop plants in association with environmental concerns is the fundamental ethical controversy surrounding CRISPR/Cas-mediated genome editing. The off-targeted mutation in a larger genome with identical sites for cleavage with CRISPR/Cas9 can cut off sites of interest, and this will create threat to environmental integrity. Off-targeted genes can be transferred to other organisms by means of gene drive. Drought-tolerant food crops may become invasive weeds if the wrong gene target is used, and another major danger or ethical problem associated with this technology is that it may be used to produce bioweapons, such as creating contagious pathogens that infect food crops (Shinwari et al., 2017). This technology also has both advantages and disadvantages as other technology, but adopting different strategies to reduce undesirable mutagenesis it can become most promising technology for crop enhancement in the near future.

## 7 Challenges and Future Prospect

Irrespective of the quantity of plant genome sequences available today, functional characterization of the bulk of genes, most importantly the multifamily genes like *CP450*, still remains unknown. Nevertheless, advances in next-generation sequencing, whole-genome sequencing technology and genome-wide identification and functional prediction of genes have helped to have insight into their possible functional biology and regulatory components during stress or diseased condition. More information regarding the same are needed to utilize CRISPR/Cas tool to the fullest for food crop trait improvement. Optimization of certain factors like promoters, delivery methods, off-targets, plant regeneration capacity and delay standard regulatory process and understanding of CRISPR-edited crops versus GM crops are critical for efficient CRISPR/Cas-mediated genome editing and its routinely use (Globus & Qimron, 2017; Ran et al., 2017; Ahmad et al., 2020).

Despite the challenges and hiccups, utilization of CRISPR/Cas's system to generate desirable traits has been growing. Numerous studies reporting success story of CRISPR-mediated gene editing either in obtaining resistance against multiple pathogens (Si et al., 2020) or in increasing overall yield and quality (Ren et al., 2021) are available now. Besides, genome editing to improve photosynthetic efficiency ultimately could enhance vegetable crop yields with rise in biomass. CRISPR/Cas may also be implemented to impart C4-plant-like characters with higher photosynthetic rate and faster $CO_2$ fixation. De novo domestication using CRISPR/Cas9 was successfully put to use in tomato with excellent productivity and yield. The same may be applied for other economically important vegetable crops which would have a significant impact on food security and malnutrition. Two other aspects into which CRISPR/Cas can be optimized are firstly utilization of two-line hybrid rather than three-line in order to boost the yield per unit area. Secondly,

insertion of large segments of DNA (multiple genes) can be targeted, as in nature traits are controlled by multiple and linked genes. CRISPR/Cas system as a whole has proven to be an excellent tool, and considering its newer variants, potential and application for food crop improvement, the system should meet zero hunger in the coming decades.

## 8 Conclusion

To overcome the spontaneous mutations or physical/chemical mutagen effects on food crops caused by traditional breeding techniques, CRISPR system was successfully introduced and employed for precise editing, especially in temperate crops and in a few tropical crops genome. However, the system needs further optimization in terms of its accessibility and practicality and globally to handle emerging problems and challenges associated with food crops. In this chapter, we have discussed the applications and advantages of CRISPR/Cas9 in the improvement of major food crops through genome editing. Its challenges and future prospects have also been demonstrated in regards to the recent literature. The progress made so far in the development of better varieties, although in a few food crops, has played a major role in initiating the process of meeting global food demand and hunger. With flexible government rules and public acceptance, the CRISPR system could be an answer to food security in the future.

## References

Abdallah, N. A., Prakash, C. S., & McHughen, A. G. (2015). Genome editing for crop improvement: Challenges and opportunities. *GM Crops & Food, 6*(4), 183–205.

Ahmad, N., Rahman, M. U., Mukhtar, Z., Zafar, Y., & Zhang, B. (2020). A critical look on CRISPR-based genome editing in plants. *Journal of Cellular Physiology, 235*(2), 666–682.

Ahmad, S., Tang, L., Shahzad, R., Mawia, A. M., Rao, G. S., Jamil, S., Wei, C., Sheng, Z., Shao, G., Wei, X., Hu, P., Mahfouz, M. M., Hu, S., & Tang, S. (2021). CRISPR-based crop improvements: A way forward to achieve zero hunger. *Journal of Agricultural and Food Chemistry, 69*(30), 8307–8323.

Ahmar, S., Gill, R. A., Jung, K., Faheem, A., Qasim, M. U., Mubeen, M., & Zhou, W. (2020). Conventional and molecular techniques from simple breeding to speed breeding in crop plants: Recent advances and future outlook. *International Journal of Molecular Sciences, 21*(7), 2590.

Al Amin, N., Ahmad, N., Wu, N., Pu, X., Ma, T., Du, Y., Bo, X., Wang, N., Sharif, R., & Wang, P. (2019). CRISPR-Cas9 mediated targeted disruption of *FAD2-2 microsomal omega-6 desaturase* in soybean (*Glycine max*. L). *BMC Biotechnology, 19*(1), 9.

Andersson, M., Turesson, H., Olsson, N., Falt, A.-S., Ohlsson, P., Gonzalez, M. N., Samuelsson, M., & Hofvander, P. (2018). Genome editing in potato via CRISPR-Cas9 ribonucleoprotein delivery. *Physiologia Plantarum, 164*, 378–384.

Atkinson, N. J., & Urwin, P. E. (2012). The interaction of plant biotic and abiotic stresses: From genes to the field. *Journal of Experimental Botany, 63*(10), 3523–3543.

Ayanoglu, F. B., Elcin, A. E., & Elcin, Y. M. (2020). Bioethical issues in genome editing by CRISPR-Cas9 technology. *Turkish Journal of Biology, 44*(2), 110–120.

Aznar-Moreno, J. A., & Durrett, T. P. (2017). Simultaneous targeting of multiple gene homeologs to alter seed oil production in *Camelina sativa*. *Plant & Cell Physiology, 58*(7), 1260–1267.

Badhan, S., Bal, A. S., & Mantri, N. (2021). First report of CRISPR/Cas9 mediated DNA-free editing of *4CL* and *RVE7* genes in chickpea protoplasts. *International Journal of Molecular Sciences, 22*, 396.

Bhowmik, P., Elison, E., Polley, B., Bollina, V., Kulkarni, M., Ghanbarnia, K., Song, H., Gao, C., Voytas, D. F., & Kagela, S. (2018). Targeted mutagenesis in wheat microspores using CRISPR/Cas9. *Scientific Reports, 8*(6502).

Bhowmik, P., Konkin, D., Polowick, P., Hodgins, C. L., Subedi, M., Xiang, D., Yu, B., Patterson, N., Rajagopalan, N., Babic, V., Ro, D. K., Tar'an, B., Bandara, M., Smyth, S. T., Cui, Y., & Kagale, S. (2021). CRISPR/Cas9 gene editing in legume crops: Opportunities and challenges. *Legume Science, 3*(3), e96.

Brooks, C., Nekrasov, V., Lippman, Z. B., & Van Eck, J. (2014). Efficient gene editing in tomato in the first generation using the clustered regularly interspaced short palindromic repeats/CRISPR-associated9 system. *Plant Physiology, 166*(3), 1292–1297.

Buiatti, M., Christou, P., & Pastore, G. (2013). The application of GMOs in agriculture and in food production for a better nutrition: Two different scientific points of view. *Genes & Nutrition, 8*, 255–270.

Butler, N. M., Atkins, P. A., Voytas, D. F., & Douches, D. S. (2015). Generation and inheritance of targeted mutations in potato (*Solanum tuberosum* L.) using the CRISPR/Cas system. *PLoS One, 10*(12), e0144591.

Cai, K., Yin, J., Chao, H., Ren, Y., Jin, L., Cao, Y., Duanmu, D., & Zhang, Z. (2018). A C3HC4-type RING finger protein regulates rhizobial infection and nodule organogenesis in *Lotus japonicus*. *Journal of Integrative Plant Biology, 60*, 878–896.

Calicioglu, O., Flammini, A., Bracco, S., Bellù, L., & Sims, R. (2019). The future challenges of food and agriculture: An integrated analysis of trends and solutions. *Sustainability, 11*(1), 222.

Cantos, C., Francisco, P., Trijatmiko, K. R., Slamet-Loedin, I., & Chadha-Mohanty, P. K. (2014). Identification of "safe harbor" loci in indica rice genome by harnessing the property of zinc-finger nucleases to induce DNA damage and repair. *Frontiers in Plant Science, 5*(302), 1–8.

Cermak, T., Baltes, N. J., Cegan, R., Zhang, Y., & Voytas, D. F. (2015). High-frequency, precise modification of the tomato genome. *Genome Biology, 16*, 232.

Chandrasekaran, J., Brumin, M., Wolf, D., Leibman, D., Klap, C., Pearlsman, M., Sherman, A., Arazi, T., & Gal-On, A. (2016). Development of broad virus resistance in non-transgenic cucumber using CRISPR/Cas9 technology. *Molecular Plant Pathology, 17*, 1140–1153.

Char, S. N., Unger-Wallace, E., Frame, B., Briggs, S. A., Main, M., Spalding, M. H., Vollbrecht, E., Wang, K., & Yang, B. (2015). Heritable site-specific mutagenesis using TALENs in maize. *Plant Biotechnology Journal, 13*(7), 1002–1010.

Chaudhary, R., & Atamian, H. S. (2017). Resistance-gene-mediated defense responses against biotic stresses in the crop model plant tomato. *Journal of Plant Pathology & Microbiology, 8*(4), 404.

Che, P., Chang, S., Simon, M. K., Zhang, Z., Shaharyar, A., Ourada, J., O'Neill, D., Torres-Mendoza, M., Guo, Y., Marasigan, K. M., Vielle-Calzada, J. P., Ozias-Akins, P., Albertsen, M. C., & Jones, T. J. (2021). Developing a rapid and highly efficient cowpea regeneration, transformation and genome editing system using embryonic axis explants. *The Plant Journal, 106*(3), 817–830.

Chen, L., Yang, D., Zhang, Y., Wu, L., Zhang, Y., Ye, L., Pan, C., He, Y., Huang, L., Ruan, Y. L., & Lu, G. (2018). Evidence for a specific and critical role of mitogen-activated protein kinase 20 in uni-to-binucleate transition of micro gametogenesis in tomato. *The New Phytologist, 219*(1), 176–194.

Chen, K., Wang, Y., Zhang, R., Zhang, H., & Gao, C. (2019). CRISPR/Cas genome editing and precision plant breeding in agriculture. *Annual Review of Plant Biology, 70*, 667–697.

Chimata, M. K., & Bharti, G. (2019). Regulation of genome edited technologies in India. *Transgenic Research, 28*, 175–181.

Christian, M., Cermak, T., Doyle, E. L., Schmidt, C., Zhang, F., Hummel, A., Bogdanove, A. J., & Voytas, D. F. (2010). Targeting DNA double-strand breaks with TAL effector nucleases. *Genetics, 186*(2), 757–761.

Climate change and food security: Risks and responses. Food and Agricultural Organization of the United Nations. (2015). Accessed 1 Jan 2022.

Conner, A. J., Glare, T. R., & Nap, J. P. (2003). The release of genetically modified crops into the environment part II. *The Plant Journal, 33*(1), 19–46.

Curtin, S. J., Tiffin, P., Guhlin, J., Trujillo, D. I., Burghart, L. T., Atkins, P., Baltes, N. J., Denny, R., Voytas, D. F., Stupar, R. M., & Young, N. D. (2017). Validating genome-wide association candidates controlling quantitative variation in nodulation. *Plant Physiology, 173*(2), 921–931.

Curtin, S. J., Xiong, Y., Michno, J. M., Campbell, B. W., Stec, A. O., Čermak, T., Starker, C., Voytas, D. F., Eamens, A. L., & Stupar, R. M. (2018). CRISPR/Cas9 and TALENs generate heritable mutations for genes involved in small RNA processing of *Glycine max* and *Medicago truncatula*. *Plant Biotechnology Journal, 16*(6), 1125–1137.

Demorest, Z. L., Coffman, A., Baltes, N. J., Stoddard, T. J., Clasen, B. M., Luo, S., Retterath, A., Yabandith, A., Gamo, M. E., Bissen, J., Mathis, L., Voytas, D. F., & Zhang, F. (2016). Direct stacking of sequence-specific nuclease-induced mutations to produce high oleic and low linolenic soybean oil. *BMC Plant Biology, 16*, 225.

Do, P. T., Nguyen, C. X., Bui, H. T., Tran, L. T., Stacey, G., Gillman, J. D., Zhang, Z. J., & Stacey, M. G. (2019). Demonstration of highly efficient dual gRNA CRISPR/Cas9 editing of the homeologous *GmFAD2–1A* and *GmFAD2–1B* genes to yield a high oleic, low linoleic andα-linolenic acid phenotype in soybean. *BMC Plant Biology, 19*(1), 311.

Ellens, K. W., Levac, D., Pearson, C., Savoie, A., Strand, N., Louter, J., & Tibelius, C. (2019). Canadian regulatory aspects of gene editing technologies. *Transgenic Research, 28*, 165–168.

El-Mounadi, K., Morales-Floriano, M. L. M., & Garcia-Ruiz, H. (2020). Principles, applications, and biosafety of plant genome editing using CRISPR-Cas9. *Frontiers in Plant Science, 11*(56).

Endo, A., Masafumi, M., Kaya, H., & Toki, S. (2016). Efficient targeted mutagenesis of rice and tobacco genomes using Cpf1 from *Francisella novicida*. *Scientific Reports, 6*, 38169.

Entine, J., Felipe, M. S. S., Groenewald, J., Kershen, D. L., Lema, M., Mchughen, A., Nepomuceno, A. L., Ohsawa, R., Ordonio, R. L., Parrott, W. A., Quemada, H., Ramage, C., Slamet-Loedin, I., Smyth, S. J., & Wray-Cahen, D. (2021). Regulatory approaches for genome edited agricultural plants in select countries and jurisdictions around the world. *Transgenic Research, 30*, 551–584.

FAO. (2009). Global Agriculture towards 2050. Food and Agricultural Organization of the United Nations. Accessed 5 Jan 2022.

FAO. (2015). Climate change and food security: Risks and responses. Food and Agricultural Organization of the United Nations. Accessed 1 Jan 2022.

FAO. (2021). The State of Food and Agriculture 2021. Making agrifood systems more resilient to shocks and stresses. Rome, FAO. https://doi.org/10.4060/cb4476en

FDA. (2020). How GMOs are regulated for food and plant safety in the United States. U.S. Food and Drug Administration. Accessed 7 Jan 2022.

Foolad, M. R. (2007). Genome mapping and molecular breeding of tomato. *International Journal of Plant Genomics*, 64358.

Gao, C. (2015). Genome editing in crops: From bench to field. *National Science Review, 2*(1), 13–15.

Garneau, J. E., Dupuis, M. E., Villion, M., Romero, D. A., Barrangou, R., Boyaval, P., Fremaux, C., Horvath, P., Magadán, A. H., & Moineau, S. (2010). The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature, 468*(7320), 67–71.

Gasparis, S., Kala, M., Przyborowski, M., Lyznik, L. A., Orczyk, W., & Nadolska-Orczyk, A. (2018). A simple and efficient CRISPR/Cas9 platform for induction of single and multiple, heritable mutations in barley (*Hordeum vulgare* L.). *Plant Methods, 14*, 111.

Global nuts and seeds market will reach USD 1345.27 billion by 2024: Zion Market Research. Zion Market Research. 2018. Accessed 5 Jan 2022.

Globus, R., & Qimron, U. (2017). A technological and regulatory outlook on CRISPR crop editing. *Journal of Cellular Biochemistry, 119*(2), 1291–1298.

Gonzalez, M. N., Massa, G. A., Andersson, M., Turesson, H., Olsson, N., Falt, A., Storani, L., Oneto, C. A. D., Hofvander, P., & Feingold, S. E. (2019). Reduced enzymatic browning in potato tubers by specific editing of a polyphenol oxidase gene via ribonucleoprotein complexes delivery of the CRISPR/Cas9 system. *Frontiers in Plant Science, 10*(1649).

Gonzalez-Avila, L. U., Vega-Lopez, J. M., Pelcastre-Rodriguez, L. I., Cabrero-Martinez, O. A., Hernandez-Cortez, C., & Castro-Escarpulli, G. (2021). The challenge of CRISPR-Cas toward bioethics. *Frontiers in Microbiology, 12*(657961).

Grebmer, K. V., Bernstein, J., Wiemers, M., Schiffer, T., Hanano, A., Towey, O., Chéilleachair, R. N., Foley, C., Gitter, S., Ekstrom, K., & Fritschel, H. (2021). *Global, regional, and national trends. Global Hunger Index. Hunger and food systems in conflict settings* (pp. 6–23).

Hanna, R. E., & Doench, J. G. (2020). Design and analysis of CRISPR-Cas experiments. *Nature Biotechnology, 38*(7), 813–823.

Haque, E., Taniguchi, H., Hassan, M. M., Bhowmik, P., Karim, K. R., Smiech, M., Zhao, K., Rahman, M., & Islam, T. (2018). Application of CRISPR/Cas9 genome editing technology for the improvement of crops cultivated in tropical climates: Recent progress, prospects, and challenges. *Frontiers in Plant Science, 9*(617).

Haun, W., Coffman, A., Clasen, B. M., Demorest, Z. L., Lowy, A., Ray, E., et al. (2014). Improved soybean oil quality by targeted mutagenesis of the fatty acid *desaturase 2* gene family. *Plant Biotechnology Journal, 12*(7), 934–940.

He, Y., & Zhao, Y. (2019). Technological breakthroughs in generating transgene-free and genetically stable CRISPR-edited plants. *aBIOTECH, 1*(1), 88–96.

Hu, B., Li, D., Liu, X., Qi, J., Gao, D., Zhao, S., Huang, S., Sun, J., & Yang, L. (2017). Engineering non-transgenic gynoecious cucumber using an improved transformation protocol and optimized CRISPR/Cas9 system. *Molecular Plant, 10*(12), 1575–1578.

Hu, J., Israeli, A., Ori, N., & Sun, T. (2018). The interaction between DELLA and ARF/IAA mediates crosstalk between gibberellin and auxin signaling to control fruit initiation in tomato. *The Plant Cell, 30*(8), 1710–1728.

Hu, C., Sheng, O., Deng, G., He, W., Dong, T., Yang, Q., Dou, T., Li, C., Gao, H., Liu, S., Yi, G., & Bi, F. (2021). CRISPR/Cas9-mediated genome editing of MaACO1 (*aminocyclopropane-1-carboxylate oxidase 1*) promotes the shelf life of banana fruit. *Plant Biotechnology Journal, 19*(4), 654–656.

Huang, Y., Cao, H., Yang, L., Chen, C., Shabala, L., & Xiong, M. (2019a). Tissue-specific respiratory burst oxidase homolog-dependent $H_2O_2$ signaling to the plasma membrane H+-ATPase confers potassium uptake and salinity tolerance in Cucurbitaceae. *Journal of Experimental Botany, 70*(20), 5879–5893.

Huang, Y., Xiao, L., Zhang, Z., Zhang, R., Wang, Z., Huang, C., Huang, R., Luan, Y., Fan, T., Wang, J., Shen, C., Zhang, S., Wang, X., Randall, J., Zheng, B., Wu, J., Zhang, Q., Xia, G., Xu, C., Chen, M., Zhang, L., Jiang, W., Gao, L., Chen, Z., Leslie, C. A., Grauke, L. J., & Huang, J. (2019b). The genomes of pecan and Chinese hickory provide insights into *Carya* evolution and nut nutrition. *Gigascience, 8*(5).

Huang, H., Cui, T., Zhang, L., Yang, Q., Yang, Y., Xie, K., Fan, C., & Zhou, Y. (2020). Modifications of fatty acid profile through targeted mutation at *BnaFAD2* gene with CRISPR/Cas9-mediated gene editing in *Brassica napus*. *Theoretical and Applied Genetics, 133*(8), 2401–2411.

Islam, S. M. F., & Karim, Z. (2019). World's demand for food and water: The consequences of climate change. In M. H. D. A. Farahani, V. Vatanpour, & A. H. Taheri (Eds.), *Desalination-challenges and opportunities* (pp. 1–27). London, UK.

Jaganathan, D., Ramasamy, K., Sellamuthu, G., Jayabalan, S., & Venkataraman, G. (2018). Crispr for crop improvement: An update review. *Frontiers in Plant Science, 9*(985).

James, C. (2011). *Global status of commercialized biotech/GM crops:2011* (ISAAA Brief 43-2011). ISAAA. Accessed 15 Jan 2022.

Jeyasri, R., Muthuramalingam, P., Satish, L., Pandian, S. K., Chen, J., Ahmar, S., Wang, X., Mora-Poblete, F., & Ramesh, M. (2021). An overview of abiotic stress in cereal crops: Negative impacts, regulation, biotechnology and integrated omics. *Plants, 10*(7), 1472.

Ji, J., Zhang, C., Sun, Z., Wang, L., Duanmu, D., & Fan, Q. (2019). Genome editing in cowpea *Vigna unguiculata* using CRISPR-Cas9. *International Journal of Molecular Sciences, 20*(10), 2471.

Jiang, W. Z., Henry, I. M., Lynagh, P. G., Comai, L., Cahoon, E. B., & Weeks, D. P. (2017). Significant enhancement of fatty acid composition in seeds of the allohexaploid, *Camelina sativa*, using CRISPR/Cas9 gene editing. *Plant Biotechnology Journal, 15*(5), 648–657.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science, 337*(6096), 816–821.

Jouanin, A., Gilissen, L. J. W. J., Schaart, J. G., Leigh, F. J., Cockram, J., Wallington, E. J., Boyd, L. A., Broeck, H. C. V. D., Meer, I. M. V. D., America, A. H. P., Visser, R. G. F., & Smulders, M. J. M. (2020). CRISPR/Cas9 gene editing of gluten in wheat to reduce gluten content and exposure-reviewing methods to screen for coeliac safety. *Frontiers in Nutrition, 7*(51).

Jun, R., Xixun, H., Kejian, W., & Chun, W. (2019). Development and application of CRISPR/Cas system in rice. *Rice Science, 26*(2), 69–76.

Jung, J. H., & Altpeter, F. (2016). TALEN mediated targeted mutagenesis of the caffeic acid O-methyltransferase in highly polyploid sugarcane improves cell wall composition for production of bioethanol. *Plant Molecular Biology, 92*(1–2), 131–142.

Jung, Y. J., Lee, H. J., Bae, S., Kim, J. H., Kim, D. H., Kim, H. K., Nam, K. H., Nogoy, F. M., Cho, Y., & Kang, K. K. (2019). Acquisition of seed dormancy breaking in rice (*Oryza sativa* L.) via CRISPR/Cas9-targeted mutagenesis of *OsVP1* gene. *Plant Biotechnology Reports, 13*(5), 511–520.

Kanazashi, Y., Hirose, A., Takahashi, I., Mikami, M., Endo, M., Hirose, S., Toki, S., Kaga, A., Naito, K., Ishimoto, M., Abe, J., & Yamada, T. (2018). Simultaneous site-directed mutagenesis of duplicated loci in soybean using a single guide RNA. *Plant Cell Reports, 37*(3), 553–563.

Kaur, N., Alok, A., Shivani, K. P., Kaur, N., Awasthi, P., Chaturvedi, S., Pandey, P., Pandey, A., Pandey, A. K., & Tiwari, S. (2020). CRISPR/Cas9 directed editing of *lycopene epsilon-cyclase* modulates metabolic flux for β-carotene biosynthesis in banana fruit. *Metabolic Engineering, 59*, 76–86.

Kelliher, T., Starr, D., Richbourg, L., Chintamanani, S., Delzer, B., Nuccio, M. L., Green, J., Chen, Z., McCuiston, J., Wang, W., Liebler, T., Bullock, P., & Martin, B. (2017). MATRILINEAL, a sperm-specific phospholipase, triggers maize haploid induction. *Nature, 542*, 105–109.

Kim, Y. G., Cha, J., & Chandrasegaran, S. (1996). Hybrid restriction enzymes: Zinc finger fusions to Fok I cleavage domain. *Proceedings of the National Academy of Sciences of the United States of America, 93*(3), 1156–1160.

Kim, S., Bengtsson, T., Olsson, N., Hot, V., Zhu, L., & Ahman, I. (2020). Mutations in two aphid regulated *β-1,3-Glucanase* genes by CRISPR/Cas9 do not increase barley resistance to *Rhopalosiphum padi* L. *Frontiers in Plant Science, 11*(1043).

Klap, C., Yeshayahou, E., Bolger, A. M., Arazi, T., Gupta, S. K., Shabtai, S., Usadel, B., Salts, Y., & Barg, R. (2017). Tomato facultative parthenocarpy results from SlAGAMOUS-LIKE 6 loss of function. *Plant Biotechnology Journal, 15*(5), 634–647.

Kumar, V. V. S., Verma, R. K., Yadav, S. K., Yadav, P., Watts, A., Rao, M. V., & Chinnusamy, V. (2020). CRISPR-Cas9 mediated genome editing of drought and salt tolerance (*OsDST*) gene in indica mega rice cultivar MTU1010. *Physiology and Molecular Biology of Plants, 26*(6), 1099–1110.

Laskowski, W., Gorska-Warsewicz, H., Rejman, K., Czeczotko, M., & Zwolinska, J. (2019). How important are cereals and cereal products in the average polish diet? *Nutrients, 11*(3), 679.

Lei, L., Gallagher, J., Arevalo, E. D., Chen, R., Skopelitis, T., Wu, Q., Bartlett, M., & Jackson, D. (2021). Enhancing grain-yield-related traits by CRISPR-Cas9 promoter editing of maize *CLE* genes. *Nature Plants, 7*(3), 287–294.

Li, B., Cui, G., Shen, G., Zhan, Z., Huang, L., Chen, J., & Qi, X. (2017b). Targeted mutagenesis in the medicinal plant Salvia miltiorrhiza. *Scientific Reports, 7, 43320.*

Li, T., Liu, B., Spalding, M. H., Weeks, D. P., & Yang, B. (2012). High-efficiency TALEN based gene editing produces disease-resistant rice. *Nature Biotechnology, 30*(5), 390–392.

Li, J. F., Norville, J. E., Aach, J., McCormack, M., Zhang, D., Bush, J., Church, G., & Sheen, J. (2013). Multiplex and homologous recombination-mediated genome editing in *Arabidopsis* and *Nicotiana benthamiana* using guide RNA and Cas9. *Nature Biotechnology, 31*(8), 688–691.

Li, M., Li, X., Zhou, Z., Wu, P., Fang, M., Pan, X., Lin, Q., Lou, W., Wu, G., & Li, H. (2016). Reassessment of the four yield-related genes *Gn1a, DEP1, GS3*, and *IPA1* in rice using a CRISPR/Cas9 system. *Frontiers in Plant Science, 7*(377).

Li, X., Zhou, W., Ren, Y., Tian, X., Lv, T., Wang, Z., Fang, J., Chu, C., Yang, J., & Bu, Q. (2017). High-efficiency breeding of early-maturing rice cultivars via CRISPR/Cas9-mediated genome editing. *Journal of Genetics and Genomics, 44*(3), 175–178.

Li, A., Jia, S., Yobi, A., Ge, Z., Sato, S. J., Zhang, C., Angelovici, R., Clemente, T. E., & Holding, D. R. (2018a). Editing of an *alpha-kafirin* gene family increases, digestibility and protein quality in sorghum. *Plant Physiology, 177*(4), 1425–1438.

Li, R., Zhang, L., Wang, L., Chen, L., Zhao, R., Sheng, J., & Shen, L. (2018b). Reduction of tomato-plant chilling tolerance by CRISPR-Cas9-mediated *SlCBF1* mutagenesis. *Journal of Agricultural and Food Chemistry, 66*(34), 9042–9051.

Li, T., Yang, X., Yu, Y., Si, X., Zhai, X., Zhang, H., Dong, W., Gao, C., & Xu, C. (2018c). Domestication of wild tomato is accelerated by genome editing. *Nature Biotechnology, 36*(12), 1160–1163.

Li, X., Wang, Y., Liu, Y., Yang, B., Wang, X., Wei, J., Lu, Z., Zhang, Y., Wu, J., Huang, X., Yang, L., & Chen, J. (2018d). Base editing with a *Cpf1-cytidine deaminase* fusion. *Nature Biotechnology, 36*(4), 324–327.

Li, H., Yang, Y., Hong, W., Huang, M., Wu, M., & Zhao, X. (2020). Applications of genome editing technology in the targeted therapy of human diseases: Mechanisms, advances and prospects. *Signal Transduction and Targeted Therapy, 5*(1).

Liang, Z., Chen, K., Yan, Y., Zhang, Y., & Gao, C. (2018a). Genotyping genome-edited mutations in plants using CRISPR ribonucleoprotein complexes. *Plant Biotechnology Journal, 16*(12), 2053–2062.

Liang, Z., Chen, K., Zhang, Y., Liu, J., Yin, K., Qiu, J. L., & Gao, C. (2018b). Genome editing of bread wheat using biolistic delivery of CRISPR/Cas9 in vitro transcripts or ribonucleoproteins. *Nature Protocols, 13*, 413–430.

Lyzenga, W. J., Harrington, M., Bekkaoui, D., Wigness, M., Hegedus, D. D., & Rozwadowski, K. L. (2019). CRISPR/Cas9 editing of three CRUCIFERIN C homoeologues alters the seed protein profile in *Camelina sativa*. *BMC Plant Biology, 19*(292).

Ma, X., Nicole, M. C., Meteignier, L. V., Hong, N., Wang, G., & Moffett, P. (2015). 4 Different roles for RNA silencing and RNA processing components in virus recovery and virus-induced gene silencing in plants. *Journal of Experimental Botany, 66*(3), 919–932.

Ma, C., Zhu, C., Zheng, M., Liu, M., Zhang, D., Liu, B., Li, Q., Si, J., Ren, X., & Song, H. (2019). CRISPR/Cas9-mediated multiple gene editing in *Brassica oleracea* var. *capitata* using the endogenous tRNA processing system. *Horticulture Research, 6*(20).

Ma, J., Sun, S., Whelan, J., & Shou, H. (2021). CRISPR/Cas9-mediated knockout of *GmFATB1* significantly reduced the amount of saturated fatty acids in soybean seeds. *International Journal of Molecular Sciences, 22*(8), 3877.

Makarova, K. S., Wolf, Y., Alkhnbashi, O. S., Costa, F., Shah, S. A., Saunders, S. J., Barrangou, R., Brouns, S. J. J., Charpentier, E., Haft, D. H., Horvath, P., Moineau, S., Mojica, F. J. M., Terns, R. M., Terns, M. P., White, M. F., Yakunin, A. F., Garrett, R. A., Oost, J. V., Backofen, R., &

Koonin, E. V. (2015). An updated evolutionary classification of CRISPR-Cas systems. *Nature Reviews. Microbiology, 13*, 722–736.

Makarova, K. S., Wolf, Y., Iranzo, J., Shmakov, S. A., Alkhnbashi, O. S., Brouns, S. J. J., Charpentier, E., Cheng, D., Haft, D. H., Horvath, P., Moineau, S., Mojica, F. J. M., Scott, D., Shah, S. A., Siksnys, V., Terns, M. P., Venclovas, C., White, M. F., Yakunin, A. F., Yan, W., Zhang, F., Garrett, R. A., Backofen, R., van der Oost, J., Barrangou, R., & Koonin, E. V. (2020). Evolutionary classification of CRISPR–Cas systems: A burst of class 2 and derived variants. *Nature Reviews. Microbiology, 18*(2), 67–83.

Malnoy, M., Viola, R., Jung, M. H., Koo, O. J., Kim, S., Kim, J. S., Velasco, R., & Kanchiswamy, C. N. (2016). DNA-free genetically edited grapevine and apple protoplast using CRISPR/Cas9 ribonucleoproteins. *Frontiers in Plant Science, 7*, 1904.

Martín-Pizarro, C., Triviño, J. C., & Posé, D. (2019). Functional analysis of the *TM6 MADS-box* gene in the octoploid strawberry by CRISPR/Cas9-directed mutagenesis. *Journal of Experimental Botany, 70*(3), 885–895.

Meng, Y., Hou, Y., Wang, H., Ji, R., Liu, B., Wen, J., Niu, L., & Lin, H. (2017). Targeted mutagenesis by CRISPR/Cas9 system in the model legume *Medicago truncatula*. *Plant Cell Reports, 36*(2), 371–374.

Miao, C., Xiaoa, L., Huaa, K., Zoua, C., Zhaoa, Y., Bressanb, R. A., & Zhua, J. K. (2018). Mutations in a subfamily of abscisic acid receptor genes promote rice growth and productivity. *PNAS, 115*(23), 6058–6063.

Michno, J., Wang, X., Liu, J., Curtin, S., Kono, T., & Stupar, R. (2015). CRISPR/Cas mutagenesis of soybean and *Medicago truncatula* using anew web-tool and a modified Cas9 enzyme. *GM Crops & Food, 6*(4), 243–252.

Mishra, R., Mohanty, J. N., Mahanty, B., & Joshi, R. K. (2021). A single transcript CRISPR/Cas9 mediated mutagenesis of CaERF28 confers anthracnose resistance in chilli pepper (*Capsicum annuum* L.). *Planta, 254*(1), 5.

Moller, I. S., Gilliham, M., Jha, D., Mayo, G. M., Roy, S. J., Coates, J. C., Haseloff, J., & Tester, M. (2009). Shoot $Na^+$ exclusion and increased salinity tolerance engineered by cell type–specific alteration of $Na^+$ transport in *Arabidopsis*. *The Plant Cell., 21*(7), 2163–2178.

Mullen, A. (2020). Fruit, nuts and seeds under environmental stress Environ. *Research Letters, 1*(15).

Mutezo, W., Sedibe, M. M., Mofokeng, A., Shargie, N., & Soko, T. (2021). The application of CRISPR/Cas9 technology in the management of genetic and nongenetic plant traits. *International Journal of Agronomy, 9993784*, 1–9.

Nakayasu, M., Akiyama, R., Lee, H. J., Osakabe, K., Osakabe, Y., Watanabe, B., Sugimoto, Y., Umemoto, N., Saito, K., Muranaka, T., & Mizutani, M. (2018). Generation of α-solanine-free hairy roots of potato by CRISPR/Cas9 mediated genome editing of the *St16DOX* gene. *Plant Physiology and Biochemistry, 131*, 70–77.

Navet, N., & Tian, M. (2020). Efficient targeted mutagenesis in allotetraploid sweet basil by CRISPR/Cas9. *Plant Direct, 4*(6).

Nekrasov, V., Staskawicz, B., Weigel, D., Jones, J. D. G., & Kamoun, S. (2013). Targeted mutagenesis in the model plant *Nicotiana benthamiana* using Cas9 RNA-guided endonuclease. *Nature Biotechnology, 31*(8), 691–693.

Nekrasov, V., Wang, C., Win, J., Lanz, C., Weigel, D., & Kamoun, S. (2017). Rapid generation of a transgene-free powdery mildew resistant tomato by genome deletion. *Scientific Reports, 7*(482).

O'Connell, M. R., Oakes, B. L., Sternberg, S. H., East-Seletsky, A., Kaplan, M., & Doudna, J. A. (2014). Programmable RNA recognition and cleavage by CRISPR/Cas9. *Nature, 516*, 263–266.

Okuzaki, A., Ogawa, T., Koizuka, C., Kaneko, K., Inaba, M., Imamura, J., & Koizuka, N. (2018). CRISPR/Cas9-mediated genome editing of the *fatty acid desaturase 2* gene in *Brassica napus*. *Plant Physiology and Biochemistry, 131*, 63–69.

Ortigosa, A., Gimenez-Ibanez, S., Leonhardt, N., & Solano, R. (2019). Design of a bacterial speck resistant tomato by CRISPR/Cas9-mediated editing of *SlJAZ2*. *Plant Biotechnology Journal, 17*(3), 665–673.

Ozseyhan, M. E., Kanga, J., Mua, X., & Lua, C. (2018). Mutagenesis of the *FAE1* genes significantly changes fatty acid composition in seeds of *Camelina sativa*. *Plant Physiology and Biochemistry, 123*, 1–7.

Peng, A., Chen, S., Lei, T., Xu, L., He, Y., Wu, L., Yao, L., & Zou, X. (2017). Engineering canker-resistant plants through CRISPR/Cas9-targeted editing of the susceptibility gene *CsLOB1* promoter in citrus. *Plant Biotechnology Journal, 15*, 1509–1519.

Pickar-Oliver, A., & Gersbach, C. A. (2019). The next generation of CRISPR–Cas technologies and applications. *Nature Reviews. Molecular Cell Biology, 20*(8), 490–507.

Prado, J. R., Segers, G., Voelker, T., Carson, D., Dobert, R., Phillips, J., Cook, K., Cornejo, C., Monken, J., Grapes, L., Reynolds, T., & Martino-Catt, S. (2014). Genetically engineered crops: From idea to product. *Annual Review of Plant Biology, 65*, 769–790.

Prihatna, C., Barbetti, M. J., & Barker, S. J. (2018). A novel tomato *Fusarium* wilt tolerance gene. *Frontiers in Microbiology, 9*(1226).

Ran, Y., Liang, Z., & Gao, C. (2017). Current and future editing reagent delivery systems for plant genome editing. *Science China. Life Sciences, 60*(5), 490–505.

Rashid, B., Tariq, M., Khalid, A., Shams, F., Ali, Q., Ashraf, F., Ghaffar, I., Khan, M. I., Rehman, R., & Husnain, T. (2017). Crop improvement: New approaches and modern techniques. *Plant Gene and Trait, 8*(3), 18–30.

Ren, Y., Li, M., Guo, S., Sun, H., Zhao, J., Zhang, J., Liu, G., He, H., Tian, S., Yu, Y., Gong, G., Zhang, H., Zhang, X., Alseekh, S., Fernie, A. R., Scheller, H. V., & Xu, Y. (2021). Evolutionary gain of oligosaccharide hydrolysis and sugar transport enhanced carbohydrate partitioning in sweet watermelon fruits. *The Plant Cell, 33*(5), 1554–1573.

Ricroch, A., Clairand, P., & Harwood, W. (2017). Use of CRISPR systems in plant genome editing: Toward new opportunities in agriculture. *Emerging Topics in Life Sciences, 1*(2), 169–182.

Rodas, A. L., Roque, E., Hamza, R., Gomez-Mena, C., Minguet, E. G., Wen, J., Mysore, K. S., Beltran, J. P., & Cañas, L. A. (2021). MtSUPERMAN plays a key role in compound inflorescence and flower development in *Medicago truncatula*. *The Plant Journal, 105*(3), 816–830.

Romero, F. M., & Gatica-Arias, A. (2019). CRISPR/Cas9: Development and application in rice breeding. *Rice Science, 26*(5), 265–281.

Sarwar, M. H., Sarwar, M. F., Sarwar, M., Qadri, N. A., & Moghal, S. (2013). The importance of cereals (Poaceae: Gramineae) nutrition in human health: A review. *Journal of Cereals and Oilseeds, 4*(3), 32–35.

Shan, Q., Wang, Y., Li, J., Zhang, Y., Chen, K., Liang, Z., Zhang, K., Liu, J., Xi, J. J., Qiu, J. L., & Gao, C. (2013). Targeted genome modification of crop plants using a CRISPR-Cas system. *Nature Biotechnology, 31*(8), 686–688.

Shan, Q., Zhang, Y., Chen, K., Zhang, K., & Gao, C. (2015). Creation of fragrant rice by targeted knockout of the *OsBADH2* gene using TALEN technology. *Plant Biotechnology Journal, 13*(6), 791–800.

Shinwari, Z. K., Tanveer, F., & Khalil, A. T. (2017). Ethical issues regarding CRISPR-mediated genome editing. *Current Issues in Molecular Biology, 26*, 103–110.

Shu, H., Luo, Z., Peng, Z., & Wang, J. (2020). The application of CRISPR/Cas9 in hairy roots to explore the functions of *AhNFR1* and *AhNFR5* genes during peanut nodulation. *BMC Plant Biology, 20*(417).

Shukla, V. K., Doyon, Y., Miller, J. C., DeKelver, R. C., Moehle, E. A., Worden, S. E., Mitchell, J. C., Arnold, N. L., Gopalan, S., Meng, X., Choi, V. M., Rock, J. M., Wu, Y. Y., Katibah, G. E., Zhifang, G., McCaskill, D., Simpson, M. A., Blakeslee, B., Greenwalt, S. A., Butler, H. J., Hinkley, S. J., Zhang, L., Rebar, E. J., Gregory, P. D., & Urnov, F. D. (2009). Precise genome modification in the crop species *Zea mays* using zinc-finger nucleases. *Nature, 459*, 437–441.

Si, X., Zhang, H., Wang, Y., Chen, K., & Gao, C. (2020). Manipulating gene translation in plants by CRISPR-Cas9-mediated genome editing of upstream open reading frames. *Nature Protocols, 15*(2), 338–363.

Soyk, S., Lemmon, Z. H., Oved, M., Fisher, J., Liberatore, K. L., Park, J., Goren, A., Jiang, K., Ramos, A., Knaap, E. V., Eck, J. V., Zamir, D., Eshed, Y., & Lippman, Z. B. (2017). Bypassing negative epistasis on yield in tomato imposed by a domestication gene. *Cell, 169*(6), 1142–1155.

Stanic, M., Hickerson, N. M. N., Arunraj, R., & Samuel, M. A. (2021). Gene-editing of the strigolactone receptor *BnD14* confers promising shoot architectural changes in *Brassica napus* (canola). *Plant Biotechnology Journal, 19*(4), 639–641.

Sun, Q., Lin, L., Liu, D., Wu, D., Fang, Y., Wu, J., & Wang, Y. (2018). CRISPR/Cas9-mediated multiplex genome editing of the *BnWRKY11* and *BnWRKY70* genes in *Brassica napus* L. *International Journal of Molecular Sciences, 19*(9), 2716.

Symington, L. S., & Gautier, J. (2011). Double-strand break end resection and repair pathway choice. *Annual Review of Genetics, 45*, 247–271.

Szczepek, M., Brondani, V., Buchel, J., Serrano, L., Segal, D. J., & Cathomen, T. (2007). Structure-based redesign of the dimerization interface reduces the toxicity of zinc-finger nucleases. *Nature Biotechnology, 25*, 786–793.

Tashkandi, M., Ali, Z., Aljedaani, F., Shami, A., & Mahfouz, M. M. (2018). Engineering resistance against tomato yellow leaf curl virus via the CRISPR/Cas9 system in tomato. *Plant Signaling & Behavior, 13*, e1525996.

Tester, M., & Langridge, P. (2010). Breeding technologies to increase crop production in a changing world. *Science, 327*(5967), 818–822.

The state of food security and nutrition in the world. Food and Agricultural Organization of the United Nations. 2021. Accessed 10 Jan 2022

Thomazella, D., Brail, Q., Dahlbeck, D., & Staskawicz, B. (2016). CRISPR-Cas9 mediated mutagenesis of a *DMR6* ortholog in tomato confers broad-spectrum disease resistance. *BioRxiv*.

Tian, S., Jiang, L., Gao, Q., Zhang, J., Zong, M., Zhang, H., Ren, Y., Guo, S., Gong, G., Liu, F., & Xu, Y. (2017). Efficient CRISPR/Cas9-based gene knockout in watermelon. *Plant Cell Reports, 36*(3), 399–406.

Tian, S., Jiang, L., Cui, X., Zhang, J., Guo, S., Li, M., Zhang, H., Ren, Y., Gong, G., Zong, M., Liu, F., Chen, Q., & Xu, Y. (2018). Engineering herbicide-resistant watermelon variety through CRISPR/Cas9-mediated base-editing. *Plant Cell Reports, 37*(9), 1353–1356.

Tomlinson, L., Yang, Y., Emenecker, R., Smoker, M., Taylor, J., Perkins, S., Smith, J., MacLean, D., Olszewski, N. E., & Jones, J. D. G. (2019). Using CRISPR/Cas9 genome editing in tomato to create a gibberellin-responsive dominant dwarf *DELLA* allele. *Plant Biotechnology Journal, 17*(1), 132–140.

Trujillo, D. I., Silverstein, K. A. T., & Young, N. D. (2019). Nodule-specific PLAT domain proteins are expanded in the Medicago lineage and required for nodulation. *The New Phytologist, 222*(3), 1538–1550.

Tuncel, A., Corbin, K. R., Ahn-Jarvis, J., Harris, S., Hawkins, E., Smedley, M. A., Harwood, W., Warren, F. J., Patron, N. J., & Smith, A. M. (2019). Cas9-mediated mutagenesis of potato starch-branching enzymes generates a range of tuber starch phenotypes. *Plant Biotechnology Journal, 17*(12), 2259–2271.

Turnbull, C., Lillemo, M., & Hvoslef-Eide, T. A. K. (2021). Global regulation of genetically modified crops amid the gene edited crop boom – A review. *Frontiers in Plant Science, 12*(630396).

Ueta, R., Abe, C., Watanabe, T., Sugano, S. S., Ishihara, R., Ezura, H., Osakabe, Y., & Osakabe, K. (2017). Rapid breeding of parthenocarpic tomato plants using CRISPR/Cas9. *Scientific Reports, 7*(507).

Uluisik, S., Chapman, N. H., Smith, R., Poole, M., Adams, G., Gillis, R. B., Besong, T. M. D., Sheldon, J., Stiegelmeyer, S., Perez, L., Samsulrizal, N., Wang, D., Fisk, I. D., Yang, N., Baxter, C., Rickett, D., Fray, R., Blanco-Ulate, B., Powell, A. L. T., Harding, S. E., Craigon, J., Rose, J. K. C., Fich, E. A., Sun, L., Domozych, D. S., Fraser, P. D., Tucker, G. A., & Seymour, G. D. (2016). Erratum: Corrigendum: Genetic improvement of tomato by targeted control of fruit softening. *Nature Biotechnology, 34*(1072).

UN Report: Nature's dangerous decline 'unprecedented'; Species extinction rates 'accelerating'. Sustainable Development Goals (United Nations). 2019. Accessed 11 Jan 2022.

Urnov, F. D., Rebar, E. J., Holmes, M. C., Zhang, H. S., & Gregory, P. D. (2010). Genome editing with engineered zinc finger nucleases. *Nature Reviews. Genetics, 11*(9), 636–646.

Wada, N., Ueta, R., Osakabe, Y., & Osakabe, K. (2020). Precision genome editing in plants: State-of-the-art in CRISPR/Cas9-based genome engineering. *BMC Plant Biology, 20*(234).

Waltz, E. (2016). Gene-edited CRISPR mushroom escapes US regulation. *Nature, 532*(293).

Wang, Y., Cheng, X., Shan, Q., Zhang, Y., Liu, J., Gao, C., & Qiu, J. L. (2014). Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. *Nature Biotechnology, 32*, 947–951.

Wang, S., Zhang, S., Wang, W., Xiong, X., Meng, F., & Cui, X. (2015). Efficient targeted mutagenesis in potato by the CRISPR/Cas9 system. *Plant Cell Reports, 34*, 1473–1476.

Wang, F., Wang, C., Liu, P., Lei, C., Hao, W., Gao, Y., Liu, Y., & Zhao, K. (2016a). Enhanced rice blast resistance by CRISPR/Cas9-targeted mutagenesis of the ERF transcription factor gene *OsERF922*. *PLoS One*.

Wang, L., Wang, L., Tan, Q., Fan, Q., Zhu, H., Hong, Z., Zhang, Z., & Duanmu, D. (2016b). Efficient inactivation of symbiotic nitrogen fixation related genes in *Lotus japonicus* using CRISPR-Cas9. *Frontiers in Plant Science, 7*(1333).

Wang, L., Chen, L., Li, R., Zhao, R., Yang, M., Sheng, J., & Shen, L. (2017). Reduced drought tolerance by CRISPR/Cas9-mediated *SlMAPK3* mutagenesis in tomato plants. *Journal of Agricultural and Food Chemistry, 65*(39), 8674–8682.

Wang, T., Deng, Z., Zhang, X., Wang, H., Wang, Y., Liu, X., Liu, S., Xu, F., Li, T., Fu, D., Zhu, B., Luo, Y., & Zhu, H. (2018a). Tomato *DCL2b* is required for the biosynthesis of 22-nt small RNAs, the resulting secondary siRNAs, and the host defense against ToMV. *Horticulture Research, 5*(62).

Wang, W., Pan, Q., He, F., Akhunova, A., Chao, S., Trick, H., & Akhunov, E. (2018b). Transgenerational CRISPR-Cas9 activity facilitates multiplex gene editing in allopolyploid wheat. *CRISPR Journal, 1*(1).

Wang, X., Tu, M., Wang, D., Liu, J., Li, Y., Li, Z., Wang, Y., & Wang, X. (2018c). CRISPR/Cas9-mediated efficient targeted mutagenesis in grape in the first generation. *Plant Biotechnology Journal, 16*, 844–855.

Wang, L., Rubio, M. C., Xin, X., Zhang, B., Fan, Q., Wang, Q., Ning, G., Becana, M., & Duanmu, D. (2019a). CRISPR/Cas9 knockout of *leghemoglobin* genes in *Lotus japonicus* uncovers their synergistic roles in symbiotic nitrogen fixation. *The New Phytologist, 224*(2), 818–832.

Wang, T., Zhang, H., & Zhu, H. (2019b). CRISPR technology is revolutionizing the improvement of tomato and other fruit crops. *Horticulture Research, 15*(6), 77.

Wang, J., Kuang, H., Zhang, Z., Yang, Y., Yan, L., Zhang, M., Song, S., & Guan, Y. (2020a). Generation of seed lipoxygenase-free soybean using CRISPR-Cas9. *Crop Journal, 8*(3), 432–439.

Wang, L., Sun, S., Wu, T., Liu, L., Sun, X., Cai, Y., Li, J., Jia, H., Yuan, S., Chen, L., Jiang, B., Wu, C., Hou, W., & Han, T. (2020b). Natural variation and CRISPR/Cas9-mediated mutation in *GmPRR37* affect photoperiodic flowering and contribute to regional adaptation of soybean. *Plant Biotechnology Journal, 18*(9), 1869–1881.

Wanga, M. A., Shimelis, H., Mashilo, J., & Laing, M. D. (2021). Opportunities and challenges of speed breeding: A review. *Plant Breeding, 140*(2), 185–194.

Wolabu, T. W., Park, J. J., Chen, M., Cong, L., Ge, Y., Jiang, Q., Debnath, S., Li, G., Wen, J., & Wang, Z. (2020). Improving the genome editing efficiency of CRISPR/Cas9 in Arabidopsis and *Medicago truncatula*. *Planta, 252*(15).

Wright, A. V., Nunez, J. K., & Doudna, J. A. (2016). Biology and applications of CRISPR systems: Harnessing nature's toolbox for genome engineering. *Cell, 164*(1–2), 29–44.

Xing, S., Jia, M., Wei, L., Mao, W., Abbasi, U. A., Zhao, Y., Chen, Y., Cao, M., Zhang, K., Dai, Z., Dou, Z., Jia, W., & Li, B. (2018). CRISPR/Cas9-introduced single and multiple mutagenesis in strawberry. *Journal of Genetics and Genomics, 45*(12), 685–687.

Xu, C., Park, S. J., Van Eck, J., & Lippman, Z. B. (2016a). Control of inflorescence architecture in tomato by BTB/POZ transcriptional regulators. *Genes & Development, 30*(18), 2048–2061.

Xu, R., Yang, Y., Qin, R., Li, H., Qiu, C., Li, L., Wei, P., & Yang, J. (2016b). Rapid improvement of grain weight via highly efficient CRISPR/Cas9-mediated multiplex genome editing in rice. *Journal of Genetics and Genomics, 43*(8), 529–532.

Xu, R., Qin, R., Li, H., Li, D., Li, L., Wei, P., & Yang, J. (2017). Generation of targeted mutant rice using a CRISPR-Cpf1 system. *Plant Biotechnology Journal, 15*(6), 713–717.

Xu, Z. S., Feng, K., & Xiong, A. S. (2019). CRISPR/Cas9-mediated multiply targeted mutagenesis in orange and purple carrot plants. *Molecular Biotechnology, 61*(3), 191–199.

Ye, J., Wang, X., Hu, T., Zhang, F., Wang, B., Li, C., Yang, T., Li, H., Lu, Y., Giovannoni, J. J., Zhang, Y., & Ye, Z. (2017). An Indel in the promoter of al-activated malate transporter 9 selected during tomato domestication determines fruit malate contents and aluminum tolerance. *The Plant Cell., 29*(9), 2249–2268.

Yin, Y., Qin, K., Song, X., Zhang, Q., Zhou, Y., Xia, X., & Yu, J. (2018). 3BZR1 transcription factor regulates heat stress tolerance through FERONIA receptor-like kinase-mediated reactive oxygen species signaling in tomato. *Plant & Cell Physiology, 59*(11), 2239–2254.

Young, N. D., & Udvardi, M. (2009). Translating *Medicago truncatula* genomics to crop legumes. *Current Opinion in Plant Biology, 12*(2), 193–201.

Yu, Q. H., Wang, B., Li, N., Tang, Y., Yang, S., Yang, T., Xu, J., Guo, C., Yan, P., Wang, Q., & Asmutola, P. (2017). CRISPR/Cas9-induced targeted mutagenesis and gene replacement to generate long-shelf life tomato lines. *Scientific Reports, 7*(11874).

Zafar, K., Khan, M. Z., Amin, I., Mukhtar, Z., Yasmin, S., Arif, M., Ejaz, K., & Mansoor, S. (2020). Precise CRISPR-Cas9 mediated genome editing in Super Basmati Rice for resistance against bacterial blight by targeting the major susceptibility gene. *Frontiers in Plant Science, 11*(575).

Zaidi, S. S., Mahas, A., Vanderschuren, H., & Mahfouz, M. M. (2020). Engineering crops of the future: CRISPR approaches to develop climate-resilient and disease-resistant plants. *Genome Biology, 21*(289).

Zhang, Y., Bai, Y., Wu, G., Zou, S., Chen, Y., Gao, C., & Tang, D. (2017). Simultaneous modification of three homoeologs of *TaEDR1* by genome editing enhances powdery mildew resistance in wheat. *The Plant Journal, 91*(4), 714–724.

Zhang, S., Wang, L., Zhao, R., Yu, W., Li, R., Li, Y., Sheng, J., & Shen, L. (2018). Knockout of SlMAPK3 reduced disease resistance to *Botrytis cinerea* in tomato plants. *Journal of Agricultural and Food Chemistry, 66*(34), 8949–8956.

Zhang, M., Liu, Q., Yang, X., Xu, J., Liu, G., & Yao, X. (2020a). CRISPR/Cas9-mediated mutagenesis of Clpsk1 in watermelon to confer resistance to *Fusarium oxysporum* f.sp. niveum. *Plant Cell Reports, 39*(5), 589–595.

Zhang, P., Du, H., Wang, J., Pu, Y., Yang, C., Yan, R., Yang, H., Cheng, H., & Yu, D. (2020b). Multiplex CRISPR/Cas9-mediated metabolic engineering increases soya bean isoflavone content and resistance to soyabean mosaic virus. *Plant Biotechnology Journal, 18*(6), 1384–1395.

Zhou, J., Wang, G., & Liu, Z. (2018). Efficient genome editing of wild strawberry genes, vector development and validation. *Plant Biotechnology Journal, 16*(11), 1868–1877.

Zong, Y., Wang, Y., Li, C., Zhang, R., Chen, K., Ran, Y., Qiu, J., Wang, D., & Gao, C. (2017). Precise base editing in rice, wheat and maize with a Cas9-cytidine deaminase fusion. *Nature Biotechnology, 35*(5), 438–440.

# Index





A. Raina et al. (eds.), *Advanced Crop Improvement, Volume 2*,
https://doi.org/10.1007/978-3-031-26669-0