Satbir Singh Gosal · Shabir Hussain Wani
Editors

Biotechnologies of Crop Improvement, Volume 1

Cellular Approaches

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Edward C. Cocking graduated from the University of Bristol, UK, in Biological Chemistry followed by research doctorates on amino acid and protein synthesis in plants, plant cell biology and nitrogen metabolism, and was a Civil Service Commission research fellow in bacterial chemistry before being appointed to a lectureship in plant physiology at the University of Nottingham. His teaching and research at Nottingham identified the key importance of the study of the plant cell in relation to all aspects of plant and cell physiology and the interaction of plants with the environment and with microorganisms. His seminal publication in 1960 of an enzymatic method using cellulases to

degrade plant cell walls releasing protoplasts facilitated the use of these single, wall-free plant cells for a new era of plant cell physiology, biochemistry and molecular biology investigations. The uptake of plasmids, viruses and nitrogen-fixing bacteria by protoplasts, and the regeneration of whole fertile plants by cell and tissue culture methodology, coupled with the fusion of protoplasts to produce hybrids between sexually incompatible species, including those of rice, attracted grant support both nationally and internationally, including the Rockefeller Foundation. This enabled him to lead a multidisciplinary, international team, with predoctoral and postdoctoral researchers from India and China that played a major role in the Rockefeller Rice Biotechnology Programme, together with a UK Research Council Group that pioneered improvements in our knowledge of plant cell biology. He was Professor of Botany and Head of the Plant Genetic Manipulation Group from 1969 to 1997 and is now Director (Emeritus) of the Centre for Crop Nitrogen Fixation. His research record has been extensive and has been highlighted by a series of highly cited publications that have represented landmark developments in the plant sciences. His national and international leadership in plant biotechnology, plant cell and tissue culture, genetic manipulations and nitrogen fixation has resulted in his recognition nationally (Fellowship of the Royal Society), within Europe (Membership Academia Europaea, Hungarian Academy of Sciences) and internationally (World Innovation

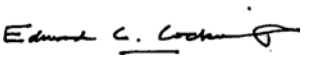
Foundation, Indian Academy of Agricultural Sciences). He has received a Life-Time Achievement Award from the University of Toledo for research on plant protoplasts and nitrogen fixation, and an Honorary D.Sc. from the University of Nottingham. In 1997, he organized a Discussion Meeting at the Rockefeller Foundation Bellagio Conference Centre on Biological Nitrogen Fixation, the Global Challenge and Future Needs, which enabled him to bring together international experts to formulate an action plan to develop nitrogen-fixing cereals and other non-legume crops; and in 2006, he took the first key step to achieve this objective by establishing nitrogen-fixing bacteria intracellularly in the root meristematic cells of crops, including cereals. This seminal publication has now resulted in numerous commercial international collaborations to establish symbiotic nitrogen fixation in crops for reduced inputs of synthetic nitrogen fertilizers, and for increased yields.

This book is dedicated to
Prof. Edward C. Cocking – father of protoplast technology.

Foreword

Biotechnology methods based on cell and protoplast culture hold significant promise for accelerated breeding and obtaining incremental improvement in crop cultivars. Plant tissue culture includes several specialized areas like micropropagation, meristem culture, micrografting, somatic embryogenesis, somaclonal variation, anther/pollen culture, embryo/ovule culture, protoplast culture/somatic hybridization, in vitro–assisted compression of breeding cycles and cryopreservation/in vitro freeze-storage of germplasm. These methods offer rich scope for creation, conservation and utilization of genetic variability for the improvement of field, fruit, vegetable, ornamental and forest plant species. Micropropagation of selected plant species is one of the best and most successful examples of the commercial application of tissue culture technology. Meristem culturing and in vitro grafting assist in developing disease-free plants. Somatic embryogenesis helps in cloning and genetic transformation resulting in transgenic crops. Production of haploids through bulbosum, anther/ pollen culture and embryo rescue from wide hybrids has been exploited for the production of haploids/doubled haploids for the early release of varieties. Embryo culture is the practical approach to obtain interspecific and intergeneric hybrids among otherwise difficult to cross parents. Somatic cell hybridization facilitates combining characteristics even from otherwise sexually incompatible species and the production of somatic hybrids/cybrids and organelle recombination not possible through conventional methods. In vitro freeze-storage and cryopreservation are imperative techniques for germplasm conservation especially of the vegetatively propagated plant species. Keeping all this in view, the editors have made strenuous efforts to include chapters covering the historical perspective and recent achievements in crop improvement using micropropagation, somatic embryogenesis, somaclonal variation, anther/pollen/embryo culture, in vitro freeze-storage and cryopreservation and somatic hybridization. Important crops such as rice, wheat, sugarcane, brassica, peanut, citrus, banana, apple, potato, eucalyptus, bamboo and medicinal and aromatic plants have been dealt with in detail. It is evident from the chapters that most of the cellular/protoplast techniques are now being routinely used in crop improvement programmes the world over. All chapters are well written by experts and will create much scientific interest not only in students but also in

teachers and researchers. I congratulate the editors of this book Dr. Satbir Singh Gosal and Dr. Shabir Hussain Wani for soliciting valuable contributions from a fine selection of experts on relevant aspects of these important crops; I am sure that this book will be highly rewarding for students, teachers and researchers in this area of cellular applications to crop improvement and biotechnology. The publisher particularly deserves congratulations for publishing this timely and useful book.



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Preface

Ever since the domestication of plants, breeders are making concerted efforts to improve crop yield and quality and developing resistance to various biotic and abiotic stresses. Using conventional breeding approaches, such as introduction, selection, hybridization, mutation, and polyploidy, an appreciable progress has been made and a series of improved cultivars have been developed, the world over. However, the breeding has been a difficult task in the presence of high level of heterozygosity, especially in the vegetatively propagated crops, cross-incompatibility, longer juvenile phase, seedlessness, complex polyploidy, lack of flowering, apomixes, polyembryony, and vegetative nature of propagation. In this regard, the innovative methods of cell and tissue culture hold significant promise to complement and supplement the conventional breeding methods for accelerated breeding. Spectacular advances in the area of plant cell, tissue, and protoplast culture offer a rich scope for creation, conservation, and utilization of genetic variability for the improvement of fields, fruits, vegetables, forest crops, and medicinal/aromatic plants. Plant breeders always look for new breeding tools to circumvent the recurring problems and to speed up the breeding process. The present book aims to describe the role of various in vitro techniques such as micropropagation, meristem-tip culture, micrografting, somatic embryogenesis, anther/microspore culture, somaclonal variation, embryo/ovule/ovary culture, protoplast culture and somatic hybridization, and cryopreservation/in vitro germplasm storage. Applications of these innovative methods for the improvement of crops such as rice, wheat, sugarcane, brassica, peanut, citrus, banana, apple, potato, eucalyptus, bamboo, and medicinal and aromatic plants have been dealt in detail in this volume. This book provides an authoritative review account of different aspects and progress in the field that has been made in the recent past. The book includes chapters prepared by specialists and subject experts on different aspects of tissue culture and crop improvement. The first chapter introduces various cell and tissue culture aspects in relation to crop improvement. Whereas six chapters deal exclusively with the micropropagation protocols for the mass production of super-elite planting material of sugarcane, banana, potato, eucalyptus, apple rootstocks, and bamboo. Two chapters on the fundamental facets of somatic embryogenesis in peanut and citrus have also

been included. The relevance of somaclonal variation and in vitro selection using stress factors has been well presented in three chapters. In vitro production of haploids/doubled haploids and their role in the accelerated breeding of world's two most important cereals, viz. rice and wheat, have been covered in two separate chapters. Likewise, a separate chapter dealing with in vitro–assisted compression of breeding cycles has been included for compressing the breeding cycle for the early release of crop varieties in the changing scenario of climate. Furthermore, the potential of cellular techniques for the improvement of medicinal and aromatic plants has been thoroughly discussed in the last chapter.

The book provides state-of-the-art information on cell and tissue culture tools to supplement and complement the conventional methods of crop improvement. We earnestly feel that this book will be highly useful for students, research scholars, and scientists working in the area of crop improvement and biotechnology at universities, research institutes, and R&Ds of agricultural MNCs for conducting research and various funding agencies for planning future strategies.

We are highly grateful to all learned contributors, each of who has attempted to update scientific information of their respective area and expertise and has kindly spared valuable time and knowledge.

We apologize wholeheartedly for any mistakes, omissions, or failure to acknowledge fully.

We thank our families (Dr. Satwant Kaur Gosal (wife of SSG); Sheikh Shazia and Muhammad Saad Wani (wife and son of SHW)) for their continuous support and encouragement throughout the completion of this book.

We highly appreciate the all-round cooperation and support of Springer International Publishing AG, Cham, for their careful and speedy publication of this book.

Ludhiana, Punjab, India — Satbir Singh Gosal
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Satbir Singh Gosal earned his B.Sc. (Med.) from PU, Chandigarh, India, and M.Sc. and Ph.D. (Plant breeding) from Punjab Agricultural University, Ludhiana, India. He was awarded fellowships by The Royal Society London and The Rockefeller Foundation (USA) for his Post-Doctoral Research at the University of Nottingham, England, and John Innes Centre Norwich, England. Dr Gosal has served Punjab Agricultural University in various capacities such as Professor Biotechnology, Director School of Agricultural Biotechnology, Additional Director Research, and Director of Research. He has also served FAO/IAEA, Vienna, Austria, and took tissue culture expert mission to Iraq during 1997. He has rigorous training on "Biosafety of GM crops" from Dan Forth Centre for Plant Science Research, St. Louis; APHIS, EPA (USDA), USTDA, Washington, DC, USA. He has been an Honorary Member of the Board of Assessors; Australian Research Council, Canberra; President Punjab Academy of Sciences; elected member (Fellow) of Plant Tissue Culture Association (India); and Fellow of Indian Society of Genetics and Plant Breeding. He is a recipient of Distinction Award by Society for the Promotion of Plant Science Research, Jaipur, India (2009); Fellow of Punjab Academy of Sciences; and Advisory member of several universities/institutes in the area of biotechnology. He served as a member of Review Committee on Genetic Manipulation (RCGM) for 3 years at Department of Biotechnology (DBT), Government of India, New Delhi, and is a member of panel of experts in area of Biotechnology for National Fund for Strategic Research of Indian Council of Agricultural Research, New Delhi. He has participated in more than 125 national/international conferences/meetings held in India, England, Scotland, Yugoslavia, Philippines, Indonesia, Thailand, The Netherlands, Malaysia, Singapore, Austria, Iraq, PR China, Australia, Mexico, Germany, and USA. He has guided more than 75 (M.Sc. and Ph.D.) students for theses research on various aspects of plant tissue culture and plant transformation. He executed more than 20 externally funded research projects funded by various national and international organizations such as Punjab State Government, ICAR, DBT, DAC NATP, FAO/IAEA, and The Rockefeller Foundation, USA. He has more than 200 research papers in refereed

journals of high repute, 135 research papers in conference proceedings, several TV/radio talks, and 30 book chapters. He has coauthored five laboratory manuals, one textbook, and two edited books.

Shabir Hussain Wani is an Assistant Professor cum Scientist, Plant Breeding and Genetics, at the Mountain Research Centre for Field Crops, Khudwani Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Srinagar, Jammu and Kashmir, India, since May 2013 till date. He received his B.Sc. in Agriculture from Bhimrao Agricultural University, Agra, India, and M.Sc. and Ph.D. in Genetics and Plant Breeding from Central Agricultural University, Manipur, and Punjab Agricultural University, Ludhiana India respectively. His Ph.D. research fetched the first prize in North zone at national level competition in India. After obtaining his Ph.D., he worked as Research Associate in the Biotechnology Laboratory, ICAR-Central Institute of Temperate Horticulture, Rangreth Srinagar, India, for two years, up to October 2011. In November 2011, he joined the Krishi Vigyan Kendra (Farm Science Centre) as Programme Coordinator (i/c) at Senapati, Manipur, India. He teaches courses related to plant breeding, seed science and technology, and stress breeding. He has published more than 100 scientific papers/chapters in peer-reviewed journals, and books of international and national repute. He has served as Review Editor of Frontiers in Plant Sciences, Switzerland, from 2015 to 2017. He is an editor of *SKUAST Journal of Research* and *LS: An International Journal of Life Sciences*. He has also edited ten books on current topics in crop improvement published by reputed publishers including CRC press; Taylor and Francis Group, USA; and Springer. He is a Fellow of the Linnean Society of London and Society for Plant Research, India. He received various awards including Young Scientist Award (Agriculture) 2015, Young Scientist Award 2016, and Young Achiever Award 2016 by various prestigious scientific societies. He has also worked as visiting scientist in Department of Plant Soil and Microbial Sciences, Michigan State University, USA, for the year 2016–2017 under the Raman Post-Doctoral Research Fellowship programme sponsored by University Grants Commission, Government of India, New Delhi. He is a member of the Crop Science Society of America.

Cell and Tissue Culture Approaches in Relation to Crop Improvement

Satbir Singh Gosal and Shabir Hussain Wani

Abstract Plant cell and tissue culture involves the growing of cells, tissues and organs on synthetic medium under closely controlled and aseptic conditions. Plant cell and tissue culture methods offer a rich scope for the creation, conservation and utilization of genetic variability for the improvement of field, horticultural and forest plant species. Micropropagation of selected plant species is one of the best and most successful examples of the commercial application of tissue culture technology. Micropropagation ensures true-to-type, rapid and large-scale multiplication. Now scores of multimillion-dollar industries around the world propagate a variety of plant species through micropropagation. Tissue culture technology offers environmental-friendly industries to flourish. It is likely that automation of multiplication systems will be commercially feasible within the next few years for several species including potato microtubers, lily bulblets and gladiolus corms. Meristem culturing and in vitro grafting help in developing disease-free plants. Improvement of somatic embryogenesis, coupled with embryo desiccation and encapsulation technology, may lead to the utilization of 'artificial seeds' for mass cloning of plants. Further induction of somatic embryogenesis in plants helps in cloning and transformation. Somaclonal variation is a potent emerging aspect for broadening the genetic base and thus obtaining incremental improvement in the commercial cultivars, more particularly, in the vegetatively propagated plant species. Using the technique of in vitro selection, many million cells/protoplasts can be screened against various biotic and abiotic stress factors in a single Petri dish which is more efficient as compared to the screening of similar number of plants in the field which requires more time and space as well. Production of haploids through bulbosum, anther/pollen culture and embryo rescue from wide hybrids has been exploited for the produc-

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tion of haploids/doubled haploids for early release of varieties. These methods ensure true-breeding (doubled haploids) plants in less than 1 year, which are otherwise obtained after seven to eight generations through conventional methods. Since the possibility of producing useful secondary products in plant cell cultures was first recognized in the 1970s, considerable progress has been made, and a number of plant species have been found to produce secondary products such as shikonin, diosgenin, caffeine, glutathione and anthraquinone. Embryo culture is the practical approach to obtain interspecific and intergeneric hybrids among otherwise difficult to cross parents. It has been successfully used to transfer desirable genes from wild relatives into cultivated varieties of several field and vegetable crops. Somatic cell hybridization helps in combining characteristics even from otherwise sexually incompatible species and to obtain cybrids and organelle recombination not possible through conventional methods. In vitro freeze storage and cryopreservation are very important techniques for germplasm conservation especially of the vegetatively propagated crops. Plants have been successfully regenerated from tissues cryopreserved at –196 °C in liquid N_2 for several months to years in several crops. During the past 25 years, the combined use of recombinant DNA technology, gene transfer methods and cell and tissue culture techniques has led to the efficient transformation and production of transgenics in a wide variety of crop plants. In fact, transgenesis has emerged as an additional tool to carry out single-gene breeding or transgenic breeding of crops.

Keywords Micropropagation · Tissue culture · Haploids · Somaclonal variation · Wide hybridization · Somatic hybridization · Secondary metabolites · Cryopreservation

1 Introduction

Crop improvement deals with changing and improving the characteristics of plants for enhancing their utility to the mankind. Crop improvement has evolved over centuries starting from cursory activities of primitive and prehistoric man to attain a status of most sophisticated technologies as of today for producing superior types of plants. Varietal decline is a common phenomenon; therefore, the crop improvement is a continuous process. In the current scenario of climate change and global warming, there is rapid emergence of new races of insect pests and new pathotypes of disease-causing agents. Minor insect pests/pathogens are rapidly emerging as major ones. Heat and drought stresses are becoming serious threats, the world over. Therefore, an efficient improvement in the existing cultivars is necessary to meet the growing food demand. Plant improvement has been focused largely on improving yield, quality, resistance to diseases and insect pests and tolerance to abiotic stresses, early maturity, nutrient-use efficiency and water-use efficiency. Most of the past and current progress has been through the sexual reproduction of the plants using selection, hybridization and backcross breeding methods. It has been difficult to improve

vegetatively propagated species using conventional methods, in this regard, plant tissue and cell culture methods offer a rich scope for the creation, conservation and utilization of genetic variability for the improvement of both sexually and asexually propagated field, vegetable, fruit, ornamental, medicinal and aromatic and forest crop species (Chahal and Gosal 2002; Kang et al. 2007). Cell and tissue culture is based on the concept of totipotency conceived by Gottlieb Haberlandt, a plant physiologist (now regarded as the father of plant tissue culture) in the year 1902. Such a property of cell has far-reaching implications to manipulate plant cells for rapid multiplication of plants, to cross plants at the level of somatic cells by overcoming limits of cross ability and also to regenerate entire plants after genetic modifications. Plant tissue culture is a technique of growing plant cells, tissues and organs on synthetic medium under closely controlled and aseptic conditions. Suitable explants, such as roots, hypocotyls, cotyledons, leaves, shoot apices, nodal segments, inflorescences, anthers, pollen, embryos and seeds, are surface sterilized with a disinfectant like sodium hypochlorite (10–50% w/v for 10–30 min) or with mercuric chloride (0.1% w/v for 5–10 min), thoroughly washed with sterile water and then aseptically cultured on a synthetic medium in culture vessels like test tubes, jars and Petri dishes. The in vitro cultures, incubated at 25 ± 1 °C, exhibit growth in 1–3 weeks depending upon the plant species, nature of explant, type of culture medium, kind and concentration of the growth regulators (hormones) used in the medium and the light intensity in the incubation room. A large number of culture media have been developed for plant tissue culture, but the most commonly used media include Murashige and Skoog (1962) (MS-1962), White (1963), Gamborg et al. (1968) (B_5) and Chu (1978) (N_6). Tissue culture medium contains macroelements, microelements, amino acids and vitamins, sugars and growth regulators (auxins, cytokinins). Auxins, such as indole acetic acid (IAA), indole butyric acid (IBA) and naphthalene acetic acid (NAA) at concentrations ranging from 0.1 to 5.0 mg/l, favour cell elongation and rooting, whereas 2,4-dichloro-phenoxyacetic acid (2,4-D) at concentrations of 0.5–4.0 mg/l usually induces callus, i.e. homogenous mass of undifferentiated cells. Likewise, cytokinins, such as 6-furfuryl amino purine (kinetin) and benzyl amino purine (BAP) at concentrations of 0.1–2.0 mg/l, cause rapid cell divisions and development of shoot buds/shoots. Whereas growth retardants such as cycocel are used to slow down the cellular growth wherever necessary, both liquid and semi-solid media are used depending upon the nature of explants and the objective of culturing. The solidification of the medium is achieved by adding chemically inert, powdered gelling agents, such as agar, agarose and gelrite before autoclaving. The medium is poured into culture vessels and sterilized in an autoclave at 121 °C, 15 lbs./sq. inch pressure for 20–25 min. Inoculation of explants in the culture vessels is done in laminar air flow cabinet fitted with HEPA filters (pore size 0.2–0.3 μm) under aseptic conditions. Culturing of explants on growth medium under suitable conditions leads to dedifferentiation, i.e. mature cells revert to meristematic state through enhanced DNA/RNA and protein synthesis. The renewed growth on agar-gelled medium gives an unorganized mass of cells, i.e. callus. The cells may be cultured in liquid medium that gives a suspension of individual cells called cell suspension culture. An increased number of cells or calli lead to the depletion of medium, and thus, the growing tissue needs to be transferred

Table 1 Various cell and tissue culture approaches to crop improvement

No.	Approach	Application
1	Micropropagation	True-to-type, rapid and mass multiplication of plants
2	Meristem culture	Production of disease-free planting material
3.	Micrografting	Production of disease-free, grafted, horticultural plants
4	Somatic embryogenesis	Mass cloning of plants and production of synthetic seeds
5	Somaclonal variation	Induction of genetic variation for selection of new clones, varieties
6	In vitro production of haploids	Production of haploids/doubled haploids for accelerated crop breeding
7	Embryo/ovule/ovary culture	Production of distant hybrids/alien gene transfer into cultivated varieties and haploid production
8	Protoplast culture and somatic hybridization	Production of somatic hybrids, cybrids and transgenic plants
9	In vitro production of secondary metabolites	Production of secondary metabolites (drugs, flavours and dyes)
10	Cryopreservation and in vitro germplasm storage	Long-term storage of germplasm especially of vegetatively propagated species
11	Genetic transformation	Production of transgenic crop varieties/hybrids.

after every 3–4 weeks to fresh medium through subculturing. Ultimately such cells/tissues are to be used to obtain organized structures like roots, somatic embryos, shoots, flower buds, etc., through the process of organogenesis. Plant tissue culture includes several specialized areas like micropropagation, meristem culture, micrografting, somatic embryogenesis, somaclonal variation, anther/pollen culture, embryo/ovule culture, protoplast culture/somatic hybridization and cryopreservation/in vitro freeze storage *of* germplasm (Table 1).

2 Micropropagation

Micro means ‘very small’; therefore, as the name suggests, micropropagation is the clonal propagation by using very small plant tissue in vitro. Micropropagation of plants is now one of the best and most successful examples of the commercial application of tissue and cell culture technology. This involves the production of plants from very small explants (0.2–10 mm) under in vitro conditions in the laboratories/greenhouses. The technique of micropropagation was developed about 55 years back, but its commercial exploitation started only during the 1970s with the commercial production of orchids. Since then, it has seen tremendous expansion globally both in number of production units and number of plants. Micropropagation industry is environment friendly and requires little raw material in the form of chemicals. Because of higher labour costs in developed countries, this industry is now being expanded in developing countries, where plant multiplication can be

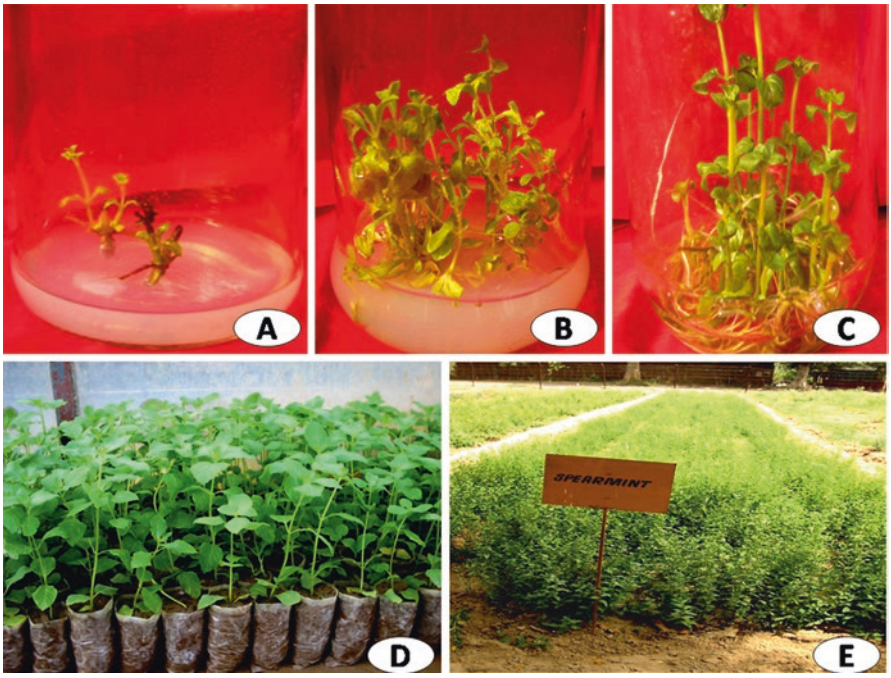

Fig. 1 Micropropagation of *Mentha.* (**a**). Establishment of shoot cultures in vitro. (**b**). Shoot multiplication in vitro. (**c**). Induction of rooting in vitro. (**d**). Transfer of plantlets to soil in greenhouse. (**e**). Micropropagated field-grown plants

done at much cheaper rates for the international markets than in developed countries. Micropropagation (Fig. 1) involves five steps:

Step 1 Establishment of aseptic cultures: This is a transitory step, in which in vivo-grown plants are made to grow under in vitro conditions in the laboratory. Thus, it requires extra care and skill. Small explants usually the meristems, shoot apices or nodal segments are taken and surface sterilized with an appropriate disinfectant like sodium hypochlorite or mercuric chloride for a specific period of time, depending upon the disinfectant used and the tissue being disinfected. Explants are then inoculated on synthetic medium, under aseptic laboratory conditions. The cultures are incubated at 25 ± 1 °C, with relative humidity of 60–80% and light intensity of about 5000 lux, when new shoot buds/shoots appear (3–4 weeks).

Step 2 Shoot bud/shoot multiplication: This is the real multiplication phase, in which cultures from the previous cycle are taken, divided into smaller parts and then re-cultured aseptically on fresh medium in separate culture vessels. This step largely makes use of growth regulator, cytokinin, which induces rapid cell division and shoot buds. This stage is considered as the game of cytokinins where different combinations and concentrations of 6-furfuryl amino purine (kinetin)

and benzyl amino purine (BAP) are used. During this step, there is about ten times multiplication per cycle (2 weeks) throughout the year. Therefore, 26 cycles can be completed in a year, generating thousands of propagules. Due to high levels of cytokinins in the culture medium, the roots generally do not develop; hence, multiplication is usually in the form of shoot buds or shoots.

Step 3 Induction of roots and hardening: The shoots obtained from Step 2 are cultured on rooting medium supplemented with rooting hormones such as indole butyric acid (IBA) and naphthalene acetic acid (NAA) at concentrations ranging from 0.1 to 5.0 mg/l, to induce the root primordia or root formation. Subsequently, hardening of plantlets is done by taking them out of the culture vessels and washing under slow-running tap water. The plantlets are then transferred onto water-moist cotton in trays, kept in the incubation room/greenhouse with daily change of tray water for a few days to get the plants acclimatized.

Step 4 Transfer of plantlets to soil: Hardened plantlets, individually or in clumps, are transferred to soil in bags kept in the greenhouse for one and a half months before their delivery to end users.

Step 5 Transfer to the field: Well-established plants are grown in the field for production of planting material or raising the commercial crop.

2.1 Significance of Micropropagation

1. Production of high-quality, disease-free, planting material, especially in the vegetatively propagated plant species.
2. Rapid spread of new varieties/clones of vegetatively propagated crops such as banana, sugarcane, potato, poplar, peppermint and other medicinal and aromatic plants.
3. Large-scale production of ornamental plants, which are otherwise difficult to multiply following conventional methods.
4. Rejuvenation of old varieties/clones of vegetatively propagated crops for improving their yield and quality.
5. Mass cloning of rootstocks in horticultural plants such as citrus, guava, peach and apple.
6. Mass cloning of genetically plus, cross-pollinated and seed-propagated trees such as eucalyptus.
7. Multiplication of male-sterile lines for hybrid seed production or the multiplication of F_1 hybrids.
8. During genetic transformation, this technique helps in increasing the plant number of elite events for precise characterization and efficient transfer of regenerated plants to greenhouse.
9. During anther/pollen culture, micropropagation of regenerants helps in minimizing the risk of losing any genotype during their hardening and transfer to soil.

10. International exchange of germplasm, avoiding the risk of pathogens and insects.
11. It possesses tremendous potential in keeping our environment clean and green.

Now scores of multimillion-dollar industries around the world propagate a variety of plant species through tissue culture. The clean planting material can certainly improve the yield potentials of vegetatively propagated crops like sugarcane, peppermint, potato, banana, strawberry, sweet potato, cassava and several ornamental plant species. Micropropagation protocols for some important crop plants have been developed and are being exploited for commercial plant production. Some of these include *Aloe vera* (Thind et al. 2007), apple rootstock (Mir et al. 2013; Castillo et al. 2015; Geng et al. 2016), banana (Cronauer and Krikorian 1984; Damasco et al. 1997; Kodym and Zapata-Arias 1999; Kalimuthu et al. 2007; Resmi and Nair 2007; Farahani et al. 2008; Prabhuling et al. 2013; Ssamula et al. 2015), brahmi (Gill et al. 2004b; Gosal and Gill 2004), carnation (Sooch et al. 1998), chrysanthemum (Liu and Gao 2007), citrus (de Oliveira et al. 2016), *Dalbergia sissoo* (Gill et al. 1997), eucalyptus (Gill et al. 1994), grapevine (Machado et al. 2007; Jaskani et al. 2008), neem (Gill et al. 2006a), poplar (Chaturvedi et al. 2004), populus (Ayesh et al. 2016), potato (Wang and Hu 1982; Gopal et al. 1998; Pereira and Fortes 2004; Ebadi et al. 2007; Badoni and Chauhan 2009), rice (Sandhu et al. 1995; Medina et al. 2004), strawberry (Chopra et al. 1993; Mohamed 2007) and sugarcane (Gosal et al. 1998; Gill et al. 2006b; Jalaja et al. 2006, 2008; Sood et al. 2006; Minarsih et al. 2013; Tarafdar et al. 2014; Kaur and Sandhu 2015; Lal et al. 2015; Singh et al. 2016) (Table 2). With the improvement of somatic embryogenesis coupled with embryo desiccation, encapsulation may lead to the utilization of artificial seeds for mass cloning of plants (Helal 2011). It is likely that automation of multiplication systems (Paek et al. 2005) will be commercially feasible in the coming years.

3 Meristem Culture

In most of the seed-propagated crops, the gametes serve as sieve against a variety of pathogens and help in production of disease-free seed, whereas in vegetatively propagated species, the pathogens keep on accumulating generation after generation, which ultimately cause varietal decline. Virus infection in plants reduces both yield and quality. Replacement of virus-infected stock with healthy stock (virus-free) has shown up to 300% yield increase (Murashige 1980; Schenck and Lehrer 2000). There are no effective chemical methods to control viral diseases. In this regard, meristem culture (Morel and Martin 1952) is a practical approach for producing disease-free plants. Shoot meristems (0.2–0.4 mm) excised from in vivo or in vitro-grown plants are aseptically cultured in vitro on suitable medium under appropriate cultural conditions. Virus indexing of meristem-derived plants is done by using electron microscopy/immunological methods for selecting pathogen-free plants (Mori and Hosokawa 1977). Disease-free plants are then micropropagated

Table 2 Application of cell and tissue culture techniques in crop improvement

Plant material	Remarks	Reference
Micropropagation		
Banana *Musa* ('Philippine', 'Lacatan' 'Grande Naine') Plantain ('Pelipita'and 'Saba')	Shoot cultures from excised shoot tips	Cronauer and Krikorian (1984), Damasco et al. (1997)
Strawberry (*Fragaria ananassa* L.)	Method for mass production of planting material	Chopra et al. (1993)
Eucalyptus tereticornis L.	In vitro clonal propagation through nodal segments	Gill et al. (1994)
Rice *Oryza sativa* L**.** var. Jaya	Propagation of *indica* rice through proliferation of axillary shoots	Sandhu et al. (1995)
Dalbergia sissoo Roxb.	Rapid in vitro propagation from mature trees	Gill et al. (1997)
Banana *Musa* cv. Grande Naine	Use of natural sunlight and rapid micropropagation	Kodym and Zapata-Arias (1999), Prabhuling et al. (2013), Karule et al. (2016)
Potato *Solanum tuberosum L.*	In vitro propagation through microtubers	Gopal et al. (1998)
Dianthus caryophyllus L. (carnation) cv. scania	In vitro propagation through axillary shoot proliferation	Sooch et al. (1998)
Sugarcane *Saccharum officinarum* L.	Micropropagation protocol for mass plant production	Gosal et al. (1998), Gill et al. (2006b), Sood et al. (2006), Jalaja et al. (2008), Minarsih et al. (2013), Kaur and Sandhu (2015), Lal et al. (2015), Rangel-Estrada et al. (2016), Udhutha et al. (2016)
Poplar *Populus deltoides*	Protocol for mass production of two important clones, viz. G_3 and G_{48}, through induced shoot differentiation of leaf, stem and root explants collected from adult trees	Chaturvedi et al. (2004)
Brahmi *Bacopa monnieri* L.	Efficient micropropagation protocol through axillary shoot proliferation	Gill et al. (2004b)
Rice *Oryza sativa* L.	Clonal propagation protocol using shoot-tip cultures, and the genetic stability of the micropropagated plants was verified by isozyme analysis	Medina et al. (2004)
Potato (*Solanum tuberosum* L.)	A protocol for production of pre-basic potato material by micro-cuttings, obtained from plants with a short period of acclimatization	Pereira and Fortes (2004)
Neem (*Azadirachta indica*)	Macro- and micropropagation protocols	Gill et al. (2006a)

(continued)

Table 2 (continued)

Plant material	Remarks	Reference
Potato (*Solanum tuberosum* L.)	An efficient micropropagation protocol	Ozturk and Yldrm (2006)
Potato (*Solanum tuberosum* L.)	Use of continuous and semi-continuous bioreactors and their functions at shoot multiplication and microtuberization of potato	Ebadi et al. (2007)
Banana (*Musa sapientum*)	Protocol for micropropagation of *Musa sapientum* using shoot meristems	Kalimuthu et al. (2007)
Grapevine (*Vitis vinifera* L.)	Best shoot development for the initial culture of rootstock VR043–43 in vitro using nodal segments and best micro-cutting multiplication using QL medium	Machado et al. (2007)
Strawberry (*Fragaria ananassa*)	Mass propagation via meristem-tip culture	Mohamed (2007)
Sugarcane (*Saccharum officinarum* L.)	Spacing of 90 cm × 60 cm was the most suitable for transplanting tissue-cultured plantlets	Ramanand et al. (2007)
Banana (*Musa* spp.)	Inflorescence apices were found more suitable for rapid in vitro propagation	Resmi and Nair (2007)
Grapevine (*Vitis vinifera*)	Micropropagation protocol for quick multiplication	Salami et al. (2007)
Sugarcane (*Saccharum officinarum* L.)	Sets obtained from micropropagated plantlets resulted in higher seed yields	Salokhe (2007)
Aloe vera	Micropropagated plants exhibited elevated levels of bioactive compounds	Thind et al. (2007)
Chrysanthemum cinerariifolium (Trev.)	Rapid micropropagation technology was established and optimized in vitro	Liu and Gao (2007)
Banana (*Musa* spp.)	Culture medium was refined	Farahani et al. (2008)
Grape vine (*Vitis vinifera* L.)	Clonal propagation of grapes for increasing plant material for cultivation	Jaskani et al. (2008)
Sugarcane (*Saccharum officinarum* L.)	No variation was detected among the regenerated plants of a particular variety	Lal et al. (2008)
Potato	Successful micropropagation was achieved using nodal segments as explants	Badoni and Chauhan (2009)
Scoparia dulcis	Micropropagation through multiple shoot induction from nodal segment and shoot-tip explants	Rashid et al. (2009)

(continued)

Table 2 (continued)

Plant material	Remarks	Reference
Apple	Rapid micropropagation protocol for apple clonal rootstock MM106	Mir et al. (2013)
Citrus	High-efficiency propagation of mature 'Washington Navel' orange and juvenile 'Carrizo' citrange using axillary shoot proliferation	de Oliveira et al. (2016)
Meristem culture		
Chrysanthemum	Production of virus-free plants	Mori and Hosokawa (1977)
Sugarcane	Elimination of yellow leaf virus from infected sugarcane plants	Fitch et al. (2001)
Sugarcane	Elimination of sugarcane mosaic virus using chemotherapy and meristem culture	Balamuralikrishnan et al. (2002)
Bananas and plantains	Eradication of mosaic disease of bananas and plantains	Gupta (1986)
Banana	Eradication of banana bunchy top virus (BBTV) and banana mosaic virus (BMV) from diseased plants	Allam et al. (2000)
Potato	Production of virus-free plantlets	Faccioli and Colombarini (1996)
Potato	Factors affecting in vitro growth of meristem-tip-derived plantlets	Thind et al. (2005)
Red raspberry (*Rubus idaeus* L.)	Elimination of apple mosaic virus and raspberry bushy dwarf virus from infected plants	Theiler-Hedtrich and Baumann (2008)
Garlic	Assessment of genetic and epigenetic changes in virus-free garlic plants obtained by meristem culture followed by in vitro propagation	Gimenez et al. (2016)
Somatic embryogenesis		
Trifolium repens	Factors influencing co-ordinated behaviour of cells as an embryogenic group	Williams and Maheswaran (1986)
Rice	Efficient plant regeneration from protoplasts through somatic embryogenesis	Abdullah et al. (1986)
Maize	Genotype specificity of somatic embryogenesis and plant regeneration	Hodges et al. (1986)
Citrus (*Citrus reticulata* Blanco)	Factors enhancing somatic embryogenesis and plantlet regeneration in mandarin	Gill et al. (1995), Gosal et al. (1995)
Sugarcane	Factors enhancing somatic embryogenesis and plant regeneration	Gill et al. (2004a)
Rice	Influence of antibiotic cefotaxime on somatic embryogenesis and plant regeneration	Grewal et al. (2006a)

(continued)

Table 2 (continued)

Plant material	Remarks	Reference
Wheat	Ammonium nitrate improves direct somatic embryogenesis and biolistic transformation of *Triticum aestivum*	Greer et al. (2009)
Sugarcane	Desiccation of callus enhances somatic embryogenesis and subsequent shoot regeneration	Kaur and Gosal (2009)
Maize	Moderate desiccation dramatically improves shoot regeneration from callus	Deng et al. (2009)
Cotton	Highly efficient plant regeneration through somatic embryogenesis in 20 elite commercial cultivars	Zhang et al. (2000, 2009)
Finger millet	Influence of plant growth regulators and spermidine on somatic embryogenesis and plant regeneration in four Indian genotypes of *Eleusine coracana* L. Gaertn	Lakkakula et al. (2016)
Peanut	Plant regeneration by somatic embryogenesis involving bulbil-like body – a new type of SE structure	Xu et al. (2016)
Triticum species	Somatic embryogenesis and plant regeneration from immature embryos of *Triticum timopheevii* Zhuk. and *Triticum kiharae* Dorof. Et Migusch, wheat species with G genome	Miroshnichenko et al. (2016)
Brahmi	Direct somatic embryogenesis and encapsulation of somatic embryos for in vitro conservation of *Bacopa monnieri* (L.) Wettst.	Khilwani et al. (2016)
Sugarcane	Putrescine induces somatic embryo development and proteomic changes in embryogenic callus	Reis et al. (2016)
Grapevine	Factors affecting somatic embryogenesis in eight Italian cultivars and the genetic stability of embryo-derived regenerants as assessed by molecular markers	Carra et al. (2016)
Somaclonal variation		
Prunus persica	Somaclones S156 and S122 resistant to leaf spot, moderately resistant to canker	Hammerschlag and Ognjanov (1990)
Apple rootstocks	S-2 (M26) performed better against *Phytophthora cactorum*	Rosati and Predieri (1990)
Banana	10 somaclones. GCTCV215–1 released for commercial planting	Hwang and Ko (2004)
Apple	16 somaclones were identified	Donovan et al. (1994)
Sugarcane (*Saccharum officinarum* L.)	Results confirmed the superiority of two somaclones, one resistant and one tolerant to eyespot disease	Leal et al. (1994)

(continued)

Table 2 (continued)

Plant material	Remarks	Reference
Potato (*Solanum tuberosum* L.)	Somaclones for heat tolerance	Das et al. (2000)
Potato (*Solanum tuberosum* L.)	Somaclones IBP-10, IBP-27 and IBP-30, infected with *Alternaria solani* and *Streptomyces scabiei*, exhibited higher resistance to the pathogen, compared to the susceptible cultivar Desiree	Veitia-Rodriguez et al. (2002)
Potato, tomato, soybean, coffee	Monomorphism was detected in tomato, coffee and soybean, indicating the genetic stability of the crops	Lara et al. (2003)
Oryza sativa L.	Four somaclones showed significantly higher degree of partial resistance when compared with the parent cultivar CICA-8	Araujo and Prabhu (2004)
Durum wheat (*Triticum durum* Desf.)	Somaclonal variation appears to induce a wide range of modifications among individual components of drought-resistance mechanisms	Bajji et al. (2004)
Sugarcane (*Saccharum officinarum* L.)	The somaclones performed better than the source plant	Khan et al. (2004)
Maize (*Zea mays* L.)	Somaclones thus derived were tolerant to NaCl	Zheng et al. (2004)
Bread wheat (*Triticum aestivum*)	Somaclones were superior to their original cultivars	Ahmed and Abdelkareem (2005)
Wheat (*Triticum aestivum* L.)	Twenty-one out of the 23 somaclones outperformed the original cultivar Sakha 61 in terms of leaf rust resistance and grain yield	Sabry et al. (2005)
Oryza sativa L.	Somaclones were obtained from anther culture of hybrid combinations INCA LP-10/C4 153, Amistad-82/C4 153, INCA LP-10 as well as Amistad-82	Cristo et al. (2006)
Sugarcane (*Saccharum officinarum* L.)	Six tissue culture-derived sugarcane somaclones were evaluated. Somaclone TC-435 gave higher cane yield at 12 months crop age over CoC 671. Somaclone TC-435 had significantly higher millable cane height and number of internodes than that of donor parent CoC671	Doule (2006)
Sugarcane (*Saccharum officinarum* L.)	A new sugarcane variety, Co 94012, was released in the name of Phule Savitri for cultivation in Maharashtra, India	Jalaja et al. (2006)

(continued)

Table 2 (continued)

Plant material	Remarks	Reference
Alfalfa (*Medicago sativa* L.)	An increase of variability was noted in important quantitative and qualitative traits compared with the initial cultivars, including productivity of the above ground mass and seeds, resistance to fungal diseases and winter hardiness	Rozhanskaya (2006)
Oryza sativa L.	The grain yield was significantly high in somaclones 'BTS 11–1', 'BTS 28', 'BTS 24', 'BTS 9–2(S)', 'BTS-17(S)', 'BTS 10–2' and 'BTS 11–7'	Elanchezhian and Mandal (2007)
Olive	Somaclonal variation could be found in olive plants regenerated through somatic embryogenesis; this appears in mature plants in the field	Leva and Petruccelli (2007)
Banana	In banana cultivars (*Musa* x *acuminata*, *Musa* x *balbisiana*), somaclonal variation can be useful in selecting for clones with improved agronomic characteristics	James et al. (2007), Oh et al. (2007)
Dieffenbachia	Potential for new cultivar development by selecting callus-derived somaclonal variants of dieffenbachia was demonstrated	Shen et al. (2007)
Sugarcane (*Saccharum officinarum* L.)	Development of somaclones resistant to red rot disease using in vitro and field selection	Singh et al. (2008), Sengar et al. (2009)
Wheat	Comparison of *Triticum aestivum* L. somaclones with their respective parents for salt tolerance	Akhtar et al. (2015)
Apple	Molecular and genome size analyses of somaclonal variation in apple rootstocks Malling 7 and Malling 9	Noormohammadi et al. (2015)
Coffea arabica	Assessment of genetic and epigenetic changes during cell culture ageing and relations with somaclonal variation in *Coffea arabica*	Landey et al. (2015)
Banana	Development of new banana variety Guijiao 9 resistant or tolerant to Fusarium wilt (*Fusarium oxysporum* f. Sp. Cubence, race 4)	Wei et al. (2016)
Rice	Genetics of yield and component characters in Pokkali somaclones a tall, traditional, photosensitive cultivar from India	Mandal et al. (2016)
Proso millet	Analysis of callus-generated somoclonal variation in *Panicum miliaceum* L. through molecular markers	Mhatre et al. (2016)

(continued)

Table 2 (continued)

Plant material	Remarks	Reference
In vitro production of haploids		
Wheat	Doubled haploid wheat variety 'florin' was developed	De Buyser et al. (1986)
Wheat	A single 2,4-D treatment given to spikes 1 day after pollination with maize enabled embryos to be recovered from all 19 varieties	Laurie and Reymondie (1991)
Rice	Revised medium was used for increasing anther culture efficacy and improved feasibility of using doubled haploids in genetic and breeding research with *indica* rice	Raina and Zapata (1997)
Rice	'Bicol' first F_1 anther culture-derived line from an *indica/indica* cross in saline-prone areas	Senadhira et al. (2002)
Rice	An improved method for pollen culture in rice	Sarao et al. (2003), Grewal et al. (2006b)
Durum wheat	Dicamba and 2,4-D was best for improving the yield of haploid plants of durum wheat through crosses with maize	García-llamas et al. (2004)
Maize (*Zea mays* L.)	Embryogenic induction of microspores within anthers in vitro conditions was the best when combination of cold treatment, TIBA (0.1 mg l^{-1}) in media and colchicine (0.02% during first 3 days of culture) was applied	Obert and Barnabas (2004)
Citrus (*Citrus clementina*)	Anther culture as a rapid and attractive method of obtaining new triploid varieties in clementine	Germanà et al. (2005)
Citrus (*Citrus clementina*)	Influence of light quality on anther culture of *Citrus clementina* Hort. ex Tan. cultivar Nules was reported	Antonietta et al. (2005)
Maize (*Zea mays* L.)	15, 10, 10 and 3 fertile doubled haploid plants were obtained in cultures treated with paraquat, t-BHP, methionine combined with riboflavin and menadione, respectively	Ambrus et al. (2006)
Wheat	Simplified wheat × maize haploid production protocol that is 100% effective across all bread wheat cultivars	Mujeeb-Kazi et al. (2006)
Maize (*Zea mays* L.)	Maize haploid plants by in vitro culture of pollinated ovaries	Tang et al. (2006)
Durum wheat *Triticum durum*	Novel pretreatment combining mannitol 0.3 M and cold for 7 days had a strong effect on the number of embryos produced and regenerated green plants	Labbani et al. (2007)

(continued)

Table 2 (continued)

Plant material	Remarks	Reference
Tomato (*Lycopersicon esculentum* L.)	Embryogenesis and plant regeneration by in vitro culture of isolated microspores and whole anthers of tomato	Segui-Simarro and Nuez (2007)
Rice	The nine DH lines could provide the basic materials for breeding on Dian-type hybrid rice with both good quality and high blast resistance in the future	Zhahg-Yi et al. (2008)
Maize	Doubled haploids should be induced from F_2 plants rather than from F_1 plants	Bernardo (2009)
Rice	Effect of cold pretreatment and different media compositions in improving anther culture response	Khatun et al. (2012)
Carrot	An improved protocol for haploid and doubled haploid plant production using induced parthenogenesis and ovule excision in vitro	Kiekowska et al. (2014)
Tomato	Haploid induction via in vitro gynogenesis in *Solanum lycopersicum L.*	Zhao et al. (2014)
Watermelon	In vitro culture of unpollinated ovary and production of haploid plants	Li et al. (2014)
Wheat	Isolated microspore culture to produce doubled haploid plants in *Triticum aestivum* L.	Scagliusi (2014)
Cucumber	Optimization of protocol for production of doubled haploids	Diao et al. (2009), Gaazka et al. (2015)
Pepper	Genotypic effects on obtaining spontaneous doubled haploid plants via anther culture	Keles et al. (2015)
Broccoli	Comparison of anther and microspore culture for androgenic embryogenesis and regeneration of *Brassica oleracea* L. var. italica plants	Qin et al. (2015)
Rice	Doubled haploids through anther culture from hybrid, CRHR32: method optimization and molecular characterization	Prachitara et al. (2016)
Rice	Agronomical performances of doubled haploid lines derived from anther culture	Fazaa et al. (2016a, b)
Wheat and barley	Enhancement in androgenesis by the addition of DMSO to the mannitol pretreatment medium	Echávarri and Cistue (2016)

(continued)

Table 2 (continued)

Plant material	Remarks	Reference
Embryo/ovule/ovary culture		
Vigna mungo X *Vigna radiata*	Interspecific hybrids between *Vigna mungo* and *Vigna radiata* were produced through embryo culture	Gosal and Bajaj (1983)
Arachis	Embryo rescue from wide crosses in *Arachis*	Moss et al. (1988)
Moricandia arvensis X *Brassica*	Intergeneric (intersubtribe) hybrids between *Moricandia arvensis* and *Brassica* A and B genome species by ovary culture	Takahata and Takeda (1990)
Vitis vinifera L.	Ovule culture of seedless grapes (*Vitis vinifera* L.	Singh et al. (1991)
Brassica X Sinapis	Production of intergeneric hybrids between *Brassica* and *Sinapis* species by means of embryo rescue techniques	Momotaz et al. (1998)
Wheat X rye	In vitro synthesis of white-grained primary hexaploid triticales	Kaur et al. (2002)
Populus euphratica Oliv.	Intraspecific hybridization of *Populus euphratica* Oliv. using in vitro technique	Calagari et al. (2004)
Sesamum	Development of interspecific hybrids between *Sesamum alatum* Thonn and *Sesamum indicum* L. through ovule culture and screening for resistance to phyllody disease	Rajeswari et al. (2010)
Peach	Ovule rescue in peaches: incubation period	Raseira and Einhardt (2010)
Citrus	Regenerating triploid plants by embryo rescue technique	Liu et al. (2010a, b), Aleza et al. (2010)
Chickpea	Poorly formed chloroplasts are barriers to successful interspecific hybridization following in vitro embryo rescue	Clarke et al. (2011)
Chrysanthemum	Embryo rescue-derived intergeneric hybrid between chrysanthemum and *Ajania przewalskii* shows enhanced cold tolerance	Deng et al. (2011)
Sunflower	Gene(s) introgression of drought resistance to *Helianthus annuus* using embryo rescue	Sauca (2010), Sauca and Lazar (2011)
Wheat	A direct hybridization approach to gene transfer from *Aegilops tauschii* Coss. to *Triticum aestivum* L.	Sehgal et al. (2011)
Cucurbits	Production of *Cucurbita* interspecific hybrids through cross-pollination and embryo rescue technique	Rakha et al. (2012a, b), Plapung et al. (2014)

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Table 2 (continued)

Plant material	Remarks	Reference
Grape	Embryo rescue of seedless grape with disease and cold resistance	Niu et al. (2012)
Radish	Transfer of auxinic herbicide resistance from wild mustard (*Sinapis arvensis*) into radish (*Raphanus sativus*) through embryo rescue	Mithila and Hall (2012)
Populous	Interspecific and intergeneric hybridization in Salicaceae (*Populus nigra* × *P. deltoides* and *P. nigra* × *Salix alba*) using an embryo rescue technique	Payamnour et al. (2013)
Mustard	Transfer of cytoplasmic male sterility from alloplasmic *Brassica juncea* and *B. napus* to cauliflower (*B. oleracea* var. *botrytis*) through interspecific hybridization and embryo culture	Chamola et al. (2013)
Sorghum	Shortening the breeding cycle of sorghum, a model crop for research	Rizal et al. (2014)
Musa species	Embryo culture and embryo rescue studies in wild *Musa* spp. (*Musa ornata*)	Dayarani et al. (2014)
Citrus	Production of common sour orange × Carrizo citrange hybrids using embryo rescue	Kurt and Ulger (2014)
Grapevine	Immature embryo rescue and in vitro development evaluation of intraspecific hybrids from Brazilian seedless grapevine 'Superior × Thompson' clones	de Menezes et al. (2014)
Lentil	Hybridization of cultivated *Lens culinaris* Medik. and wild *Lens tomentosus* Ladizinsky	Suvorova (2014)
Protoplast culture and somatic hybridization		
Potato (*S. brevidens x S. tuberosum*)	Somatic hybrids were produced by electrofusion	Fish et al. (1988)
Brassica (*B. juncea, B. nigra and B. carinata X B. napus*)	Resistance to *Phoma lingam* was expressed in all symmetric hybrids and in 19 of 24 toxin-selected asymmetric hybrids	Sjödin and Glimelius (1989)
Potato (*S. brevidens x S. tuberosum*)	20 hybrids tested expressed a high level of resistance to PVY	Rokka et al. (1994)

(continued)

Table 2 (continued)

Plant material	Remarks	Reference
Brassica (*Brassica napus x Brassica oleracea*)	Inoculations *Xanthomonas campestris* pv. *campestris* identified four somatic hybrids with high resistance	Hansen and Earle (1995)
Brassica (*Brassica oleracea x Brassica rapa*)	Disease assays showed that most somatic hybrids had lower disease severity ratings against bacterial soft rot	Ren et al. (2000)
Citrus (*Citrus sinensis* L. *Osbeck x C. volkameriana Pasquale*, *C. reticulata Blanco*)	Somatic hybrids combined characteristics from both sources and have potential for tolerance to blight and citrus tristeza virus (CTV)	Mendes et al. (2001)
Banana ('Maçã'(*Musa AAB group*) *x* Lidi' *Musa* sp. *AA group*)	Somatic hybrids were identified by using RAPD markers	Matsumoto et al. (2002)
Citrus (mandarin *x* pummelo, sweet orange *x* pummelo)	Somatic hybrids were confirmed by leaf morphology, ploidy analysis via flow cytometry and RAPD analysis	Ananthakrishnan et al. (2006), Aleza et al. (2016)
Potato (*Aminca-Cardinal x Cardinal-Nicola*)	Complete resistance to PVY was noted for one somatic hybrid line (CN2). All other hybrids also showed improved tolerance to *Pythium aphanidermatum* infection during tuber storage or after plant inoculation	Nouri-Ellouz et al. (2006)
Potato (*Solanum tuberosum x Solanum tuberosum*)	Tetraploid intraspecific somatic hybrids between 16 different diploid breeding lines of *Solanum tuberosum* L. were produced by PEG-induced fusion	Przetakiewicz et al. (2007)
Brassica (*Brassica oleracea x Brassica rapa*)	Calli were screened by RAPD analysis for their hybrid character. This is the first report about a hybrid formation between two haploid protoplasts	Liu et al. (2007)
Brassica napus (2n = 38) **X** *Orychophragmus violaceus* (2n = 24)	Symmetric fusions of mesophyll protoplasts and subsequent development of *B. napus-O. violaceous* chromosome addition lines	Zhao et al. (2008)
Citrus	Production and molecular characterization of two new citrus somatic hybrids for scion improvement and rootstock improvement	Cai et al. (2010), Dambier et al. (2011)
Ginger	Regeneration of somatic hybrids via chemical protoplast fusion	Guan et al. (2010)

(continued)

Table 2 (continued)

Plant material	Remarks	Reference
Mango	Intraspecific somatic hybridization of *Mangifera indica* L. through protoplast fusion	Rezazadeh et al. (2011)
Brassica oleracea	Production and characterization of interspecific somatic hybrids between *Brassica oleracea* var. *botrytis* and *B. nigra* and their progenies for the selection of advanced pre-breeding materials	Wang et al. (2011a, b)
Brassica	Protoplast isolation and culture for somatic hybridization of rapid cycling *Brassica rapa* with 'Anand' CMS and *Brassica juncea* and production of sterile somatic hybrids	Lian et al. (2012)
Citrus	Production and characterization of somatic hybrids combining sweet orange and mandarin/mandarin hybrid cultivars for citrus scion improvement	Soriano et al. (2012), Abbate et al. (2012), Guo et al. (2013), Xu et al. (2014)
Cotton	Characteristics of fertile somatic hybrids of *G. hirsutum* L. and *G. trilobum* generated via protoplast fusion	Sun et al. (2011), Yu et al. (2012)
Potato	Resistance to common scab developed by somatic hybrids between *Solanum brevidens* and *Solanum tuberosum* and resistance to *Phytophthora infestans* from *Solanum villosum*	Ahn and Park (2013), Tarwacka et al. (2013), Smyda et al. (2013), Tiwari et al. (2013)
Chrysanthemum	Intergeneric hybrids between *Chrysanthemum morifolium* 'Nannongxiaoli' and *Artemisia vulgaris* 'Variegata' show enhanced resistance against both aphids and *Alternaria* leaf spot	Zhu et al. (2014)
In vitro production of secondary metabolites		
Capsicum annum	Accumulation of capsaicin in callus cultures	Varindra et al. (1997)
Secondary metabolites, like vincristine and vinblastine, and recombinant proteins	Plant suspension cells are an in vitro system that can be used for recombinant protein production	Fischer et al. (1999)
Secondary metabolites	Hairy root culture for mass production of high-value secondary metabolites	Srivastava and Srivastava (2007)
Secondary metabolites	Production of ginsenoside and polysaccharide by two-stage cultivation of *Panax quinquefolium* L. cells	Wang et al. (2012)

(continued)

Table 2 (continued)

Plant material	Remarks	Reference
Secondary metabolites	Production of medicinally important secondary metabolites (stigmasterol and hecogenin) from root cultures of *Chlorophytum borivilianum* (Safed musli)	Gayathri and Archana (2012)
Secondary metabolites	In vitro plant regeneration, secondary metabolite production and antioxidant activity of micropropagated *Aloe arborescens* Mill.	Amoo et al. (2012)
Secondary metabolites	In vitro culture of lavenders (*Lavandula* spp.) and the production of secondary metabolites.	Gonçalves and Romano (2013)
Secondary metabolites	Influence of L-tryptophan and salicylic acid on secondary metabolite production from leaves-induced callus of *Catharanthus roseus* L. G. Don in vitro	Jassim and Ameen (2014)
Secondary metabolites	Withanolides from *Withania somnifera* Dunal: development of cellular technology and their production	Sangwan et al. (2014)
Cryopreservation and in vitro germplasm storage		
Rice (*Oryza sativa* L.)	Rice plants (cv. Taipei 309) were regenerated from different cryopreserved calli, and cryopreservation was declared to be a reliable way to store transformation-competent rice lines	Moukadiri and Cornejo (1996)
Potato (*Solanum tuberosum* L)	Shoot tips of in vitro grown potato (*Solanum tuberosum* L.) plants were cryopreserved by a modified vitrification method	Halmagyi et al. (2004)
Potato (*Solanum tuberosum* L)	In vitro plants of potato were cold acclimated, cryopreserved and regenerated in the regeneration medium	Zhao et al. (2005)
Rubus spp. (blackberry and raspberry)	Encapsulation-dehydration and PVS2-vitrification cryopreservation protocols were found successful for preserving diverse *Rubus* germplasm (shoot tips)	Gupta and Reed (2006)
Arabidopsis	*Arabidopsis* can be successfully cryopreserved using either plant vitrification solution 2 (PVS2) or plant vitrification solution 3 (PVS3) as cryoprotectants prior to rapidly cooling shoot tips in liquid nitrogen (LN)	Volk and Casperson (2007)

(continued)

Table 2 (continued)

Plant material	Remarks	Reference
Potato (*Solanum tuberosum* L.)	Protocol was tested with 12 selected cultivated varieties and wild species, and the survival percentages obtained ranged between 64.0 and 94.4%	Yoon-Ju et al. (2007)
Citrus	Up to 98.1% of the plants obtained by cryopreservation were free from HLB bacterium, as compared with a sanitation rate of 25.3% yielded by conventional meristem-tip culture	Ding et al. (2008)
Date palm	In vitro culture techniques for conservation of germplasmin	Bekheet et al. (2010)
Wheat	Effect of cryopreservation in liquid nitrogen on germination rate and vigour of seeds	Wang et al. (2011a, b)
Date palm	Optimizing in vitro cryopreservation of *Phoenix dactylifera* L.	AlBahrany and AlKhayri (2012)
Mentha	Cryopreservation of germplasm and analysis of genetic variation	Guo et al. (2012)
Actinidia, *Diospyros*, *Malus*, *Olea*, *Prunus*, *Pyrus and Vitis*	Cryopreservation of shoot-derived germplasm	Benelli et al. (2013)
Garlic	A technique of cryopreservation of germplasm and virus eradication	Liu and Cheng (2013)
Teak	Cryopreservation of *Tectona grandis* L. seeds	Hine Gómez et al. (2013)
Apple	Cryopreservation of winter-dormant buds using two-step freezing	Yi et al. (2013)
Pear	Growth medium alterations improve in vitro cold storage of germplasm	Kovalchuk et al. (2014)
Wheat	Somatic embryogenesis and cryopreservation of South African bread wheat (*Triticum aestivum* L.) genotypes	Roux et al. (2016)
Genetic transformation		
Direct gene transfer to plants	Particle-mediated genetic transformation	Yang and Christou (1994)
Rice	Analysis of transgene integration patterns, expression levels and stability	Maqbool and Christou (1999)
Various plant species	Various methods for genetic transformation and production of transgenic plants	Gosal and Gosal (2000)
Agrobacterium-mediated transformation of basmati rice (*Oryza sativa* L.)	Expression of synthetic Cry1AB and Cry1AC genes for the control of European corn borer	Ahmad et al. (2002)
Transgenic crop varieties	Transgenic crop varieties commercialized in more than 28 countries	James (2015)

in vitro for developing super-elite planting material which is further multiplied under greenhouse conditions for production of commercial planting material. This technique is well established for the production of disease-free planting material of apple (Theiler-Hedtrich and Baumann 2008), banana and plantains (Gupta 1986; Allam et al. 2000; Karule et al. 2016), potato (Faccioli and Colombarini 1996; Thind et al. 2005), sugarcane (Fitch et al. 2001; Balamuralikrishnan et al. 2002; Udhutha et al. 2016) and tomato (Koeda et al. 2015).

4 Micrografting

Micrografting involves the scion meristem or shoot tip, grafting onto rootstock plantlet, grown in vitro through shoot multiplication or the seed culturing. It is also called in vitro shoot-tip grafting (STG). This technique combines the advantages of meristem culture and conventional grafting and is used to produce virus-free plants. The pioneer work of Navarro et al. (1975) on citrus has led to the development of successful micrografting protocols for various plant species such as cherry, chestnut, *Citrus*, grapes, mulberry, olive, peach, pear, pistachio and walnut (Navarro et al. 1982; Navarro 1988; Singh et al. 2008; Hussain et al. 2014). It has been used on commercial scale for production of virus-free plants in some fruit crops and viroid-free plants in *Citrus*. Two viruses (apple chlorotic leaf spot virus, Prunus necrotic ringspot virus) and a viroid (peach latent mosaic viroid) have been eliminated in almost 100% of the micrografted plants using bud explants from infected plants, grafted onto rootstocks forced in vivo at 35 °C, as shoot-tip source. The subsequent development of healthy plants for tested peach and Japanese plum cultivars demonstrates the suitability of this method for safe transport of plant material (Conejero et al. 2013).

5 Somatic Embryogenesis

Somatic embryogenesis is the process by which somatic cells cultured in vitro develop into differentiated plants through characteristic embryological stages without the fusion of gametes (Williams and Maheswaran 1986). The developmental stages (ontogeny) of somatic embryos resemble with the ontogeny of zygotic embryos that helps in their characterization using histological approach. Somatic embryos are uniparental, whereas the zygotic embryos are biparental. Plants produced from somatic embryos in those species which exhibit nucellar embryony or apomixes are genetically similar to nucellar seedlings or apomictic seedlings of such plant species. During in vitro culturing, a number of factors, such as genotype of donor plant, composition of culture medium, nature and concentration of auxins, sugars, amino acids, growth retardants, desiccation, etc., influence the process of somatic embryogenesis and subsequent plant regeneration. Embryogenic cultures

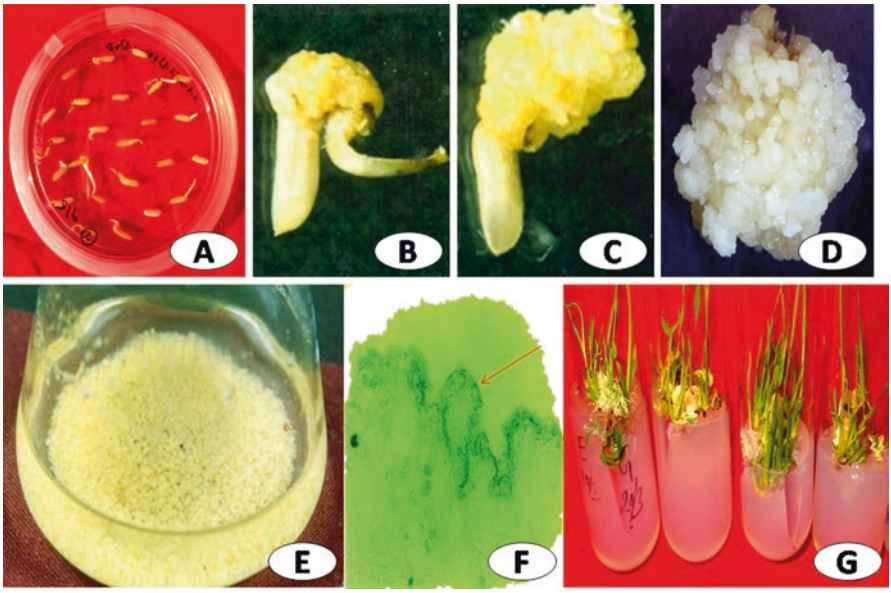

Fig. 2 Induction of somatic embryogenesis in rice. (**a**). Mature seeds cultured in vitro. (**b**). Callus initiation from scutellar tissue of germinating embryo. (**c**). Scutellar callus proliferation. (**d**). Separated embryogenic callus. (**e**). Embryogenic cell suspension culture. (**f**). Histology of embryogenic callus showing developing of embryos. (**g**). High-frequency plant regeneration from embryogenic callus

can be maintained and multiplied through periodic subculturing on suitable medium, usually the fresh medium of the same composition. Embryogenic callus/cell suspension cultures (Fig. 2) ensure high-frequency plant regeneration upon their transfer to shoot regeneration medium. Therefore, such cultures are preferred for genetic transformation. Further, somatic embryos can be encapsulated in a suitable gel, containing nutrients and suitable growth regulators, antibiotics, etc., needed for the development of a complete plant to make what are known as synthetic or artificial seeds that can be stored for several years and can be sown like natural seeds. Frequent somatic embryogenesis has been reported in citrus (Gill et al. 1995; Gosal et al. 1995; Cardoso et al. 2016), cotton (Zhang et al. 2000, 2009), finger millet (Lakkakula et al. 2016), grapevine (Carra et al. 2016), maize (Hodges et al. 1986; Deng et al. 2009), peanut (Xu et al. 2016), rice (Abdullah et al. 1986; Grewal et al. 2006a), sugarcane (Gill et al. 2004a; Kaur and Gosal 2009; Manchanda and Gosal 2012; Rani et al. 2012; Cardoso et al. 2016) and wheat (Williams and Maheswaran 1986; Greer et al. 2009; Gill and Gosal 2015; Miroshnichenko et al. 2016). Improvement of somatic embryogenesis coupled with embryo desiccation and encapsulation technology (Helal 2011; Ravi and Anand 2012; Khilwani et al. 2016) may lead to the utilization of artificial seeds for mass cloning of plants. Synthetic seeds have been produced in several plants such as *Brassica oleracea* (Yussof et al. 2012; Qamar et al. 2014), *Cassia angustifolia* (Bukhari et al. 2014), cauliflower

(Rihan et al. 2012), cucumber (Bushra et al. 2010), stevia (Aamir et al. 2012) and *Ziziphus* (Sudhersan et al. 2013).

6 Somaclonal Variation

The success of plant breeder largely depends upon the nature and extent of genetic variability available in the base population. It is an established fact that the greater is the genetic variability, the larger is the success of the plant breeder. Unlike seed-propagated crops, it has been difficult to create enough genetic variation in the vegetatively propagated plant species. In this regard, the phenomenon of somaclonal variation is an additional tool to create, genetic variation, especially in the vegetatively propagated plant species. Variation among tissues or plants derived from the in vitro somatic cell cultures, i.e. callus and suspension cultures, is called somaclonal variation. It may be genetic or may result from culture-induced epigenetic changes (Larkin and Scowcroft 1981). The epigenetic changes are expressed at cell culture stage, but these usually disappear when plants are regenerated or reproduced sexually. Variation arising out of anther/pollen culture is more precisely known as gametoclonal, from cultured apical meristems as mericlonal and through protoplast culture is called protoclonal variation. It, therefore, provides a novel mechanism to generate new genetic variation for crop improvement (Jain et al. 1998; Krishna et al. 2016).

6.1 Induction of Somaclonal Variation

Somatic cell (callus and cell suspension) cultures are established from suitable explants and multiplied through periodic subculturing on the fresh and suitable culture medium for several times. Cell suspension cultures can be established by transferring actively growing callus to constantly agitated liquid medium and can be maintained/multiplied through periodic subculturing. Plants are regenerated usually from long-term maintained (old) callus/cell suspension cultures and transferred to soil and screened for variation, in the glasshouse or field. In vitro selection at cellular level can be carried out for some traits by growing cells from cell suspensions and calli on a medium supplemented with elevated levels of various biotic and abiotic stress factors (only the variant cells survive). This is similar to the selection of antibiotic-resistant cells by growing them on antibiotic-containing medium. Using this technique, many million cells (potential plants) can be screened in a single Petri dish, which is practically difficult, if not impossible, to be adopted at whole-plant level in the greenhouse/field. Moreover, in vitro selection at cellular level also reduces the chances of diplontic selection (in multicellular tissues, the normal cells mask the growth of variant cell), but it requires high level of correspondence between the trait(s) selected in vitro and expressed in vivo. Somaclonal variations

can be easily identified in the field for traits like flower colour, leaf colour, seed colour, plant height, tillering ability, stem girth, etc. Further, somaclonal variants can be identified through screening, which involves assessment of regenerated plants for characters like yield that cannot be evaluated at single-cell level or through cell selection that involves application of suitable selection pressure like that of some toxins to permit preferential survival of variant cells.

6.2 Causes of Somaclonal Variation

Somaclonal variation may be genetic or epigenetic; the genetic variation is heritable, whereas the epigenetic variation, caused by cultural conditions, is not heritable and hence of no significance in the sexually propagated plants. Genetic variation may result from the following causes:

6.2.1 Chromosomal Changes

During in vitro culturing, cells multiply at a much faster rate that leads to several chromosomal changes. These changes have been observed with respect to chromosome number and their structure as well. Besides polyploidy, aneuploidy (monosomics and trisomics) has been observed in oats, ryegrass, wheat, triticale and potato. There have been a number of reports of modified chromosome structure in cultured plant cells (Lee and Phillips 1988). Duplications, deletions, inversions and translocations have been frequently observed in barley, wheat, potato and maize. While large changes in chromosome structure have been detected, it is likely that less dramatic structural changes that were not detected occur quite frequently. Small changes in chromosome structure could alter expression and genetic transmission of specific genes. In addition, recombination or chromosome breakage may occur in preferential regions or 'hot spots' of particular chromosomes, thereby affecting some regions of the genome in a disproportionally higher frequency (Evans et al. 1984).

6.2.2 Mitotic Crossing Over

The somatic cells cultured in vitro divide and redivide through the process of mitosis. Therefore, mitotic crossing over may account for some of the genetic variation that leads to the recovery of homozygous recessive single-gene mutations in some regenerated plants. Dulieu and Barbier (1982) regenerated plants from *Nicotiana tabacum* with specific chlorophyll deficiency markers present in heterozygous condition. A high frequency (9.6%) of variant regenerants at the 'a_1' and 'y' loci has been ascribed to the combination of deletion and mitotic recombination.

6.2.3 Apparent 'Point' Mutations

Genetic changes resembling single-gene mutations have now been detected in numerous crops. The recessive single-gene mutations are suspected if variant does not express itself in the regenerant (Ro) plant but the self-fertilized R_1 progeny segregates in an expected 3:1 Mendelian ratio for a morphological trait. This type of analysis has been completed for several tomato somaclones and used to map somaclones to specific loci (Evans and Sharp 1986). Such a phenomenon has also been noted in maize, tobacco, rice and wheat.

6.2.4 Cytoplasmic Genetic Changes

Cytoplasmic genetic interactions are known to cause a kind of male sterility in plants. Cytoplasmic genetic changes involving mitochondrial DNA (mtDNA) have been described in maize by evaluating plants for two cytoplasmic traits (Gengenbach et al. 1977). Sensitivity to host-specific toxin of *Drechslera maydis* race T, the causal agent of southern corn leaf blight, is associated with all genotypes containing Texas male-sterile (cms-T) cytoplasm. Such traits are tightly linked and controlled by mitochondrial DNA. Gengenbach et al. (1977) selected in vitro for resistance to toxin and regenerated resistant plants with the aim of recovering toxin-resistant cytoplasmic male-sterile lines, but among the regenerants, resistance was associated with reversion to male fertility. Restriction endonuclease pattern of mtDNA revealed significant changes in mtDNA. This mutation to male fertility and toxin insensitivity has been shown to be a frameshift mutation in mitochondrial DNA.

6.2.5 Amplifications and Deamplifications

Deficiencies in ribosomal DNA (rDNA deamplifications), although not associated with change in plant morphology, have been observed at the molecular level in tissue-cultured flax, potato and triticale. On the other hand, gene amplifications, i.e. duplications, have been observed in somaclones of tobacco and tomato (Santoso and Thornburg 2002).

6.2.6 Transposable Element Activation

Activation of transposons, otherwise silent, controlling elements (mutator genes), has been observed following plant cell cultures of alfalfa, maize and tobacco. Chromosome breakage and fusion, which occur during culture, and genomic stress caused by cultural conditions are major causes of transposable element activation. Transposable elements are known to cause phenotypic changes in plants, and their activation during in vitro culture induces somaclonal variation (Kaeppler et al. 2000).

6.2.7 *Methylation*/Demethylation of DNA

De novo methylation and demethylation events are part of differential genomic changes. Tissue-specific DNA methylation of different sequences has been reported for several plants. Genome activity, i.e. transcription, replication, rearrangements and the structural organization of chromatin, somehow seems to be related to DNA methylation (Bardini et al. 2003; Bednarek et al. 2007).

6.2.8 Altered Expression of Multigene Families

It has been postulated that the cultural conditions may regulate the expression of the multigene family in a way that a member gene that previously expressed some agronomically important genes, including those for gliadins, zeins, glutenins and α-amylase, is coded on multigene families. Heritable somaclonal variation has been obtained for gliadin – a storage protein and β-amylases in wheat (Larkin et al. 1989).

6.3 Nature of Somaclonal Variation

Somaclonal variation has been reported in several crops for both qualitative and quantitative traits, including male sterility in maize; improved protein content in rice and triticale; high sucrose content in sugarcane; early tasseling in corn; changed plant height, awns, tiller number, grain colour, heading date, gliadin proteins and α-amylase in wheat; herbicide tolerance in tomato; disease resistance in maize, sugarcane, mustard and potato; and salt tolerance in rice (Jain et al. 1989). However, such somaclonal variations have not been frequently utilized because, in many cases, these include either the already existing types or there were desirable changes accompanied by several undesirable changes.

6.4 Significance of Somaclonal Variation in Crop Improvement

Several interesting and potentially useful traits have been recovered using this method in alfalfa (Rozhanskaya 2006), apple (Noormohammadi et al. 2015), apple rootstocks (Rosati and Predieri 1990; Donovan et al. 1994), banana (Oh et al. 2007; Molina et al. 2016; Wei et al. 2016), garlic (Gimenez et al. 2016), maize (Zheng et al. 2004), millet (Mhatre et al. 2016), peach (Hammerschlag and Ognjanov 1990), potato (Das et al. 2000; Veitia-Rodriguez et al. 2002; Lara et al. 2003), *Prunus persica* (Hammerschlag and Ognjanov 1990), rice (Araujo and Prabhu 2004; Cristo et al. 2006; Elanchezhian and Mandal 2007; Mandal et al. 2016), sugarcane (Leal

et al. 1994; Kaur et al. 2001; Khan et al. 2004; Doule 2006; Jalaja et al. 2006; Singh et al. 2008; Sengar et al. 2009) and wheat (Ahmed and Abdelkareem 2005; Sabry et al. 2005; Akhtar et al. 2015). Recovery of novel variants that either do not exist or are rare in the natural gene pool, for example, atrazine resistance in maize, glyphosate resistance in tobacco, improved lysine and methionine contents in cereals, increased seedling vigour in lettuce, jointless pedicels in tomato and *Fusarium* resistance in alfalfa, is of much significance (Evans and Sharp 1986; Jain et al. 1989) Genetic, cytogenetic and molecular evidences for increased recombination frequency through cell culture have now been provided (Larkin et al. 1993). Tissue culturing of wide hybrids also helps in breaking undesirable linkages and achieving introgression from alien sources. Several new varieties have been developed through somaclonal variation in tomato, sugarcane, potato, celery, brassica and sorghum. This simple and cost-effective technique possesses a huge potential for the improvement of apomictic and vegetatively propagated plant species and, of course, seed-propagated crop plants with narrow genetic base. In India, a somaclonal variant of a medicinal plant, *Citronella java*, has been released as a commercial variety, 'B-3', which gives higher yield and oil content than the original variety. Likewise, 'Pusa Jai Kisan' is a variety of *B. juncea* which has been released as a somaclonal variant of 'Varuna' variety. However, under several situations, low plant regeneration ability and the lack of correspondence in expression of the trait in field-grown plants are the major problems (Karp 1995).

7 In Vitro Production of Haploids

In self-pollinated crops, an inordinately long period is required to assemble desirable gene combinations from different sources in homozygous form. Generally, it takes eight to ten generations to develop stable, homozygous and ready-to-use materials from a fresh cross of two or more parental lines. In cross-pollinated crops, because of inbreeding depression, it becomes difficult to develop vigorous true-breeding lines (inbreds) for hybrid seed production programmes. In this regard, haploids possessing gametic chromosome number are very useful for producing instant homozygous true-breeding lines that save several years and resources and help in early release of varieties. In addition, haploids constitute an important material for induction and selection of mutants, particularly for recessive traits. During conventional breeding, the early segregating-generation populations (F_2, F_3 and F_4) exhibit variation attributable to both additive and nonadditive gene effects (Khush and Virk 2002), whereas doubled haploid (DH) lines exhibit variation only of additive genetic nature, including additive x additive type of epistasis, which can be easily fixed through a single cycle of selection. The elimination of dominance effects leads to high narrow-sense heritability, and availability of sufficient seed of each DH line allows for replicated testing. Thus, in contrast to relatively large

segregating populations in conventional breeding, fewer DH breeding lines are required for the purpose of selection of desired recombinants. For instance, in rice, about 150 DH breeding lines derived from F_1 plants, instead of 4000–5000 F_2 plants, are sufficient for selecting desirable genotypes. Production of haploids has also been exploited during wide hybridization for the development of addition and substitution lines (plants carrying additional or substituted chromosomes). Production of haploids/doubled haploids through anther culture from F_1 rice plants results in true-breeding plants in less than 1 year, which otherwise takes seven to eight generations through conventional methods (Gosal et al. 1996). Commonly used methods for in vitro production of haploids include:

(i) Anther culture
(ii) Isolated microspore/pollen culture
(iii) Unpollinated ovary culture
(iv) Embryo rescue from wide crosses

7.1 Anther Culture

The technique of anther culture was first developed in *Datura* by Guha and Maheshwari (1964, 1966). Anther culture has now become an attractive alternative for developing haploids (sporophytes with gametophytic chromosome number). The microspore/pollen in the cultured anthers, instead of forming pollen tubes, starts developing directly into pollen embryos and pollen plants. Anthers are cultured in liquid or on semi-solid agar medium (Bajaj et al. 1980; Gill et al. 2003), where the cultured anthers containing pollen exhibit the development of pollen embryos/callus formation. The pollen embryos directly develop into haploid plantlets or doubled haploids in some crops (because of spontaneous doubling of chromosomes during callus proliferation). Haploid plantlets are treated with a chemical, colchicine, to obtain fertile, doubled haploid homozygous plants for field testing and selection. Flower buds at an appropriate stage are collected from healthy greenhouse or field-grown plants. The collected flower buds are usually wrapped in plastic bags and kept in a refrigerator at 4 °C for 7–10 days for cold pretreatment. Flower buds are surface sterilized with 0.1% $HgCl_2$ for 9–10 min, and anthers are carefully dissected out of the flower buds and inoculated on the medium. Cultures are incubated at 25 °C ± 1 °C under diffused light conditions. In general, the cultured anthers exhibit callusing after 2–6 weeks. About a month-old calli are made to regenerate into plants. The factors affecting anther culture success include (i) growth conditions of donor plant, (ii) genotype of donor plant, (iii) pretreatment of anthers, (iv) developmental stage of anthers/microspores, (v) composition of culture medium and (vi) physical conditions during incubation.

7.1.1 Significance of Anther Culture

The most important use of haploids often advocated is their use in the production of instant homozygous lines, which may be directly used as cultivars or may be used in breeding programmes. Anther culture systems have been developed (Table 2) for several important crop plants, such as apple (Höfer and Flachowsky 2015), barley (Sriskandarajah et al. 2015), broccoli (Qin et al. 2015), citrus (Germanà et al. 2005; Antonietta et al. 2005; Wang et al. 2015), cucumber (Gaazka et al. 2015), loquat (Blasco et al. 2015), maize (Obert and Barnabas 2004; Ambrus et al. 2006; Bernardo 2009), papaya (Gyanchand et al. 2015), pepper (Lantos et al. 2009; Bhattacharya et al. 2016; Keles et al. 2015), rice (Raina and Zapata 1997; Senadhira et al. 2002; Sarao et al. 2003; Grewal et al. 2006b; Zhahg-Yi et al. 2008; Khatun et al. 2012; Usenbekov et al. 2014; Fazaa et al. 2016a, b; Karaoglu et al. 2016; Prachitara et al. 2016), tomato (Segui-Simarro and Nuez 2007; Ahmadi et al. 2015) and wheat (De Buyser et al. 1986; Laurie and Reymondie 1991; García-llamas et al. 2004; Mujeeb-Kazi et al. 2006; Tadesse et al. 2012; Echávarri and Cistue 2016). Several cultivars either are in tests or have been released in rice, wheat, maize, rapeseed, mustard and tomato in China, Canada, Denmark, the USA and France (Jain et al. 1996a, b; Guzmán and Zapata-Arias 2000). Further, the doubled haploid approach is increasingly being used for rapid development of populations for QTL mapping and construction of genetic linkage maps for traits of interest.

7.2 Isolated Microspore/Pollen Culture

Unlike anther culture, microspore/pollen culture eliminates the participation of diploid tissues such as anther wall and connective tissues, in the callus formation and subsequent plant regeneration. Microspore culture (Nitsch and Nitsch 1969) has several advantages over anther culture because microspores are haploid single cells that can readily be genetically manipulated. The two methods of pollen isolation are (a) naturally shed pollen in the culture medium after a pre-culture of anthers and (b) mechanical means by crushing or magnetic stirring. The naturally shed-cultured pollen are known to result in more calli and plantlets than mechanically isolated pollen of rice, barley and tobacco. In case of rice, large microspores (50–58 μm) with thin pink-coloured outer walls produced embryos, whereas the division of small (40 μm) microspores with thick cell walls was not observed (Cho and Zapata 1990). Addition of glutamine, proline at 1 mM concentration and ficoll 10% (w/v) into the culture medium has shown beneficial effects during the isolated microspore culture in the liquid medium. Most of the factors affecting anther culture success also affect the success of pollen culture. Microspore/pollen culture has been well demonstrated for haploid production in *Brassica juncea* (Agarwal and Bhojwani 1993, 2004; Chanana et al. 2005; Agarwal et al. 2006), *Brassica napus* (Huang et al. 1990; Binarova et al. 1993; Chen et al. 1994; Malik et al. 2007), *Hordeum vulgare*

(Hoekstra et al. 1993), oat (Sidhu and Davies 2009), pepper (Lantos et al. 2009) and wheat (Hu and Kasha 1997; Scagliusi 2014).

7.3 *Unpollinated Ovary Culture*

Ovary/ovule culture is an alternative approach to develop haploids when anther/pollen culture fails to produce haploids (Chen et al. 2011; Rakha et al. 2012a, b). In contrast to anther culture, ovary culture is inefficient because there is only one or few embryo sacs per ovary as compared to thousands of microspores per anther. Using this technique, haploids are induced from megaspores, through gynogenesis as in *Allium* (Bah et al. 2012), carrot (Kiekowska et al. 2014), strawberry (Wang et al. 2011a, b), tomato (Zhao et al. 2014), watermelon (Li et al. 2014) and wheat (Getahun et al. 2013). It is well demonstrated in maize by culturing unpollinated ovaries (Tang et al. 2006). Subsequently, the technique has also been extended to other crops, including barley and rice. Genotypic differences have been observed for the development of gynogenic calli. The success rate has been highly genotypically oriented. In rice, *Japonica* types have been found to be more responsive than genotypes of *indica* types. The success of ovary culture is mainly dependent on the developmental stage of the ovary. Success has been reported with ovaries ranging from uninucleate to mature embryo-sac stages. Use of growth regulators to promote gynogenesis and to inhibit the proliferation of somatic tissues has been very critical for ovary culture (Zhou and Yang 1981). Rice ovaries failed to enlarge in the absence of MCPA (2-methyl, 4-chlorophenoxyacetic acid). An increase in MCPA concentration from 0.125 to 8 mg/l favoured ovary swelling. However, the rate of induced embryo sacs has generally been higher than for microspores, and the frequency of green plant regeneration has also been higher than from anther cultures. But ovary culture has been successful only in a few species.

7.4 *Embryo Rescue from Wide Crosses*

Embryo rescuing from wide crosses in some crops serves as an alternative route to haploidy. Moreover, the system is less prone to gametoclonal variation owing to the absence of callus phase. The phenomenon is based on the elimination of a full set of chromosomes of one of the parents during in vitro embryo development.

7.4.1 Bulbosum Method

This method has been named after the use of *Hordeum bulbosum* as pollinator, the chromosomes of which get eliminated during embryo development. This method was first developed for production of haploids in diploid barley by Kasha and Kao

(1970). The haploids are produced from interspecific crosses between *Hordeum vulgare* (female) and *H. bulbosum* (male). Zygote induction is fairly high, and the chromosomes of *H. bulbosum* are rapidly eliminated from the developing embryos. Developing endosperm also aborts after about 2–5 days of growth, which necessitates the rescuing of embryos in order to complete their development. Embryo culture using nutritionally rich medium results in complete haploid plants of *H. vulgare*, and chromosome doubling is induced in the established plants. Barclay (1975) extended this method to hexaploid wheat where haploids of wheat were produced through embryo culture from *Triticum aestivum* cv. Chinese Spring x *H. bulbosum* cross. However, this method is restricted to wheat varieties such as Chinese Spring possessing 'kr' crossability genes that are responsible for the elimination of *H. bulbosum* chromosomes.

7.4.2 Wheat Haploids from Wheat × Maize Crosses

Zenkteler and Nitzsche (1984, 1985) were the first to report microscopic early-stage embryos in crosses between wheat and maize. Subsequently, Laurie and Bennett (1986a, b) demonstrated the presence of both wheat and maize chromosomes in zygotes and found that maize chromosomes were eliminated during initial cell divisions. Endosperm development ceases early or never occurs, and embryos fail to develop to a size that can be readily rescued. Complete wheat haploid plants using wheat × maize system by employing in vitro culture of wheat spikelets, 2 days after pollination, were obtained (Laurie and Reymondie 1991). This method has been successfully extended to durum wheat (O'Donoughue and Bennett 1994) and to field-grown bread wheat by daily injecting 2,4-D (125 ppm) into pollinated tillers (Fig. 3) for 3 days, followed by embryo culturing 15 days after pollination with maize (Bains et al. 1995; Verma et al. 1999).This method is now commercially exploited for transfer of yellow rust resistant to popular wheat varieties. Likewise, another system, wheat x *Imperata cylindrica* – a new chromosome elimination mediated system – has also been developed for production of wheat haploids using cogon grass (*Imperata cylindrical*) as pollen parent (Chaudhary et al. 2002, 2005, 2013*;* Mahato and Chaudhary 2015). The doubled haploid (DH) system has been widely used in wheat improvement, and several varieties of DH origin have been released in Europe, the USA, Canada, Brazil and China.

8 Embryo/Ovule/Ovary Culture for Wide Hybridization

Hybridization following selection has been a routine conventional technique for crop improvement. But the problem arises when the gene(s) of agronomic importance are not available in the related (cross compatible) species. Under such situations, embryo/ovule/ovary culture techniques are being exploited (Table 2) to transfer useful genes from the donor (usually the wild species). During wide

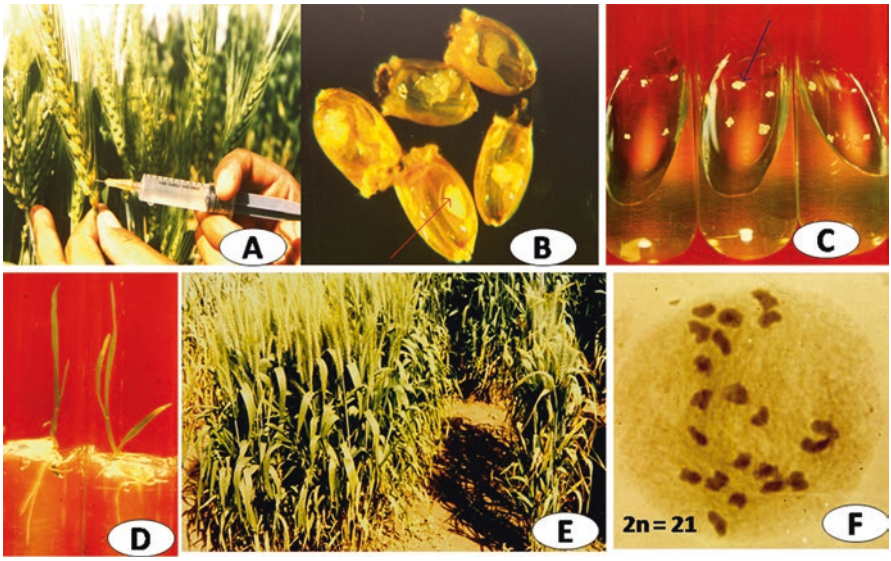

Fig. 3 Production of wheat haploids from wheat × maize crosses using embryo culture. (**a**). 2,4-D injection at the base of emasculated and maize pollinated floret. (**b**). Developing grains showing embryonic tissue. (**c**). Immature embryos cultured in vitro. (**d**). Plant regeneration from cultured embryos. (**e**). Field-grown plants from embryo culture. (**f**). Haploid chromosome number (2n = 21) in the embryo-derived field-grown plants

hybridization, when parents are genetically diverse, endosperm degeneration leads to embryo abortion and the failure of the cross. Thus, following pollination, developing embryos, ovules and even ovaries are aseptically excised and cultured in vitro using suitable culture medium and appropriate incubation conditions. The plantlets thus obtained are transferred to greenhouse and characterized using morphological, cytogenetic, biochemical and molecular approaches. In case of seed sterility, the plantlets are treated with colchicine for production of amphiploids through chromosome doubling. It has been a practical approach (Sharma et al. 1996) to obtain interspecific and intergeneric hybrids in *Arachis* (Moss et al. 1988), banana (Dayarani et al. 2014), *Brassica* (Takahata and Takeda 1990; Momotaz et al. 1998; Gupta et al. 2010; Ding et al. 2011; Mithila and Hall 2012; Chamola et al. 2013; Sun et al. 2014), chickpea (Clarke et al. 2011), citrus (Aleza et al. 2010; Liu et al. 2010a; Kurt and Ulger 2014), cucumber (Plapung et al. 2014), *Cucurbita* (Rakha et al. 2012a, b), grapevine (Wang et al. 2010; Donici and Tardea 2012; Niu et al. 2012; de Menezes et al. 2014), *Helianthus* (Chandler and Beard 1983; Sauca 2010; Sauca and Lazar 2011), *Lens* (Cohen et al. 1984; Suvorova 2014), *Lilium* (Van Tuyl et al. 1991), *Populus* (Calagari et al. 2004; Payamnour et al. 2013), sesamum (Rajeswari et al. 2010), sorghum (Rizal et al. 2014), *Vigna* (Gosal and Bajaj 1983) and wheat (Kaur et al. 2002; Sehgal et al. 2011). Interspecific and intervarietal hybrids have been generated in seedless citrus, seedless grape, mango and papaya using embryo rescue method. Culture of embryos has also been demonstrated in capsicum, hot

pepper, onion, rose and tomato (Sahijram et al. 2013). Ovule culture in peach (Raseira and Einhardt 2010) and grapes (Singh et al. 1991) has been attempted for developing hybrids. These methods have been successfully used to transfer desirable genes from wild relatives into cultivated varieties of several field and vegetable crops.

9 Somatic Hybridization

9.1 Protoplast Culture and Somatic Hybridization

Somatic hybridization is an effective approach to hybridize sexually incompatible species. Complete fusion of nuclei and cytoplasms of somatic cells from both species leads to the formation of somatic hybrid cell and plant. Likewise, the fusion of cytoplasm from two species and nuclear genes from any one leads to the development of a cybrid. The plant cells are surrounded by a thick cell wall that does not allow cells to fuse to get somatic hybrid cell/plant. However, protoplasts can be easily fused and employed in several other experiments aimed at the genetic modification of plants. A protoplast is a naked cell without cell wall, surrounded by plasma membrane and potentially capable of cell wall regeneration, growth and division. The techniques of isolation, culture and regeneration of protoplasts have been established in more than 100 plant species, including major field, vegetable and fruit crops. Protoplast technology basically involves five steps: (1) isolation of protoplasts, (2) fusion of protoplasts, (3) culturing of protoplasts, (4) regeneration of plants and (5) characterization of protoplast-derived plants.

Since the production of the first somatic hybrid between *Nicotiana glauca* and *N. langsdorfii* in 1972, numerous intraspecific, interspecific and intergeneric hybrids have been produced. Somatic hybrids fall into two categories, viz. symmetric and asymmetric (Hinnisdaels et al. 1988). Symmetric hybrids consist of complete sets of chromosomes from both the parents, whereas asymmetric hybrids possess full chromosome complement of only one parent. Earlier, efforts were made to obtain somatic hybrids among closely related and cross-compatible species where somatic hybrids resembled the sexual hybrids. For instance, the somatic hybrid between *Brassica campestris* and *B. oleracea* resembled *Brassica napus*. With refinements in the techniques of protoplast isolation, fusion and culture, people thought to produce novel hybrids by fusing protoplasts from remote species. However, somatic incompatibility has been observed to operate at various levels, and different growth patterns have been observed as hybrid cells underwent only few divisions, fusion products grew successfully as undifferentiated cells and morphogenesis resulted in teratomas (highly abnormal and sterile plants). Asymmetric hybrids carry partial genomes from the donor species. Asymmetrization occurs spontaneously or it can be induced artificially. The final product of protoplast fusion among phylogenetically remote species is usually an asymmetric combination of two genomes, with

parts of one or both the genomes being lost during the in vitro passage. The extent and direction of asymmetrization are largely random, hence unpredictable. The phenomenon of asymmetrization is essential for improving plant regeneration, but it is random and gradual; hence, it may not be potentially desirable at least for the applied objectives. Like nuclear genes, asymmetrization also occurs in cytoplasmic genes. Now, there is growing interest in artificial production of asymmetric cytoplasmic hybrids (cybrids) for a single-step transfer of useful cytoplasmic traits like male sterility, disease resistance and herbicide resistance (Lakshmanan et al. 2013). The protoplasts of the donor species are X-irradiated (9 kr-50 kr) or treated with iodoacetate to inactivate the nuclear genome. In addition, mini-protoplasts (protoplasts lacking nucleus) from donor species are also being increasingly used. In the case of somatic hybrids combining full nuclear genomes, the parental genomes usually remain spatially separated within the nucleus. Effects of spindle-disturbing chemicals like colchicine, chlorosopropyl-N-phenyl-carbamate and aminoprophosmethyl (APH) are being investigated to induce translocations/recombinations.

9.2 Significance in Crop Improvement

Protoplast culture and somatic cell hybridization, involving fusion of protoplasts from different plant species, are important approaches for combining characteristics even from otherwise sexually incompatible species. Furthermore, cybrids (cytoplasmic hybrids) and organelle recombinants, not possible through conventional methods, can also be developed (Hinnisdaels et al. 1988). Therefore, researchers' interest has moved from creation of novel somatic hybrids to the production of cybrids, chromosome transfer and gene introgression (Cui et al. 2009). It is well known that alloplasmic association (nucleus in alien cytoplasm) leads to male sterility as a consequence of interactions between nuclear and mitochondrial elements. Male sterility has been developed by fusing protoplasts of *Nicotiana tabacum* with X-irradiated protoplasts (cytoplasts) of *N. africana*. Using this approach, male sterility has been transferred from *Raphanus sativus* into *Brassica napus*. Moreover, resistance to triazine herbicide has been combined with male sterility by fusing *Brassica napus* protoplasts from a male-sterile line with *B. napus* protoplasts from a triazine-resistant parent. Protoplast-to-plant system developed in basmati rice (Jain et al. 1995; Kaur et al. 1999) and cotton (Sun et al. 2011; Yu et al. 2012) is being exploited for single-step transfer of male sterility from one line to another for the production of hybrid. Likewise, resistance against *Phoma lingam* disease in *Brassica s*pecies (Sjödin and Glimelius 1989) has been transferred to agronomically superior genotypes. A large number of intraspecific, interspecific and intergeneric hybrids have been produced in brassica (Wang et al. 2011a), chrysanthemum (Zhu et al. 2014), citrus (Grosser 2003; Mendes et al. 2001; Grosser et al. 2007; Cai et al. 2010; de Bona et al. 2011; Dambier et al. 2011; Abbate et al. 2012; Soriano et al. 2012; Satpute et al. 2015; Aleza et al. 2016), eggplant (Yu et al. 2013), ginger (Guan et al. 2010), mango (Rezazadeh et al. 2011) and potato (Sonntag et al. 1997; Ahn and

Park 2013; Smyda et al. 2013; Tarwacka et al. 2013; Tiwari et al. 2013; Yu et al. 2013; Nouri-Ellouz et al. 2016; Smyda-Dajmund et al. 2016). Resistances to some diseases like potato leafroll virus, PVX and PVY have been incorporated into *Solanum tuberosum* from *Solanum brevidens* and *Solanum phureja* through protoplast fusion (Rokka et al. 1994; Nouri-Ellouz et al. 2006). Autotetraploidazation during protoplast culturing led to the development of tetraploids (4×) of *Iris fulva* which were sexually hybridized with *Iris laevigata.* Since *Iris fulva* has unique brown flowers, this trait could be very useful for flower colour improvement in *Iris laevigata*, which lacks this colour (Inoue et al. 2006). Furthermore, pollen protoplasts (haploid protoplasts) have also been fused, using pollen protoplasts of *Brassica oleracea* var. *italica* and haploid mesophyll protoplasts of *Brassica rapa* (Liu et al. 2007).

10 In Vitro Production of Secondary Metabolites

Several plant species are known to produce bioactive compounds (secondary metabolites) in vivo or in vitro. These metabolites do not perform vital physiological functions, but some act as potential predators and attract pollinators. Further, these metabolites act as a valuable source of a vast array of chemical compounds, including fragrances, flavours, natural sweeteners and industrial feedstocks. Cultured cells/organs (roots) produce a wide range of secondary products. For producing secondary metabolites in vitro, mainly three approaches have been followed: (i) raising cell suspension cultures in large volumes, (ii) immobilization of plant cells and (iii) growing hairy root cultures in vitro. There are several advantages of in vitro culture systems over the conventional cultivation for the production of secondary metabolites, e.g. (1) independence from various environmental factors, (2) any plant cell can be multiplied to yield specific metabolite and (3) culture of cells may prove suitable where plants are difficult or expensive to grow in the field because of their long life cycles. Since the 1970s, when the possibility of producing useful secondary products in plant cell cultures was first recognized, considerable progress has been made, and a number of plant species have been found to produce secondary products, such as shikonin, diosgenin, caffeine, glutathione, capsaicin and anthraquinone (Havkin-Frenkel et al. 1997; Varindra et al. 1997, 2000; Fischer et al. 1999; Sandhu et al. 2003; Pandhair et al. 2006; Mishra et al. 2011; Amoo et al. 2012; Gayathri and Archana 2012; Wang et al. 2012; Amoo et al. 2013; Gonçalves and Romano 2013; Jassim and Ameen 2014; Sangwan et al. 2014). Large-scale production of such compounds (molecular farming) is increasingly becoming popular with the industry where some physical and chemical conditions for growth and product formation have been optimized. Hairy root cultures, which are considered genetically more stable, are now increasingly being used for the production of secondary metabolites in vitro (Srivastava and Srivastava 2007).

11 Cryopreservation and In Vitro Germplasm Storage

The 'cryopreservation' refers to the storage of plant cells, tissues and organs at the ultralow temperature of liquid nitrogen (−196 °C) in cryocans. The genetic materials in the form of in vitro cultures (cells, tissues or organs) can be preserved as gene banks for long-term storage under suitable conditions. The aim of germplasm conservation is to ensure the ready availability of useful germplasm for scientific research. In seed-propagated crops, seed is extensively used for conservation of germplasm through conventional storage methods. However, in vegetatively propagated species, where conventional techniques are used, it is very difficult to store germplasm on a long-term basis. The conservation of plant parts in vitro has a number of advantages over in vivo conservation, e.g. in vitro techniques allow conservation of plant species that are in danger of becoming extinct. In vitro storage of vegetatively propagated plants can result in great savings in storage space and time, and sterile plants that cannot be reproduced generatively can be maintained in vitro. Complete plants have been successfully regenerated from tissues cryopreserved at −196 °C in liquid nitrogen for several months to years in several crops such as date palm (Bekheet et al. 2010; AlBahrany and AlKhayri 2012), fruit trees (Benelli et al. 2013), garlic (Liu and Cheng 2013), mentha (Guo et al. 2012), orchids (Merritt et al. 2014), pear (Ford et al. 2000; Panis et al. 2001; Gupta 2014; Kovalchuk et al. 2014), teak and other woody species (Hine Gómez et al. 2013; Corredoira et al. 2011). This method is now being practically used at several national and international germplasm banks. Successful cryopreservation of plant shoot tips is dependent upon effective desiccation through osmotic or physical processes. Cryoprotective treatments, which favour survival of small, meristematic and young leaf cells, are most likely to produce high survival rates after exposure to liquid nitrogen. Further, microscopy techniques have been used to determine the extent of cellular damage and plasmolysis that occurs in peppermint (*Mentha piperita*) shoot tips during the process of cryopreservation, using cryoprotectant plant vitrification solution 2 (PVS2) (30% glycerol, 15% dimethyl sulfoxide, 15% ethylene glycol, 0.4 M sucrose) prior to liquid-nitrogen exposure (Volk and Casperson 2007). *Arabidopsis*, which is increasingly being used in genomic studies, can be successfully cryopreserved using either PVS2 or PVS3 as cryoprotectants prior to rapidly cooling shoot tips in liquid nitrogen (LN). PVS3 contains 50% glycerol as compared with PVS2 that contains 30% glycerol. PVS3 was less injurious than PVS2. All of the shoot tips regrew after LN exposure when cryoprotected with PVS3 for 60 min at 22 °C (Towill et al. 2006). The high levels of shoot formation after LN exposure of *Arabidopsis* shoot tips make this a desirable system, in which molecular tools can be used to examine how alterations in biochemical, metabolic and developmental processes affect regrowth after cryoprotective treatments. The method has been used for cryopreservation of blackberry and raspberry (Gupta and Reed 2006), citrus (Ding et al. 2008; Malik et al. 2015), potato (Halmagyi et al. 2004; Zhao et al. 2005; Yoon-Ju et al. 2007), rice (Moukadiri and Cornejo 1996) and wheat (Roux et al. 2016).

12 Genetic Transformation for Developing Transgenic Crop Varieties

During the past 25 years, the combined use of recombinant DNA technology, gene transfer methods and tissue culture techniques has led to the efficient transformation and production of transgenic plants in a wide variety of plants. In fact, transgenesis has emerged as an additional tool to carry out single-gene breeding or transgenic breeding of crops. Unlike conventional breeding, only the cloned gene(s) of agronomic importance is/are being introduced without cotransfer of undesirable genes from the donor. The recipient genotype is least disturbed, which eliminates the need for repeated backcrosses. Above all, the transformation method provides access to a large gene pool, as the useful gene(s) may come from viruses, bacteria, fungi, insects, animals, human beings, unrelated plants and even from chemical synthesis in the laboratory. Various gene transfer methods based on cell, tissue and protoplast culture such as *Agrobacterium*, physicochemical uptake of DNA, liposome encapsulation, electroporation of protoplasts, microinjection, use of laser microbeam, electroporation into tissues/embryos, silicon carbide fibre method and 'particle bombardment' have been developed (Yang and Christou 1994; Gosal and Gosal 2000). Among these, *Agrobacterium* and 'particle gun' methods are being widely used. Using different gene transfer methods, transgenes and strategies, transgenics carrying useful agronomic traits have been developed and released, the word over. Attempts are being made to develop transgenic varieties resistant to abiotic stresses, such as drought (Gosal et al. 2010), low and high temperature, salts and heavy metals, and also to develop transgenic varieties possessing better nutrient-use efficiency and better keeping, nutritional and processing qualities. Genetically modified foods, such as tomato containing high lycopene, tomato with high flavonols as antioxidants, proteinaceous potatoes and edible vaccines, are leading examples of genetically engineered crops. More than 20 transgenic crops including maize, cotton, soybean, potato, tomato, papaya and rice carrying mainly insect resistance, herbicide resistance or both are now being grown over an area of 181.5 million hectares spread in over 28 countries of the world (James 2015).

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Micropropagation and Somatic Embryogenesis in Sugarcane

Ajinder Kaur, Pawan K. Malhotra, Pooja Manchanda, and Satbir Singh Gosal

Abstract Sugarcane propagation through conventional means does not provide sufficient planting material of a variety, particularly desirable in case of newly released varieties to achieve large-scale dissemination; this is attributed to slow rate of seed multiplication by conventional sett planting. On the other hand, micropropagation technique of tissue culture ensures production of disease-free and true-to-type planting material of popular (new as well as old) varieties in an abundant quantity in a short period of time. The cultures of meristematic buds or spindle leaves, collected from healthy plants, are established aseptically under controlled nutritional and environmental conditions in vitro, followed by multiplication of shoots and induction of roots; the plantlets are hardened and supplied to growers. Somatic embryogenesis is the process of embryo formation and development from somatic cells of an explant under in vitro conditions. The somatic cells in culture can follow two pathways for somatic embryogenesis, either direct or indirect. The plants regenerated through direct somatic embryogenesis are often uniform; thus, the pathway finds use in clonal propagation and genetic transformation of sugarcane genotypes. In indirect somatic embryogenesis pathway, first callus is induced from cultured explants under the influence of an auxin (mostly 2, 4-D) which is then regenerated into plants; such plants may exhibit somaclonal variation.

Keywords Micropropagation · Organogenesis · Plant regeneration · Root formation · Shoot multiplication · Somatic embryogenesis · Sugarcane

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1 Micropropagation: An Introduction

Sugarcane (*Saccharum* spp.) is a perennial monocot grass grown in the tropical and subtropical regions of the world (Wekesa et al. 2015) for its sweet stalk and is a commercially important sugar crop worldwide. India ranks second in the world for the production of sugarcane (Salokhe 2016). Besides sugar, sugarcane is a source of useful byproducts such as bagasse, molasses, bioethanol, press mud, biofertilizer and green tops for livestock feed (Jalaja et al. 2008; Salokhe 2016). The crop is conventionally propagated vegetatively through cane cuttings called 'setts or billets' containing one to three buds. The major drawback of conventional propagation is that it cannot provide enough planting material required for large-scale cultivation, due to low rate of seed multiplication (1:6 to 1:8 in a year) leading to slow dissemination of new high-yielding cultivars [2–3 years for small-scale spread, 7–8 years for large-scale spread (Geijskes et al. 2003)]. Further, during conventional propagation, there is build-up of pathogens causing diseases, such as red rot, smut, wilt, grassy shoot, ratoon stunting, yellow leaf and leaf scald, in seed canes over a period of time, leading to the deterioration of newly released varieties. Hence, non-availability of good quality, disease-free planting material is a main problem in sugarcane production and improvement. Besides this, a proper record about the different categories of sugarcane seed, viz. breeder seed, foundation seed and certified seed, is not maintained by the various sugarcane-growing states in India, leading to reduced seed replacement rate in the different states (Directorate of Sugarcane Development, Lucknow, India 2013) that affects the productivity potential of the varieties (Salokhe 2016). It has been reported that the planting material of a sugarcane variety should be replaced every 4 years to maintain its productivity potential and vigour (Sundara 2000; Sawant et al. 2014).

The micropropagation technique helps in the sustained production of quality planting material in an abundant quantity and is thus one of the finest and most successful examples of the commercial application of tissue culture technology. Although the first definition of micropropagation reads as 'any aseptic procedure involving the manipulation of plant organs, tissues or cells that produces a population of plantlets thereby making it possible to bypass conventional sexual or vegetative propagation' (Krikorian 1982), now it is widely defined as clonal propagation of plants from very small plant parts (0.2–10.0 mm) under in vitro conditions in a tissue culture laboratory. The plants raised through micropropagation are usually disease-free, so the vigour of a newly developed variety is maintained and that of an old variety is restored leading to its rejuvenation (Lal et al. 2015). This is due to the fact that healthy meristematic regions are cultured aseptically and shoot number is increased under controlled chemical and physical conditions resulting in quick bulking of the planting material of new varieties and enabling rapid coverage of a region with rejuvenated material, in contrast to 2–3 years using conventional means of propagation. In the field, the micropropagated sugarcane plants have been reported to exhibit better tillering, cane yield, juice content and quality in contrast to conventionally propagated plants (Gosal et al. 1998; Jalaja et al. 2008). The micropropagated plants are largely identical, apart from some rare off-type plants

showing abnormal morphology, which should be uprooted and discarded in the first generation itself (Sreenivasan and Jalaja 1981). The tissue culture-raised sugarcane plants are used as breeder seed, and seed obtained from tissue culture regenerated plants is used as foundation seed (Nerkar 2006; Tawar 2006; Sawant et al. 2014).

2 Micropropagation of Sugarcane: Historical Perspective

The technique of micropropagation was developed by Ball (1946) who obtained complete plants of *Tropaeolum majus* and *Lupinus albus* by in vitro culture of shoot tips and is regarded as the father of micropropagation (Gautheret 1985). Rotor (1949) was the first to develop in vitro method for clonal propagation of orchids by culturing nodal cuttings (bearing buds) of *Phalaenopsis* inflorescences. Micropropagation of sugarcane has been obtained through the culture of shoot tips (Hendre et al. 1975, 1983; Hu and Wang 1983; Lee 1986, 1987; Nagai 1988; Burner and Grisham 1994; Siddiqui et al. 1994; Singh et al. 2001; Ali et al. 2008; Jalaja et al. 2008), apical/axillary buds (Sauvaire and Galzy 1978; Taylor and Duke 1993; Taylor 1994; Chattha et al. 2001) and leaf segments (Gosal et al. 1998; Gill et al. 2006; Lakshmanan et al. 2006; Kaur and Sandhu 2014). The regeneration of adventitious shoots from leaf segments can occur either through direct somatic embryogenesis (Gill et al. 2006; Lakshmanan et al. 2006) or indirect somatic embryogenesis through callus (Ho and Vasil 1983a; Lee 1987; Chowdhury and Vasil 1993; de Alcantara et al. 2014) or organogenesis (Gill et al. 2006; Lakshmanan et al. 2006). Leva et al. (2012) reported that plants regenerated from shoot tips or buds or adventitious shoots possess high genetic fidelity, whereas plants regenerated from callus cultures exhibit somaclonal variation (Lee 1987).

2.1 Micropropagation Through Shoot-Tip Culture

Sugarcane can be successfully freed from pathogens especially viruses using shoot-tip culture, and the plants so produced are identical to the mother plant (Lee 1987). The shoot tips should be excised from actively growing tops (Fig. 1a) of primary shoots or secondary shoots (used if a plant is disease-free) as these possess high regeneration capacity and contain no or very low virus concentration. Micropropagation through shoot-tip culture can be of two types, viz. (a) micropropagation through apical meristem culture and (b) micropropagation through shoot apex culture. The apical meristem in sugarcane is present in the apical bud, observed after removing the leaf whorls from the apical top. The apical meristem measures approximately 0.1 mm in diameter and 0.25–0.30 mm in length (Chawla 2009) and is obtained by cautiously taking off the leaf sheaths from the apical bud. The meristem cells are genetically stable, thus producing plants alike the mother plant (Hendre et al. 1983; Sreenivasan and Jalaja 1992). Coleman (1970) and

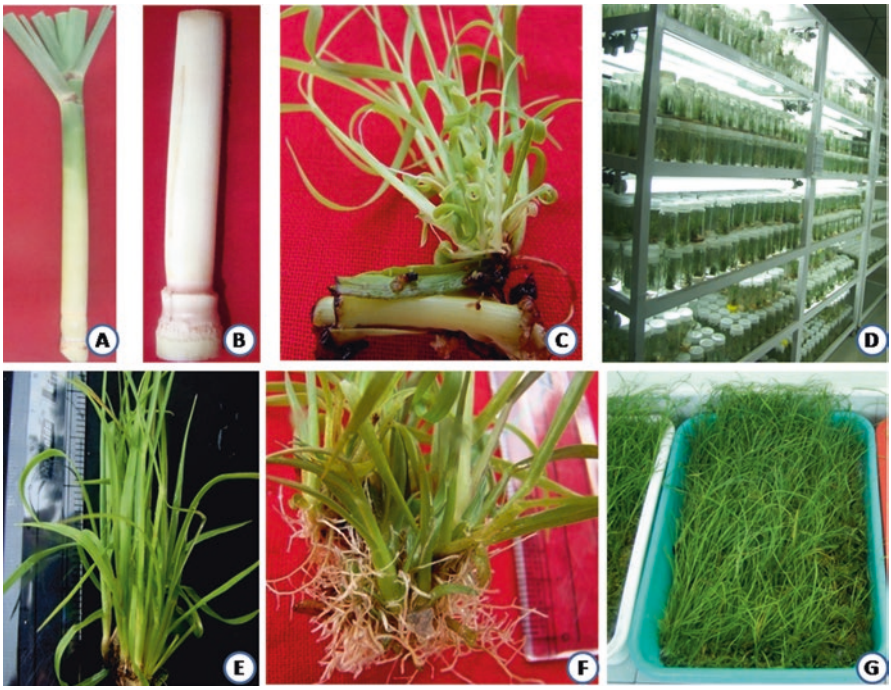

Fig. 1 Micropropagation of sugarcane-laboratory. (**a**) Actively growing top. (**b**) Spindle. (**c**) Establishment of shoot cultures in vitro. (**d**) Rapid shoot multiplication through subculturing. (**e**) Rate of shoot multiplication per cycle. (**f**) Root formation in shoots. (**g**) Hardening of plantlets ex vitro

Hendre et al. (1975) cultured apical meristem to develop sugarcane mosaic virus-free plants. Hendre et al. (1983) optimized an apical meristem culture procedure for regeneration and multiplication of sugarcane mosaic virus-free plants of variety Co 740. A shoot apex measures 0.1 mm in diameter and 0.5 mm in length since it comprises a couple of young leaf primordia also (Chawla 2009) and is easy to excise as compared to apical meristem coupled with high frequency of organogenesis (Jalaja et al. 2008). Sreenivasan and Jalaja (1981) used shoot apex for sugarcane micropropagation, and Jalaja et al. (2008) reported that a large number of plantlets (1,80,000) could be produced from one shoot apex in a time period of 372 days. The shoot tips measuring 2–3 mm in size give higher frequency of shoot regeneration (Dhumle et al. 1994) as compared to shoot apex. Hendre et al. (1983) documented that nearly 2 lakh plants can be regenerated in a period of 6 months from one shoot tip. Anita et al. (2000) reported that a single shoot tip can produce about 15 lakh plants in 6 months through micropropagation. Ali and Afghan (2001) observed that a shoot tip measuring 4 mm in size was best for sugarcane micropropagation producing plants morphologically similar to the mother plants. The Philippine Sugar Research Institute Foundation, Inc., Philippines, has a commercial tissue culture

laboratory that generates 40,000 plantlets per week using shoot tips as explants. MS (Murashige and Skoog 1962) medium supplemented with cytokinins such as BAP and kinetin has been found to be suitable for shoot multiplication from shoot-tip explants (Naritoom et al. 1993; Ali and Afghan 2001; Baksha et al. 2003).

2.2 Micropropagation Through Apical/Axillary Bud Culture

The axillary buds also contain meristems and can be used for initiating tissue culture of sugarcane. The outer scale leaves of dormant axillary buds are first wiped with 70% ethanol before removal; thereafter, the buds are surface sterilized, excised and cultured (Jalaja et al. 2008). Sauvaire and Galzy (1978) produced genetically similar clones in a number of sugarcane varieties through axillary bud culture. Cheema and Hussain (2004) reported sugarcane micropropagation through the use of both apical and axillary buds and observed that the different genotypes responded differently on different MS media compositions with respect to establishment of explants, shoot differentiation, shoot multiplication rate, shoot elongation and root length. Thus, an efficient micropropagation method needs to be worked out for every variety or elite clone. Wagih et al. (1995) developed axillary bud culture technique for sugarcane cultivar NCo 310 on half strength MS medium containing naphthalene acetic acid, NAA (2 mg/l) and malt extract (500 mg/l). Mulleegadoo and Dookun (1999) found that axillary buds showed poor growth response as compared to apical buds. Chattha et al. (2001) and Khan and Rashid (2003) micropropagated sugarcane by culturing apical and axillary buds on MS medium containing cytokinin and gibberellic acid. Biradar et al. (2009) documented that axillary bud is the most appropriate explant for culture initiation on MS medium supplemented with 6-benzylaminopurine, BAP (2 mg/l). Wagih et al. (2009) established a tissue culture method for overcoming contamination from mature axillary buds of sugarcane. Godheja et al. (2014) standardized sugarcane micropropagation protocol from apical buds for large-scale production of shoots. In general, the apical/axillary buds have an edge over apical meristems since these produce shoots on a wide range of media, besides shoot development and multiplication from apical/axillary buds is also faster than apical meristems (Sauvaire and Galzy 1978; Hendre et al. 1983; Taylor 1994).

2.3 Micropropagation Through Spindle Leaf Segment Culture

The young leaf segments of sugarcane have an immense regeneration potential and are widely used for commercial plant production. The primary cultures of young leaf segments exhibit regeneration of multiple shoots in contrast to regeneration of one to two shoots in the primary cultures of apical/axillary buds. Gosal et al. (1998)

developed an effective protocol for large-scale establishment of shoot cultures from spindle explants (0.5–1.0 cm) on semi-solid MS + 0.5 mg/l IAA (indole-3-acetic acid) + 0.5 mg/l BAP + 0.5 mg/l kinetin medium. Geijskes et al. (2003) developed SmartSett™ micropropagation technology for quick clonal propagation of sugarcane (with up to 35-fold shoot multiplication rate) from immature leaf whorl explants, resulting in plant production in 3–4 weeks with up to 95% survival and a production cost of US $ 0.50 per plant. The cane yield (101 t/ha) and cane sugar (15.17%) of SmartSett™ seedlings were parallel to cane yield (104 t/ha) and cane sugar (15–15.5%) of plants raised conventionally from one-eye setts (Mordocco 2006). The SmartSett™ process takes 12–14 weeks for producing plants, making it possible to produce thousands of plants in a year. Gill et al. (2006) developed a distinctive one-step method for direct plant regeneration (without callus interphase) from immature leaf roll segments of sugarcane through culturing on semi-solid MS + NAA (5 mg/l) + Kin (0.5 mg/l) medium, where the shoots regenerated either through organogenesis or direct somatic embryogenesis or both; the method is highly sought-after for mass cloning of newly bred varieties/elite planting material of sugarcane. Lakshmanan et al. (2006) established developmental and hormonal basis of high-frequency in vitro regeneration ($\geq$ 20 shoots per explant) through adventitious shoot production and somatic embryogenesis from transverse thin layer sections (1–2 mm thick) of sugarcane spindle leaf rolls producing a large number of plantlets directly and rapidly. Kaur and Sandhu (2014) developed a cost-effective high-throughput in vitro micropropagation protocol in sugarcane for use in agribusiness industry. The protocol comprising of five stages led to production of complete plants at a high frequency in a period of 157 days with 97% survival rate. The shoots were generated through direct adventitious shoot regeneration without an intervening callus phase and comprised six subculture passages with up to 25-fold shoot multiplication rate. The practicability of the protocol lied in its cheap per plant production cost (US$ 0.13) effected through incorporation of low-cost options. A detailed review on sugarcane micropropagation is presented in Table 1.

2.4 Micropropagation of Sugarcane for Commercial Plant Production

The methodology involves four stages:

2.4.1 Establishment of Aseptic Cultures of Explants In Vitro (Stage 1)

This is a stage in which field-grown plants are brought in the laboratory to grow under in vitro conditions, thus requiring additional concern and skilfulness. It comprises of the following steps:

Table 1 Micropropagation of sugarcane

Plant material/cultivar	Remarks	References
Saccharum sp.	Plant differentiation from callus induced on shoot apices, leaves and inflorescences	Heinz and Mee (1969)
Saccharum sp.	Sugarcane mosaic virus-free plants through apical meristem culture	Coleman (1970), Hendre et al. (1975)
Saccharum sp.	Root and shoot development from callus	Nadar and Heinz (1977)
Saccharum sp.	Micropropagation from callus culture within 9½ months	Barba et al. (1978)
Saccharum sp.	Production of disease-free plants through meristem culture	Leu (1978)
Saccharum sp.	Micropropagation using axillary buds	Sauvaire and Galzy (1978)
Saccharum sp.	Standardized micropropagation using meristem tip culture	Sreenivasan and Jalaja (1981)
IJ76-316	Plant regeneration from embryogenic callus cultures established from primordial leaves and apical meristems	Ahloowalia and Maretzki (1983)
Co 740	Standardized apical meristem culture technique for quick multiplication of mosaic virus-free plants	Hendre et al. (1983)
NA56-79	Mass propagation through shoot-tip culture	Lee (1986)
H75-8776	Micropropagation through apical meristem culture	Nagai (1986)
RB735275	Micropropagation through shoot-tip culture is better than from indirect somatic embryogenesis	Lee (1987)
NC0310, NC0376, Nl2	Compared tissue culture-derived seed cane with conventional seed cane	Bailey and Bechet (1989)
CP 65-357, CP 70-321	Comparison of in vitro propagation efficiency by direct regeneration from leaf tissue and shoot-tip culture	Grisham and Bourg (1989)
Saccharum sp.	Shoot vigour and multiplication rate was maximum at 4% sucrose	Lal (1993)
Breeding lines, viz. 87-588, 87-693, 87-696	Plant regeneration from callus induced from immature inflorescences	Liu (1993)
CP 74-383	Shoot-tip culture induced phenotypic variation	Burner and Grisham (1994)
CoC 671	In vitro regeneration using 2- to 3-mm-long shoot tips	Dhumle et al. (1994)
Saccharum sp.	In vitro clonal propagation on modified MS media	Shukla et al. (1994)
BL4, AEC81-8415	Rapid multiplication of plantlets using apical meristem	Siddiqui et al. (1994)
Saccharum sp.	High-frequency in vitro plant regeneration	Alam et al. (1995)

(continued)

Table 1 (continued)

Plant material/cultivar	Remarks	References
NCo 310	Elimination of Fiji disease virus by thermotherapy and axillary bud culture	Wagih et al. (1995)
Co. Se. 92423, U.P.22	Crop raised from mericlone-derived seed cane gave higher yield than conventionally derived seed cane	Lai and Krishna (1997)
CoJ 64, CoJ 83, CoP 84-211	Protocol for commercial plant production from spindle explants	Gosal et al. (1998)
CoS 91269, CoS 687	Plantlet regeneration from callus cultures raised from young leaf explants	Lal and Singh (1999)
CoLK 8001	Effect of medium composition on in vitro establishment and growth of sugarcane meristem	Patel et al. (1999)
Twelve cultivars	Plantlets (80–100%) derived from meristem culture were free from sugarcane mosaic virus	Visessuwan et al. (1999)
Saccharum sp.	Addition of BAP and coconut water to MS media resulted in multiple shoot formation	Geetha et al. (2000)
Quarantine material	Regeneration of sugarcane yellow leaf virus-free plants through apical meristem culture of infected plants	Chatenet et al. (2001)
H 62-4671, H 65-7052, H 73-6110, H 77-4643, H 87-4094, CP 65-127	Eradication of sugarcane yellow leaf virus from infected canes using meristem tip culture	Fitch et al. (2001)
C-1051-73	Close relationship exists between shoot formation and phenolic excretion during micropropagation	Lorenzo et al. (2001a)
Saccharum sp.	Field performance of temporary immersion bioreactor-derived sugarcane plants	Lorenzo et al. (2001b)
Isd 28	In vitro shoot-tip culture of sugarcane	Baksha et al. (2002)
Isd 31	In vitro clonal propagation from callus induced on leaf sheath explants	Karim et al. (2002a)
Isd 16, Isd 28	Micropropagation from callus culture	Karim et al. (2002b)
SPF-213, CPF-237	Plantlet production from callus induced on young leaves	Niaz and Quraishi (2002)
CC 8527, CC 8215, R 830288, R 831592, R 830395, R 832065, R 840653, R 832276, G 75368, N 27, Q 159, Q 135, Q 155, Q 127, SP 80185, ROC 14, ROC 13, SP 803390, SP 792233	Production of disease-free plants from callus induced on young leaf rolls	Parmessur et al. (2002)
Saccharum sp.	Half MS media supplemented with elevated sucrose is better than full MS medium for rooting	Pawar et al. (2002)

(continued)

Table 1 (continued)

Plant material/cultivar	Remarks	References
RB83-5486, SP80-185	Sugarcane plants derived from meristem cultures exhibit somaclonal variation detected using molecular markers	Zucchi et al. (2002)
Isd 31	Effect of auxin, sucrose and pH on in vitro rooting of shoots induced from callus	Baksha et al. (2003)
Co 740, CoC 671	Elimination of sugarcane mosaic virus using chemotherapy and meristem culture	Balamuralikrishnan et al. (2003)
Q196[A], Q205[A], 85 N1205, 87A1413	Production of SmartSett™ seedlings using immature leaf whorl explants	Geijskes et al. (2003)
CP 70-321, LCP85-384, HoCP 85-845	Yield components were similar for micropropagated plants derived from apical meristem and plants derived from conventional bud propagation	Hoy et al. (2003)
CO-975, CP-77400, HSF-240	Rapid clonal propagation using meristem and axillary buds	Khan and Rashid (2003)
B.O. 91	High-frequency plant regeneration from callus formed on subapical slices and leaf roll explants	Lal (2003)
N32	Production of virus-free plants from immature leaf roll discs	Pillay et al. (2003)
CP-48-103, CP-57-614, CP-69-1062, NCO-310	Micropropagation through shoot-tip and axillary bud culture	Ramin (2003)
HSF-240, SPF-213, SPF-234, CP43/33, CP77/400, CPF237	Micropropagation through apical and axillary buds	Cheema and Hussain (2004)
CoPant 94,211	Efficient micropropagation protocol from shoot-tip explants	Ramanand and Lal (2004)
Erianthus 3854, SES 089	Micropropagation through bud culture	Razi-ud-din Shah et al. (2004)
CP 84-1198	In vitro plant regeneration from sugarcane seed-derived callus	Chengalrayan et al. (2005)
CP59-73, CP63-588, CP80-314, SP71-1081, F160, L62-96, CP70-321, CP57- 614, clone III	Effect of genotype on callus induction and plant regeneration from leaf explants	Gandonou et al. (2005)
CoJ 64, CoJ 83	Tissue culture-raised plants were superior in agro-morphological and quality traits as compared to conventionally raised plants	Lal and Singh (2005)
N14, N27, N30, N32, NCo376	Simultaneous removal of viruses, bacteria from diseased plants and large-scale micropropagation through NOVACANE®	Snyman et al. (2005)
CoJ 83	Direct plant regeneration from young leaf segments	Gill et al. (2006)
NIA-2004, BL4, NIA-98, AEC82-223	Effect of sucrose and growth regulators on micropropagation	Khan et al. (2006)

(continued)

Table 1 (continued)

Plant material/cultivar	Remarks	References
Saccharum sp.	Transverse thin cell layer culture system from young leaf spindle rolls for mass propagation of commercial varieties	Lakshmanan et al. (2006)
S-3807/99	Plantlet regeneration through callus culture induced on young meristematic leaf sheath explants	Ramanand et al. (2006)
Co 89003, Co 91010, Co 96258, Co 97017, CoP 84211, CoP 84212, CoP 90223, CoS 767	Effect of genotype on micropropagation using shoot-tip explants	Singh et al. (2006)
CoJ 64	The tissue culture-raised plants were better in cane height, number of buds, cane yield and sugar recovery as compared to conventionally raised plants	Sood et al. (2006)
CoS 99259	Spacing of 90 cm × 60 cm was most suitable for transplanting tissue-cultured plantlets	Ramanand et al. (2007)
Isd 32	In vitro mass propagation through shoot tips and folded leaves culture	Roy and Kabir (2007)
S. officinarum L.	Micropropagation through axillary buds	Warakagoda et al. (2007)
CP-77400, BL-4	Efficient protocol for large-scale plant production using shoot apical meristem	Ali et al. (2008)
Co 419, Co 740, Co 6907, Co 7219, Co 7717, Co 8014, Co 8021, Co 8122, Co 8208, Co 85007, Co 85019, Co 86010, Co 86032, Co 86249, Co 87025, CoC 671, CoC 86062, CoC 90063, 85 R 186, CoJ 64	Micropropagation through meristem tip culture for seed production	Jalaja et al. (2008)
HSF-240, CP-77- 400, CPF-237	Rapid micropropagation by shoot-tip culture	Khan et al. (2008)
S. officinarum L.	The regenerated plants did not exhibit any variation from each other as well as from the mother plant on the basis of molecular markers	Lal et al. (2008)
HSF-243, HSF-245	Somatic embryogenesis and plant formation in sugarcane	Naz et al. (2008)
N19, N23, N25, N32, N40, N41	Micropropagation of sugarcane via NOVACANE® using apical leaf rolls	Snyman et al. (2008)
SP-241	Efficient regeneration from meristematic explant callus through inclusion of amino acids in regeneration medium	Asad et al. (2009)
Thatta-10	Optimized protocol for callus induction, regeneration and acclimatization	Ather et al. (2009)
Nayana	Rapid in vitro micropropagation through callus culture from young meristem explants	Behera and Sahoo (2009)

(continued)

Table 1 (continued)

Plant material/cultivar	Remarks	References
CoC-671	Direct shoot regeneration without intervening callus phase by using shoot-tip culture	Biradar et al. (2009)
Q117, Q165, Q205	Development of a temporary immersion system (RITA®) for mass production of sugarcane	Mordocco et al. (2009)
CoS 99,259, CoSe 01235	Effect of growth regulators on in vitro shoot multiplication and rooting	Pathak et al. (2009)
NCo 310	Plant regeneration from in vitro decontaminated mature axillary bud culture	Wagih et al. (2009)
S96SP-302, S96SP- 571, S96SP-574, HSF-240, CP72–2086, CP77–400, SPF-213, S97US-183, S97US- 102	Genotype-independent plant regeneration from calli induced from young leaf roll discs	Ali et al. (2010)
CoC 671	Effect of the different auxins and cytokinins on callus induction, shoot regeneration and root regeneration induced from innermost leaf whorls	Gopitha et al. (2010)
Saccharum sp.	Influence of hormonal supplementations on callus induction, somatic embryoid induction and plantlet regeneration	Jahangir et al. (2010)
NCo 376	Elimination of *sugarcane mosaic virus* and sugarcane yellow leaf virus using thermotherapy and apical meristem culture	Ramgareeb et al. (2010)
Saccharum sp.	Mass propagation via shoot-tip culture	Dash et al. (2011)
GT54-9 (C9)	Plant regeneration through direct organogenesis and indirect somatic embryogenesis using young leaf segments	Eldessoky et al. (2011)
Co 94032, CoC 671, Co 86032, SNK 754, SNK 61, SNK 44	Thidiazuron-induced callus formation, somatic embryogenesis and plant regeneration using leaf explants of different varieties	Malabadi et al. (2011)
CoS 96268	Cost reduction in sugarcane micropropagation through direct adventitious regeneration and ex vitro rooting	Pandey et al. (2011)
Saccharum sp. hybrid	Development of sugarcane streak mosaic virus-free plants from infected plants using meristem-tip culture	Reddy and Sreenivasulu (2011)
CoS 96258, CoS 99259	Effect of in vitro environmental conditions on micropropagation	Sengar et al. (2011)
Saccharum sp.	Elimination of sugarcane grassy shoot disease through apical meristem culture	Tiwari et al. (2011)
SPF- 213, HSF-240	Single-step in vitro direct regeneration of plantlets from immature leaf explants	Ali et al. (2012)
S-2003-us-359, S-2006-sp-30, S-2003-us-165	Regeneration through callus induction from innermost young leaf rolls	Ijaz et al. (2012)

(continued)

Table 1 (continued)

Plant material/cultivar	Remarks	References
CoS 8820	Standardized sterilization protocol and established callus culture for in vitro regeneration	Tiwari et al. (2012)
S. officinarum	Efficient in vitro regeneration from bud explants	Zamir et al. (2012)
HSF-242, SPF-213, HSF-240, CP-77-400, CP-43-33	Effect of hormones on shoot multiplication in different genotypes	Abbas et al. (2013)
RB 872552	Micropropagation using light-emitting diodes	da Rocha et al. (2013)
CoS 8820, CoS 767	Enhanced in vitro regeneration from young leaf explants through callogenesis and organogenesis	Tiwari et al. (2013)
CoSe 01235	Micropropagation from callus cultures induced on immature leaf rolls	Yadav and Ahmad (2013)
RB855156, RB72454	Plant regeneration from meristematic leaf segments through somatic embryogenesis	de Alcantara et al. (2014)
Co-86032, Co-94012	Protocol for mass multiplication of plants using shoot tips containing axillary meristems	Godheja et al. (2014), Tarafdar et al. (2014)
CoPb 91	High-throughput in vitro micropropagation from spindle leaf roll segments	Kaur and Sandhu (2014)
Co-91017	Micropropagation through shoot-tip culture	Dinesh et al. (2015)
Saccharum sp.	Constraints and remedies of commercial sugarcane micropropagation	Lal et al. (2015)
PS-881	Elimination of sugarcane mosaic virus and rapid propagation of virus-free plants through the use of apical bud explants	Dewanti et al. (2016)
B4906, Pr1013	Half MS medium plus NAA produced profuse and elongated roots	Getnet et al. (2016)
Co 86032	Sets obtained from micropropagated plantlets resulted in higher seed yields	Salokhe (2016)
N52, N53	Optimized protocol for in vitro rooting and acclimatization	Tesfa et al. (2016)
RB98710	Plants obtained through somatic embryogenesis and grown under light-emitting diode had a high multiplication rate	Ferreira et al. (2017)

Collection of Tops and Excision of Spindles

The actively growing sugarcane tops are excised from 3-month-old field-grown healthy mother plants. The tops (Fig. 1a) can be collected from primary shoots or secondary axillary shoots, and the outer leaf sheaths of tops are removed one after the other to obtain spindles of about 6 cm length (Fig. 1b) by giving cuts at both ends.

Sterilization of Spindles and Preparation of Explants

The spindles (six in number) are rinsed in Teepol detergent solution contained in a culture vessel (500 ml) for 2 min for eliminating wax from leaf sheaths and then washed under running tap water. Thereafter, the surface sterilization of spindles is done in a laminar air flow cabinet by treating with 5% (v/v) sodium hypochlorite solution for 30 min with vigorous manual shaking followed by washing thrice in sterile distilled water. The spindle ends and an outer leaf whorl are removed using sterilized forceps and scalpel by giving gentle cuts in a longitudinal fashion so that the inner leaf layers of spindle are not damaged. The procedure is repeated till tender spindles are obtained, which are transversely cut to obtain 1.0-cm-long spindle leaf segments referred to as explants. The forceps and scalpel are made red hot and cooled in ethanol after culturing each spindle.

Inoculation and Incubation of Spindle Leaf Segments

The explants are cultured on semi-solid MS medium supplemented with growth regulators and incubated under aseptic laboratory conditions at a temperature of 25 ± 2 °C, relative humidity of 60–80% and light intensity of 5000 lux till the appearance of new shoots or callus. Initially, the growth is slow and it takes about 3–4 weeks for new shoots to appear (Fig. 1c). The explants are slightly embedded into the medium during inoculation to ensure accessibility of the nutrients to the explants. In some sugarcane varieties, the cut ends of leaf segments secrete phenols, which are oxidized by plant phenol oxidases; the oxidation products hinder the uptake of nutrients by the explants resulting in their death. Incorporation of activated charcoal in the medium avoids the browning problem due to release of phenols. Otherwise, the explants are shifted on fresh medium after 1 week of culturing. In case browning reoccurs, another shifting on fresh medium is carried out. The lowermost nodal portion of the spindle is retained and cultured on a different medium to obtain shoot formation from apical buds; axillary buds can also be used for shoot regeneration in vitro; however, it is difficult to excise them.

2.4.2 Shoot Multiplication (Stage 2)

This is the actual multiplication stage in which developing shoot cultures from stage 1 are excised, divided and subcultured aseptically in separate culture vessels containing fresh medium for shoot multiplication (Fig. 1d). This step indeed is the game of cytokinins, and there is about ten times shoot multiplication (Fig. 1e) per cycle of 2 weeks depending on the variety. A variety showing high rate of shoot multiplication may be required to be subcultured once a week. Likewise, frequent subculturing is done if medium gets exhausted or there is secretion of phenols or drying of leaves. A total of 26 shoot multiplication cycles can be carried out in 1 year resulting into production of lacs of propagules. However, according to tissue

culture standards of Department of Biotechnology, Government of India, New Delhi, one should not carry out more than seven shoot multiplication cycles in sugarcane to avoid somaclonal variation. Some shoot cultures may show vitrified (ball-like) growth due to intense shoot multiplication; this can be prevented by reducing the number of subculture cycles and lowering concentration of cytokinin in the medium. In this stage, due to high cytokinin level in the culture medium, roots are not formed on the shoots.

2.4.3 Induction of Roots and Hardening of Plantlets In Vitro (Stage 3)

The well-elongated shoots/shoot clumps from stage 2 are taken and cultured on semi-solid or liquid rooting medium to obtain root formation (Fig. 1f). Prior to transfer on rooting medium, the dry leaves are taken off from the shoot clumps, and green clumps are divided so that each clump being transferred contains five to seven shoots. Care is taken not to injure the base of shoots while removing the dry leaves or dividing the shoot clumps. Roots emerge in a period of 10–15 days; once these are 5–10 mm in length, the plantlets are taken out from the culture vessels and washed thoroughly under slow-running tap water to remove the culture medium adhered to the roots. The plantlets are then hardened by keeping on water-moist cotton in plastic trays that are placed in the incubation room with daily change of water for 3–4 days (Fig. 1g).

2.4.4 Transfer of Plantlets to Soil (Stage 4)

The hardened plantlet clumps are separated, and individual plantlets are transferred to potting mixture (field soil + farm yard manure in 3:1 ratio) in polythene bags kept in the greenhouse (Fig. 2a). The humidity is maintained in the greenhouse for the first 2 weeks during which new leaves emerge. The initial growth of plants is facilitated by spraying urea (0.05% w/v) once a week and mixture of $FeSO_4$ and $ZnSO_4$ (0.1% w/v) once in 2 weeks. The plants are kept in the greenhouse for 45 days before delivery to farmers or transfer to field (Fig. 2b). The soil-grown plants branch profusely with thin tillers indicating the residual effect of cytokinin in the shoot multiplication medium. It is for this reason that the tissue-cultured plants are used for seed multiplication and not for commercial use. We have so far developed three micropropagation protocols for mass multiplication of elite planting material (Table 2).

2.5 Transplanting of Micropropagated Plants in the Field

The micropropagated plants are grown at a plant-to-plant distance of 60 cm and row-to-row distance of 75 cm in a disease-free field without taking off the root-soil mass. The irrigation is applied immediately after transplantation, and the subsequent irrigations are applied at regular intervals. There is more than 95% survival of

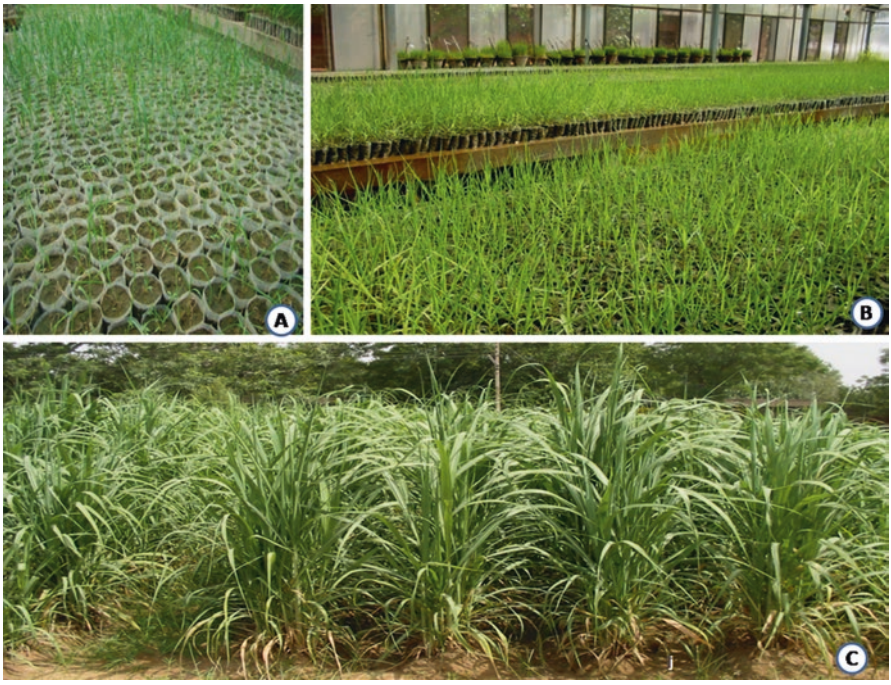

Fig. 2 Micropropagation of sugarcane-greenhouse. (**a**) Transfer of individual plantlets to potting mixture. (**b**) Established plants ready for transfer to field. (**c**) Micropropagated plants 120 days after planting in the field

the hardened plants under field conditions if proper care is taken. The formation of new leaves on the transplanted plants gives an indication of their successful establishment in the field. First dose of nitrogen (20 kg/acre) is applied after 3 weeks of transplantation along with irrigation, and remaining amount of nitrogen is applied in two doses at monthly intervals (Sandhu et al. 2009). Care is taken to rogue out the off-type plants that are observed only during the first year. The plants grow uniformly, possess thin cane stalks and are free from pathogens and insect pests (Fig. 2c). Gosal et al. (1998) observed that the micropropagated plants displayed better cane number and mean cane yield in comparison with the conventionally propagated crop. All intercultural operations are performed on time, and canes obtained from tissue-cultured plantlets are harvested within a period of 10 months.

2.6 Production of Commercial Seed

The stalk canes obtained from the micropropagated plants growing in the field form the breeder seed. The breeder seed canes are cut into two-budded or three-budded setts and sown to obtain foundation seed; the canes are again cut into setts which are

Table 2 Efficient protocols developed for micropropagation of sugarcane in our laboratories

Explant	Media used	% Efficiency	No. of plants formed	References
Spindle leaf explants (0.5–1.0 cm length)	MS salts +0.5 mg/l IAA + 0.5 mg/l BAP + 0.5 mg/l kin +3% (w/v) sucrose +0.8% (w/v) agar for establishment of shoot cultures; liquid MS + 0.5 mg/l BAP + 0.5 mg/l kin +3% (w/v) sucrose for shoot multiplication; liquid half strength MS for shoot elongation; liquid MS + 5 mg/l NAA + 7% (w/v) sucrose for root induction; tap water for hardening in vitro; field soil in polythene bags for transfer of plants	Culture establishment, 75%; root induction, 95%; survival in soil, 90%	More than 15,000 involving one shoot multiplication cycle	Gosal et al. (1998)
Spindle leaf explants (1.0–1.5 cm length)	MS salts +5 mg/l NAA + 0.5 mg/l kin +3% (w/v) sucrose +0.8% (w/v) agar for establishment and proliferation of shoot cultures and root induction; tap water for hardening in vitro; field soil and fly ash (1:1) in polythene bags for transfer of plants	Culture establishment, 74.37–83.12%; survival in soil, 95%	–	Gill et al. (2006)
Spindle leaf explants (1.0 cm length and 0.5 cm diameter)	MS salts +5 mg/l NAA + 0.5 mg/l kin +100 mg/l myo-inositol +3% (w/v) sucrose +0.8% (w/v) agar for culture initiation; MS salts +0.5 mg/l BAP + 0.5 mg/l kin +0.5 mg/l IAA + 100 mg/l myo-inositol +3% (w/v) sucrose +0.8% (w/v) agar for shoot multiplication; liquid MS + 0.5 mg/l BAP + 0.5 mg/l GA_3 + 100 mg/l myo-inositol +3% (w/v) sucrose for shoot multiplication and elongation; liquid MS + 3 mg/l IBA + 3 mg/l NAA + 200 mg/l myo-inositol +3% (w/v) sucrose for root induction; tap water for hardening in vitro; farmyard manure + field soil (1:3) in polythene bags for transfer of plants	Culture establishment, 97%; root induction, 100%; survival during hardening, 94%; survival in soil, 97%	1,01,434 involving five shoot multiplication cycles	Kaur and Sandhu (2014)

then used to raise commercial crop (Sinha 2006). The breeder seed grown on an area of 0.125 acre provides planting material for 1–1.5 acres in the second year and for 15–20 acres in the third year (Kaur et al. 2014). The breeder seed should be changed after every 4 years of propagation (Jalaja et al. 2008).

2.7 Precautions to Be Taken During Micropropagation

The following points should be kept in mind to maintain the quality of micropropagated plants:

- In order to raise genetically pure stock plants, the plant material should be procured from the breeder or the research institute that has developed the variety.
- The tops should be collected only from healthy stock plants.
- The tissue culture laboratory should be accredited by a competent authority to ensure that the infrastructure is according to the guidelines.
- The protocol to be followed for micropropagation should not result in somaclonal variation in the regenerated plants.
- Only well-established plants with a good shoot and root system should be handed over to the end users.
- The tissue-cultured plants should be tested for genetic purity through molecular markers and indexed to be virus-free through ELISA.

2.8 Scenario of Sugarcane Micropropagation

Production of superelite planting material of sugarcane through micropropagation is now done in India, Pakistan, Australia, the Philippines, Bangladesh, Indonesia, Thailand and Sri Lanka. India has more than 100 commercial micropropagation units with a yearly production capacity of 0.2–5.0 million plants (Singh and Shetty 2011). The Department of Biotechnology (DBT), Government of India, has recognized a total of 95 commercial tissue culture units under the National Certification System for Tissue Culture Raised Plants (NCS-TCP) located in states of Assam, Andhra Pradesh, Bihar, Chattisgarh, Gujarat, Haryana, Himachal Pradesh, Karnataka, Maharashtra, Madhya Pradesh and Orissa that are involved in the micropropagation of potato, apple, bamboo, sugarcane, vanilla, banana, black pepper and citrus (www.dbtncstcp.nic.in). DBT along with Biotech Consortium India Limited, New Delhi, has made available handy information on norms and procedure of sugarcane micropropagation (www.dbtncstcp.nic.in). The University of Agricultural Sciences, Bangalore, has been accredited for virus indexing and genetic fidelity of tissue culture-raised plants including sugarcane under NCS-TCP. The micropropagation-based seed production technology is also widely accepted by the farmers (Kaur and Sandhu 2014). In general, it is the responsibility of the regional

sugar mills to establish and popularize this technology among the farmers so that the superior planting material is available in abundant amount contributing to enhanced sugarcane productivity and income of the farmers. The micropropagation-raised crop can be used for multiratooning due to freedom from sett-borne diseases (Jalaja et al. 2008).

2.9 Significance of Micropropagation

The micropropagation of sugarcane offers the following advantages:

- A new variety can be quickly multiplied in large numbers in a short period of time through micropropagation (due to 1:10 shoot multiplication rate per cycle of 2 weeks each), thus making it possible to disseminate the seed material of the new variety to the farmers' fields and diversify the cropping pattern. On the contrary, it takes several years to produce the same number of plants through conventional vegetative propagation method.
- Only a small number of starting plant material (apical tops) is required for generating a large number of clonal plants.
- Disease-free plants can be obtained by culturing the shoot apices/apical buds of diseased plants as the apices/buds are devoid of pathogens. Hence, old varieties/clones can be rejuvenated for improving their yield and quality. Thus, micropropagation helps to increase sugarcane production potential in a sustainable manner.
- The micropropagated plants obtained through axillary bud proliferation approach and direct adventitious shoot regeneration approach have high genetic fidelity and are true to type, i.e. identical to mother plant.
- The in vitro-maintained mother stocks can be quickly proliferated at any time of the year, thus providing year-round nursery for different varieties and exhibiting independence from seasonal and raw material availability constraints.
- The micropropagated plants are easy to transport, in contrast to setts which are voluminous posing difficulty in transportation.
- The field-grown micropropagated plants exhibit better agronomic and biochemical characters such as cane yield, sucrose, juice content and quality as compared to conventionally propagated plants. Gosal et al. (1998) reported that the micropropagated plants revealed up to 22.9% increase in mean cane yield per plot in comparison with conventionally propagated sugarcane using three-budded setts.
- The micropropagated plants grown in the field display better tillering (an after-effect of growth regulators added in the culture medium) and hence provide more seed material (setts) for planting next clonal generation as compared to that provided by conventionally propagated plants. Gosal et al. (1998) reported an increase up to 44.96% in the number of canes per plot of micropropagated plants and 22.9% increase in mean cane yield per plot as compared to conventionally propagated sugarcane using three-budded setts. Further, the setts obtained from

micropropagated plants exhibit high germination frequency since these are disease-free.
- The commercial crop raised from tissue culture-derived seed display synchronous cane maturity, thus enabling harvesting at a particular time.
- There is fast interstate/international exchange of plant material as it is free from pathogens and insects; thus, the period of quarantine is bypassed and time is saved.
- Artificial seeds can be produced by encapsulating the somatic embryos, buds, etc., in a hydrogel for supply to the farmers.
- The elite transgenic events/plants obtained through genetic transformation can be multiplied through micropropagation, thus providing enough clonal plants for precise characterization and transfer to greenhouse.

2.10 Problems Associated with Micropropagation

Various problems are encountered while carrying out in vitro micropropagation, an account of which is given below:

- For carrying out micropropagation, proper infrastructure, costly equipments and skilled workers are required; consequently, the initial investment cost is very high.
- The tissue culture regenerated plants may contain somaclonal variants (Rani and Raina 2000; Zucchi et al. 2002) at a low frequency. The somaclones have abnormal morphology affecting crop uniformity and productivity; thus, in vitro shoot multiplication through callus cultures should be avoided. The axillary branching approach and direct adventitious shoot regeneration from young leaf segments ensures the production of genetically stable and true-to-type plants. The number of shoot multiplication cycles should be kept to a minimum so that the there is no problem of hyperhydration (morphological, physiological and metabolic defects) in the regenerated plants.
- The field-grown micropropagated plants exhibit epigenetic changes such as excessive tillering, slender canes and short internodes due to exogenous application of cytokinin in the culture medium. As a result, the breeder seed so produced is not morphologically identical to the mother plant; however, these changes are temporary, and plants may relapse to the regular phenotype quite easily (Smulders and de Klerk 2011). Lourens and Martin (1987) observed a few transient morphological changes in the sugarcane plants raised from 1.5-month-old callus due to epigenetic effects, which disappeared in the second year of their vegetative propagation.
- Contamination in the cultures appearing soon after culturing due to latent bacteria and fungi could result in considerable losses in a short duration. To avoid systemic infection, explants are treated with an antibiotic solution before culturing, or the culture medium is supplemented with an antibiotic.

- Many genotypes of sugarcane, e.g. CoJ 85, are loaded with polyphenols (e.g. gallic acid, p-coumaric acid, etc.); these are released from the cut ends of explants and oxidized by polyphenol oxidases (Chawla 2009). The oxidation products are responsible for browning of the culture medium and obstructing the uptake of nutrients by the explants resulting in their death. The majority of phenolic excretion occurs after 11–20 days of culturing and prior to shoot formation period of 21–30 days (Lorenzo et al. 2001a). Incorporation of antioxidants such as activated charcoal or ascorbic acid in the medium avoids the browning problem due to release of phenols. Otherwise, the explants are shifted onto fresh medium as and when the phenols are excreted in the medium. In case browning reoccurs, another shifting of explants on fresh medium is carried out. Incubation of the explants under partial light or dark conditions also prevents browning problem as the formation of oxidation products takes place under light conditions (Chawla 2009).
- Per unit plant production cost is high that should be reduced by the incorporation of low-cost options during micropropagation (Kaur and Sandhu 2014). Such low-cost options are incorporated during sugarcane micropropagation in Cuba (Ahloowalia 2004). Automation of micropropagation using bioreactors can also help in lowering per plant production cost by cutting the labour costs; however, asepsis is necessary for making bioreactor-based micropropagation a success. Further, the farmers should be encouraged to buy planting material for a small area and produce their own seed.

In conclusion, micropropagation has proven a practical and victorious technology for quick production of quality and true-to-type sugarcane seed in large numbers. The newly bred varieties, old varieties popular among farmers and diseased varieties – all can be benefitted from micropropagation in terms of quick multiplication, rejuvenation and freedom from pathogen, respectively. The use of micropropagated plantlets by farmers helps in enhancing crop productivity in a sustainable manner. The Government of India has identified micropropagation as the main concern area for further research, development and commercialization.

3 Somatic Embryogenesis

Somatic embryogenesis is defined as the process of embryo initiation and development from somatic cells or plant tissues grown under in vitro conditions, by undergoing some changes in the developmental pathways under the influence of plant growth regulators. During this developmental process, the differentiated cells undergo dedifferentiation by active cell division, reprogram their physiological and metabolic pathways by changing gene expression (Yang and Zhang 2010; Bajpai et al. 2016) and develop into proembryonic cell mass, leading to formation of somatic embryos, their maturation and regeneration (Hussein et al. 2006; Widuri

et al. 2016). Somatic embryo is a bipolar structure that has no vascular connection with the parental tissue and undergoes different stages typical for zygotic embryo development (Sharp et al. 1980). The underlying principle of somatic embryogenesis is cellular totipotency, which demonstrates how somatic cells undergo genetic changes for complete plant development without fertilization (Zimmerman 1993). Besides the occurrence of somatic embryos, new embryos emerge from primary somatic embryos called as secondary somatic embryos that have several applications in plant biotechnology including rapid and mass in vitro clonal propagation of plants, genetic transformation, induction of mutations and cryopreservation (Litz and Gray 1995; Raemakers et al. 1995). The phenomenon of somatic embryogenesis has been reported in some plant species of angiosperms and gymnosperms including sugarcane (Raza et al. 2012; Widuri et al. 2016). In tissue culture, the initiation and development of somatic embryos were first reported by Steward et al. (1958) in *Daucus carota*. Sharp et al. (1980) illustrated two routes to somatic embryogenesis, viz. direct somatic embryogenesis and indirect somatic embryogenesis. In direct somatic embryogenesis, the somatic embryos initiate directly from an explant in the absence of callus phase under the influence of a stimulator or removal of an inhibitor; such explant cells are referred to as 'pre-embryogenic determined cells' (PEDC) as these are committed to embryonic development and need only to be released. PEDC are present in embryonic tissues, e.g. scutellum of cereals. In indirect somatic embryogenesis, explant cells first proliferate to form callus, e.g. leaf of coffee; this is followed by embryo development near the callus surface or inside the callus (Widuri et al. 2016). Such explant cells are called as 'induced embryogenic determined cells' (IEDC). In majority of instances, somatic embryogenesis occurs through indirect method.

3.1 Regulation of Somatic Embryogenesis

For a somatic cell to develop into a complete plant through tissue culture, there is a prerequisite to convert the highly differentiated cell into an undifferentiated stage, i.e. callus, which can be achieved by using different growth regulators in the culture medium. Generally, an auxin is used for callus induction, e.g. in members of Poaceae family to which sugarcane belongs; 2,4-dichlorophenoxyacetic acid (2,4-D) is the most potent auxin used for callus induction. Several studies have reported high concentration of 2,4-D (3–4 mg/l) to be the best for callus initiation in sugarcane, whereas low concentration of 2,4-D or a combination of 2,4-D and cytokinin helps in the development of somatic embryos in the callus (Naz et al. 2008; Jahangir et al. 2010). Ho and Vasil (1983a) used 0.5 mg/l of 2,4-D for obtaining maximum frequency of embryogenic callus; besides nitrogen in ammonia form was observed to be better than the nitrate form for embryogenic callus development. Brisibe et al. (1994) observed that dicamba was better than 2,4-D and NAA in maintaining embryogenic potential of callus cultures for a long period. They reported that

somatic embryogenesis can also be prolonged by supplementing the culture medium with high concentration of maltose (6%) or corn syrup (6–9%). Some other culture medium factors like source of carbohydrate, proline, activated charcoal, abscisic acid (ABA) and antibiotic (cefotaxime) have been found to be related to somatic embryo development in sugarcane. Replacement of sucrose (3%) with maltose (3%) in MS medium resulted in high percentage of embryogenic calli (Gill et al. 2004) with increased callus mass (Kaur and Kapoor 2016); this is because maltose is slowly metabolized in the cultures and remains available to the cells for a longer period as compared to other carbohydrate sources (Orshinsky et al. 1990). Similar observations on increased frequency of embryogenic calli have been noted in separate experiments with proline (560 mg/l) alone (Gill et al. 2004) and proline in combination with maltose (Kaur and Kapoor 2016). Proline provides buffering to the medium and resists any change in the pH of medium during culture. Addition of activated charcoal (2.0 g/l) in the culture medium has also been reported to increase the embryogenic response in callus cultures (Kaur and Kapoor 2016) by reducing the effect of phenolic substances released from the cut ends of explants into the medium. ABA along with some other growth adjuvants like corn syrup and casein hydrolysate affect both embryogenic callus formation and frequency of embryogenesis in long-term callus cultures of sugarcane (Brisibe et al. 1994; Gill et al. 2004). Although ABA is a growth retardant, but its effect on somatic embryogenesis is profound; it was observed that sugarcane callus grew as well as differentiated in presence of ABA in the culture medium (Kaur and Kapoor 2016). Desiccation conditions caused by addition of high concentration of agar (10 g/l) in the culture medium induced compact and whitish embryogenic callus in sugarcane (Himanshu et al. 2000). Liu (1993) observed that cold treatment of sugarcane callus cultures induced from inflorescence explants at 13 °C for a few days improved their embryogenic potential. Age of explant is also an important factor determining the embryogenic potential of callus cultures.

3.2 Effect of Source of Explant on Somatic Embryogenesis

Generally, callus can be produced from any part of the plant. In sugarcane, practically all plant parts produce callus; however, immature leaves (Ho and Vasil 1983a) and young inflorescences produce embryogenic callus (Table 3). Ho and Vasil (1983a) divided callus into two types: (i) hard, compact and embryogenic and (ii) soft, friable, translucent and non-embryogenic. The embryogenic callus induced from leaf sheath was found to be better than that induced from leaf blade. Further, the leaf explants taken from field-grown and in vitro plants showed different percentages of non-embryogenic and embryogenic calli. In the leaf explants prepared from field-grown plants, callus initiation was observed after 7 days of culturing; the non-embryogenic calli turned into 70% non-embryogenic callus and 30% embryogenic callus after 45 days of culturing. In case of leaf explants taken from

Table 3 Selected examples of different morphogenic pathways in sugarcane using different explants

Morphogenic pathway	Explant	Plant regeneration[a]	References
Indirect somatic embryogenesis	Leaf roll	R, T, A	Barba et al. (1978), Lourens and Martin (1987), Burner and Grisham (1994), Nkwanyana et al. (2010)
	Leaf roll	R	Lee (1987)
	Leaf roll	R	Meyer et al. (2009)
	Apical meristem	R	Ramgareeb et al. (2010)
	Shoot apices from in vitro plants	R	Garcia et al. (2007)
	Immature inflorescence	R	Liu (1993), Desai et al. (2004), Snyman et al. (2006)
Direct somatic embryogenesis	Immature inflorescence	R	Desai et al. (2004)
	Leaf roll with pre-emergent inflorescence	R	Snyman et al. (2006)

[a]*R* Plant regeneration, *T* field transfer, *A* phenotypic or genotypic analysis

in vitro-raised plants, origin of callus was observed after 10 days of culturing, and 60-day-old cultures consisted of 60% non-embryogenic callus and 40% embryogenic callus. Further, the cell suspension cultures were established from the two types of callus cultures. The embryogenic cells were small (30 μm diameter), rich in cytoplasm, actively dividing, had prominent nucleus and conspicuous starch grains. In contrast, the non-embryogenic cells were large, elongated (70 μm × 30 μm) and vacuolated with scanty cytoplasm and a few starch grains.

3.3 Evidence for Somatic Embryogenesis and Proteins Produced from Callus

The experimental evidence for occurrence of somatic embryogenesis in sugarcane was first given by Ahloowalia and Maretzki (1983) and Ho and Vasil (1983a, 1983b). Ho and Vasil (1983a) reported somatic embryogenesis in callus cultures, cell suspension cultures and protoplasts. Histologically, callus induction in sugarcane can be divided into two types, viz. mucilaginous and nodular (de Alcantara et al. 2014). The mucilaginous callus is soft and watery, does not undergo embryogenesis or develop into shoots and turns necrotic. On the other hand, cells of whitish nodular callus have dense cytoplasm with high nuclear to cytoplasmic ratio revealing their meristematic nature. These cells develop into somatic embryos followed by regeneration of shoots. Different stages of somatic embryogenesis in sugarcane include the development of proembryos, which divide and turn into globular-stage embryos

enclosed by protoderm. The globular embryos either separate themselves from the rest of the callus cells or remain attached to the surface of callus by suspensor. The globular embryos develop into scutellum by formation of scutellar node. The scutellar cells are rich in cytoplasm, irregular in shape, and divide further to form coleoptiles, apical shoot and root vascular system. The embryogenic callus of sugarcane has more protein than non-embryogenic callus; this could be related to their cytological characters. The cells of embryogenic calli are small and actively dividing and have dense cytoplasm with prominent nuclei, so their metabolic activity is higher than the non-embryogenic cells; consequently, these have higher level of proteins, mRNAs and other cytoplasmic components as compared to non-embryogenic cells. The electrophoretic analysis of protein pattern showed polypeptides of 38–44 kDa and another polypeptide of 23 kDa in embryogenic calli; however, these polypeptides were absent, and another polypeptide of 35 kD was present in non-embryogenic calli. Thus, sugarcane has a complex protein pattern and could be due to the effect of 2,4-D on embryogenesis (Oropeza et al. 2001). In another study, activated charcoal was used in the culture medium at different concentrations (0.0, 0.75, 1.5 and 2.0 g/l). The data on somatic embryo development and protein expression was recorded at 0 and 21 days using shotgun proteomic analyses. Use of activated charcoal (1.5 g/l) resulted in faster maturation of somatic embryos in embryogenic callus and showed no effect on non-embryogenic callus. Embryogenic callus showed 65 exclusive proteins on day 0 and 14 exclusive proteins on maturation at day 21. Non-embryogenic callus expressed 23 exclusive proteins on day 0 and 10 exclusive proteins after 21 days (Heringer et al. 2015).

3.4 Somatic Embryogenesis from Cell Suspension Cultures

Cell suspension cultures are prepared by growing callus cultures in liquid medium under constant agitation and maintained by selection of embryogenic cells; this is accomplished by allowing the cell suspension cultures to settle for some time, removing the supernatant followed by replacing it with fresh culture medium every 7 days. The cell suspension growth is measured by the number of embryogenic cells, packed cell volume and cell fresh and dry weight (Falco et al. 1996). Ho and Vasil (1983b) were the first to report plant regeneration from embryogenic cell suspension cultures of sugarcane. The small, actively dividing cells of suspension cultures became embryogenic upon supplementing the medium with high concentration of sucrose (6–10%). The embryogenic potential of the suspension cultures was maintained by addition of coconut water and casein hydrolysate in the culture medium. Such suspension cultures produced embryogenic callus when plated on solidified MS medium supplemented with coconut water, casein hydrolysate and 2,4-D; the callus subsequently showed plant regeneration when cultured on a suitable medium. Falco et al. (1996) observed plant regeneration only in juvenile cell suspension cultures and in culture media containing low or no 2,4-D.

3.5 Somatic Embryogenesis and Cryopreservation

Cryopreservation is the storage and preservation of cells, tissues and organs by immersion in liquid nitrogen (−196 °C). It is used for the conservation of plant genetic resources and as a technique for preserving the regeneration capacity of somatic embryos in cell suspension or callus cultures. In vitro conservation has several advantages over in vivo conservation, e.g. it saves space and time and can be used to conserve endangered species, sterile plants and in vitro material produced using tissue culture. Slow cooling (0.5–1 °C/min) up to 40 °C followed by immersion of samples in liquid nitrogen is usually employed for conservation of cell suspensions and embryogenic calli (Kartha and Engelmann 1994). During cryopreservation, there is no cell division, so the chances of somaclonal variation are limited, which otherwise increase with culture duration. With the establishment of tissue culture in sugarcane, considerable efforts were made to develop cryopreservation protocols for germplasm conservation using various explants like apices of in vitro plantlets, cell suspensions and calli (Eksomtramage et al. 1992). During initial attempts, the success was limited to obtaining viable cultures but not in regenerating plants from the frozen suspension and callus cultures (Finkle and Ulrich 1982; Ulrich et al. 1984). Gnanapragasam and Vasil (1990) developed a procedure for successful regeneration of sugarcane plants with 92% efficiency from cryopreserved cells and reported that enrichment of cell suspension culture with embryogenic cells is important to get high plant regeneration during re-culture. Martinez-Montero et al. (1998) compared three vitrification techniques to preserve somatic embryos of sugarcane and concluded that there is a need to develop different cryopreservation protocols for different genotypes.

3.6 Somatic Embryogenesis and Synthetic Seeds

Somatic embryos encapsulated in hydrated or desiccated gel coating which helps in their protection and germination are called synthetic or artificial seeds. Besides somatic embryos, shoot tips and axillary buds can also be used for encapsulation. There is a great scope for the production of synthetic seeds in asexually propagated crops like sugarcane, where these can be used for large-scale production of better quality plants (Aamir et al. 2013), and thus synthetic seed technology is an advancement in micropropagation (Naik and Chand 2006). In sugarcane, the survival rate of synthetic seeds is controlled by the concentration of gelling matrix components, i.e. sodium alginate and calcium chloride, which affects the type, colour and quality of encapsulation. Treatment of somatic embryos with 3% sodium alginate and 100 mM calcium chloride for 15 min resulted in the formation of isodiametric and compact beads. Decrease in treatment time (10 min or less) formed fragile and soft beads, whereas increase in treatment time (20 min or more) resulted in hard and whitish

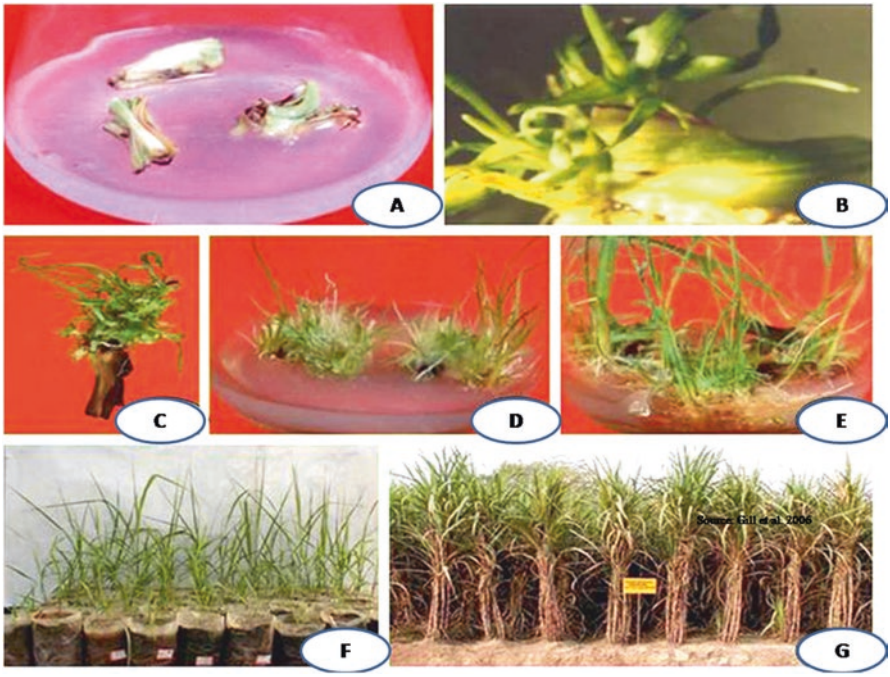

Fig. 3 Plant regeneration in sugarcane via direct somatic embryogenesis or organogenesis. (**a**) Cultured young leaf segments showing swelling and unwhorling during first week of incubation. (**b**, **c**). Stereo micrographs of direct shoot regeneration from cut ends of cultured segments. (**d**) Profuse direct shoot regeneration. (**e**) Profuse rooting from base of shoots during fifth week of incubation. (**f**) Tissue culture-derived hardened plantlets transferred to soil in the greenhouse. (**g**) Micropropagated plants in the field exhibiting normal growth and better tillering

coloured beads, which hampered germination potential of somatic embryos (Aamir et al. 2013). Concerted efforts are required to develop synthetic seeds in sugarcane for propagation.

3.7 Somatic Embryogenesis and Plant Regeneration

Direct plant regeneration in sugarcane from spindle leaf culture occurs either through direct somatic embryogenesis or organogenesis (Gill et al. 2006; Lakshmanan 2006) and has decreased chance of somaclonal variation (Chowdhury and Vasil 1993) due to minimum culture duration and callus formation. The direct somatic embryogenesis or organogenesis system (Fig. 3) has become routine protocol for mass propagation (Kaur and Sandhu 2014) and genetic transformation (Taparia et al. 2012). Nickell (1964) was the first to report plant regeneration from callus cultures of sugarcane. The plants regenerated through callus formation

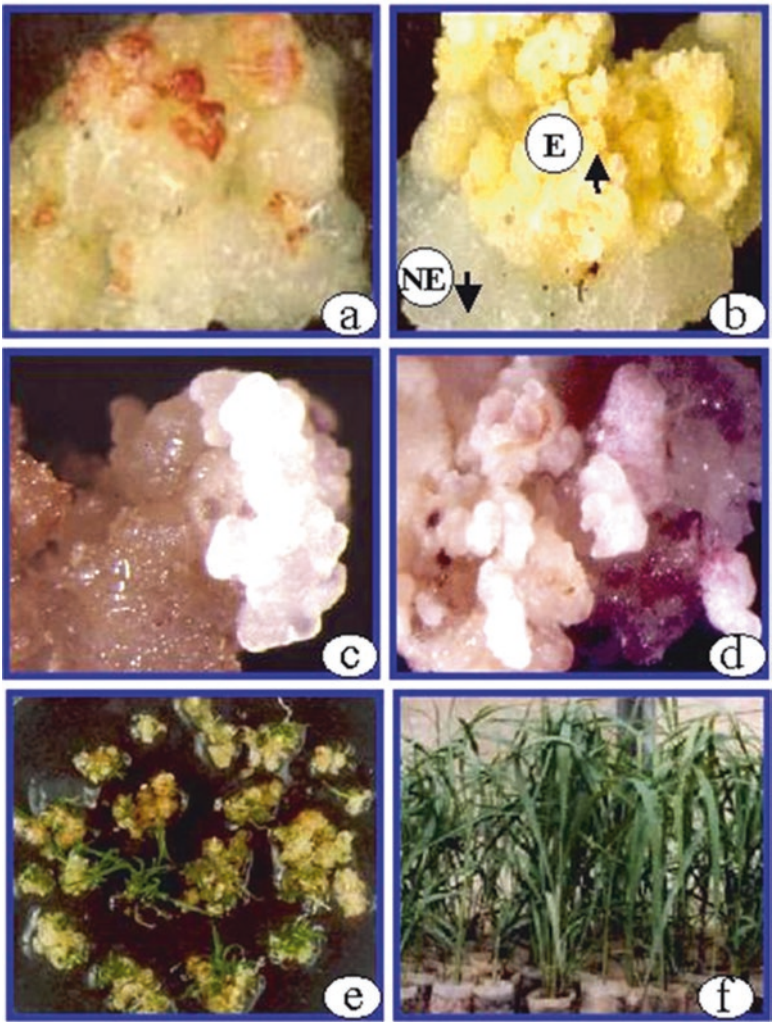

Fig. 4 Plant regeneration in sugarcane through indirect somatic embryogenesis. (**a**) Callus showing initiation of somatic embryos (anthocyanin pigmentation is a marker for somatic embryogenesis). (**b**–**d**) Nodular embryogenic callus (E) developing on non-embryogenic callus (NE). (**e**) Shoot regeneration from embryogenic callus. (**f**) Embryogenic callus-derived plants growing in the greenhouse

(Fig. 4) possess significant somaclonal variation (Vickers et al. 2005). However, the callus cells can be screened in vitro for various biotic and abiotic stresses to develop new resistant cultivars of sugarcane (Geijskes et al. 2003).

Thus, somatic embryogenesis reveals the totipotency of plant cells (Raghavan 1997) and has significant importance in combining efficient multiplication of desirable genotypes with genetic modification (Sharp et al. 1980) for sugarcane improvement.

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Production of Superelite Planting Material Through In Vitro Culturing in Banana

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Abstract Different procedures of tissue culture for clonal propagation of banana on large scale are required to meet an ever-increasing demand of genetically uniform plants of banana. As banana is a vegetatively propagated plant, therefore, propagation of banana directly through suckers or corms attached at the base of field-grown plants (conventional means) increases the chances of transmission of diseases from one generation to the next. Therefore, this chapter reviews the literature as well as describes the approaches involved in the applications of plant tissue culture techniques for mass multiplication of banana through micropropagation, somatic embryogenesis and induction of variations through gamma irradiation. As banana is a parthenocarpic and triploid fruit, therefore, micropropagation technology ensures true-to-type, rapid and disease-free multiplication of plants for quick bulking up of new clones/varieties and rejuvenation of old clones/varieties. It also serves ideal for the gene pool conservation. Therefore, an efficient protocol of micropropagation for banana cv. Grand Naine and direct somatic embryogenesis for banana cvs. Grand Naine and Robusta was standardized. In vegetatively propagated crops, the induction of genetic variation through gamma irradiation under in vitro conditions and the multiplication of desirable mutants/variants provide an alternative means to widen the scope of genetic as well as epigenetic variations. Thus, for in vitro mutagenesis, shoot tips were used as an explant, and mutation was induced using gamma irradiation. The results of this study can be used for the mass propagation of banana and for obtaining banana plants with improved quality and resistance to various biotic and abiotic stresses.

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1 Introduction

Banana (*Musa* spp., $2n = 22–44$) is the largest evergreen, perennial and monocotyledonous fruit plant. It is an important staple commodity for many developing countries, together with wheat, rice or corn. It is also a dessert fruit for millions and is a rich source of vitamin B_6, vitamin C, potassium, iron and easily digestible carbohydrates with 95 calories per 100 g fruit. Millions of people in the world depend for their livelihood on dessert and cooking (plantain) bananas. According to the newsletter published by International Network for the Improvement of Banana and Plantain (INIBAP 2003) in the USA, a natural extract from the pseudostem of banana patented under the name 'CellQuest' is being sold as a dietary supplement and as an aid to prevent cancer. The banana cultivars are mostly diploid, triploid and tetraploid because the edible banana plants belong to section *Eumusa* with basic haploid chromosome number of 11 (Robinson 1996). Cultivated banana are mostly triploid ($2n = 3x = 33$), and most of the edible varieties are polyploid (Stover and Simmonds 1987). The edible bananas are derived from two wild species of banana: *Musa acuminata* (A genome) and *Musa balbisiana* (B genome) (UNCST 2007). Due to its triploid nature, banana exhibits a marked degree of sterility. Further, it is a parthenocarpic (seedless) fruit; therefore, it does not produce viable seeds and is propagated vegetatively through suckers. Conventional vegetative propagation has disadvantages like low multiplication rate (5–10 in number/year depending upon variety), lack of uniform plant size and transmission of pathogens from one generation to the next generation which results in varietal decline. The application of micropropagation and somatic embryogenesis techniques for mass multiplication of banana are effective and superior alternatives to conventional method of banana propagation through suckers. The tissue culture techniques enable the plantlets to be regenerated from normal and genetically modified cells and tissues in an efficient way under sterile conditions.

Micropropagation of plants is one of the best and most successful examples of commercial application of tissue culture technology (Gosal et al. 2010). In this method, over a million plants can be produced from a small or even a microscopic piece of plant tissue within a year (Mantell et al. 1985). Moreover, the shoot multiplication cycle is very small (2–6 weeks), each cycle resulting in an exponential increase in the number of shoots, and plant multiplication can be continued throughout the year irrespective of the seasonal constraints (Razdan 1993). There are numerous reports on micropropagation of banana (Table 1). The first report of banana tissue culture came in the early 1970s from Taiwan by Ma and Shii (1972) who developed in vitro adventitious buds from cultured shoot apices following decapitation of Cavendish banana (*Musa acuminata* L.). These were shortly followed by the production of virus-free banana plants through meristem culture combined with thermotherapy by Berg and Bustamante (1974). Due to the presence of high level of cytokinin on pre-existing leaf-opposed buds, it was observed

Table 1 Micropropagation and somatic embryogenesis in banana

Technique	Genotype/variety	Explant	Composition of medium	Remarks	Reference
Micropropagation	*Musa* spp. cv. 'Gonja-Horn plantain' (AAB) and three East African highland cooking banana (AAA) cultivars, viz. 'Kibuzi', 'Mbwazirume' and Namwezi	Shoot tips	Eriksson (ER), Gamborg's B-S (B-S), MS + 4.5 mgl^{-1} BAP with or without NAA	Shoot proliferation occurred better on ER medium due to higher ammoniacal nitrogen content in ER	Talengera et al. (1994)
	Musa spp., viz. Dwarf Cavendish (DC), Amruthapani (AMP), Telia chakkerakeli (TCK) and Robusta	Shoot apices	MS + BAP (6 mgl^{-1}) + IAA (2 mgl^{-1}) + adenine sulphate (200 mgl^{-1})	DC showed better response of shoot-bud initiation (93.7%) as compared to TCK (58.8%) and Robusta (93.7%)	Sudhavani and Reddy (1999)
	Musa spp. cv. 'Grand Naine'	Shoot tip	Modified MS (20 µM + 40 mgl^{-1} cysteine + 1 mgl^{-1} thiamine + 4% (w/v) sugar + 0.2% (w/v) Gelrite, used sunlight instead of artificial light, replaced sucrose and gelrite of tissue culture grade with locally available commercial sugar and a starch or gelrite medium	Production cost reduced to 90% and white and light brown sugars with low electrical conductivity gave best results	Kodym and Zapata-Arias (2001)
	Musa spp. cv. 'Sabri'	Shoot tip	MS + BAP (5 mgl^{-1})	During in vitro shoot multiplication of banana, 3.11 shoots/explant were obtained	Rahman et al. (2002)
	Musa spp. cv. 'Basrai'	Meristematic stem cuttings	MS + 10.0 µM BA +15.0 µM IAA + phytagel (1–4 gl^{-1})	Micropropagation efficiency was increased to 60% on phytagel-solidified medium	Haq and Dahot (2007)

(continued)

Table 1 (continued)

Technique	Genotype/variety	Explant	Composition of medium	Remarks	Reference
	Musa acuminata cv. 'Sannachenkadali', AA, and 'red banana', AAA	Male inflorescence apices	MS with different combinations of BAP	Multiple shoots (maximum in number) were obtained on MS + 8.9 µM BAP in Sannachenkadali as compared with the triploid cv. red banana (AAA) in which, multiple shoots were obtained on MS + 17.8 µM BAP	Resmi and Nair (2007)
	Musa spp. cv. 'Amritasagar'	Meristem from 4-month-old banana suckers	MS + BAP (0, 3, 4 and 5 mgl^{-1}) + NAA (0.0, 1.0, 1.5, 2.0, and 2.5 mgl^{-1})	4.58 shoots per explant were obtained on MS + 4 mgl^{-1} BAP + 1.5 mgl^{-1} NAA	Shiragi et al. (2008)
	Musa acuminata cv. 'Dwarf Cavendish' and 'Valery'	Suckers	MS + sucrose (30 gl^{-1}) + N-phenyl-N-1, 2, 3-thiadiazol 5-yl urea (0.5 mgl^{-1}) + IAA (2 mgl^{-1})	After 45 days, the multiplication rate was evaluated to be 10.85–12.88 in both the cultivars	Farahani et al. (2008)
	Musa spp. cvs. 'Berangan Intan', 'Berangan' (AAA), 'Rastali', 'Nangka' (AAB) and 'Baka Baling' (ABB)	Shoot tips	MS + BAP (0.0, 11.1, 22.2, 33.3 and 44.4 µM) + TDZ (0.0, 0.5, 2.0, 5.0, and 7.5 µM)	Lower occurrence of abnormal shoot formation was observed on MS + BAP (22.2 µM) + TDZ (2 µM)	Shirani et al. (2009)
	Musa spp. cvs. 'Berangan', 'Rastali', 'Nangka' and 'Abu' belonging to three (AAA, AAB and ABB)	Male inflorescences	MS + different combinations and concentrations of TDZ, BAP, Kin, 2-ip and zeatin (0–1 mgl^{-1})	TDZ at 0.4, 0.6 and 0.8 mgl^{-1}, appeared to be optimum for shoot induction in 'Berangan (AAA)', 'Rastali (AAB)' and 'Nangka (AAB)' and 'Abu (ABB)'	Darvari et al. (2010)

	Musa acuminata cv. 'Berangan'	Shoot apices derived from suckers	MS + BAP (11, 22, and 33 μM)	Frequency of bud formation from the base of the explant increased about four times in medium supplemented with BAP (33 μM)	Jafari et al. (2011)
	Musa spp. cvs. 'Virupakshi' and 'Sirumalai'	Male floral meristems	MS supplemented with different combinations and concentrations of BAP, NAA, GA_3 and additional supplements such as coconut water	MS + BAP (5 mgl^{-1}) + coconut water (15%) was the best medium for shoot initiation and multiplication	Mahadev et al. (2011)
	Musa balbisiana cv. 'Kluai Hin'	Apical and lateral buds	MS + BAP (22 μM) + coconut water (15% – v/v)	MS + BAP (44 μM) gave maximum number of shoots and roots were induced on basal MS medium	Kanchanapoom and Promsom (2012)
	Musa spp. cv. 'Mas", 'Nangka', 'Berangan' and 'Awak'	Suckers	MS + BAP (1–14 mgl^{-1}) + IAA (0.2 mgl^{-1})	Each shoot tip produced maximum of five shoots on MS + BAP at 5 mgl^{-1} (Pisang Nangka), BAP at 6 mgl^{-1} (Pisang Mas and Pisang Berangan) and BAP at 7 mgl^{-1} (Pisang Awak)	Sipen and Davey (2012)
	Musa beccarii	Suckers and male buds inflorescence	MS + BAP (0.014 mgl^{-1})	Multiple shoot formation occurred prolifically from suckers as well as male flower buds	Rashid et al. (2012)

(continued)

Table 1 (continued)

Technique	Genotype/variety	Explant	Composition of medium	Remarks	Reference
	Musa laterita	Suckers	MS supplemented with different concentrations and combinations of IAA and BAP	MS + BAP (3 mgl^{-1}) + IAA (1 mgl^{-1}) was found to be the best medium for in vitro multiplication	Dayarani et al. (2014)
	Musa spp. cvs. 'Poovan' and 'Monthan'	Suckers	Mixed nutrients + sucrose (30 gl^{-1}) + agar (8 gl^{-1}) instead of MS (basal) (served as control)	61.4% reduction in the cost of the nutrients used in the media preparation	Dhanalakshmi and Stephan (2014)
	Musa acuminata (AAA) cv. Vaibalhla	Immature male flowers	MS + BAP (2 mgl^{-1}) + NAA (0.5 mgl^{-1}) + Kin (2 mgl^{-1})	Highest number of white budlike structures per explant (4.30), highest percentage (77.77) and number (3.51) of shoot formation from each explant	Hrahsel et al. (2014)
	Musa sapientum cvs. 'Anupam' and 'Chini Champa'	Meristematic part of suckers	MS + different combinations and concentrations of BAP + Kin + IAA + IBA	MS + BAP (4.5 mgl^{-1}) and MS + BAP (2 mgl^{-1}) sprouted buds for 'Chini Champa' and 'Anupam', respectively. Roots were induced on MS + IBA (1 mgl^{-1})	Mahdi et al. (2014)
	Musa spp. cv. Mzuzu	Suckers	MS + BAP (5 mgl^{-1}) + ascorbic acid (0, 50, 100, and 200 mgl^{-1})	Lethal browning was reduced, and survival rate of explants was increased due to ascorbic acid (acting as an antioxidant)	Ngomuo et al. (2014)
	Musa spp. cv. 'Grand Naine'	Suckers	MS + BAP (4 mgl^{-1}) + IAA (2 mgl^{-1})	Maximum establishment of cultures obtained in lesser time with minimum contamination	Ahmed et al. (2014)

	Musa cv. 'Udhayam' (ABB)	Shoot tips	MS + BAP + additional supplements	Shoot establishment was obtained on MS + ascorbic acid (50 mgl^{-1}) + myo-inositol (100 mgl^{-1}) + BAP (4 mgl^{-1}), while maximum no. of shoots was obtained in 7.5 days on MS + BAP (3 mgl^{-1}) + coconut water (5%)	Saraswathi et al. (2014)
	Mixed diploid banana (*Musa* spp., AB) cv. 'Elakki Bale'	In vitro propagules carrying single and double buds	MS supplemented with different concentrations and combinations of four different cytokinins: BAP, 2-iP, mT (8.89, 17.78 and 35.56 μM) and TDZ (1.0, 2.0, and 4.0 μM)	Higher shoot multiplication was observed on MS + TDZ. Higher levels of BAP + IAA improved shoot multiplication	Bohra et al. (2014)
	Musa spp. cv. Malbhog	Shoot tips	MS + IAA (0.57 μM) + BAP (17.74 μM)	Maximum differentiation of shoots occurred (92.05%)	Suman and Kumar (2015)
	Musa spp. cvs. GCTCV-215 (AAA), 'Yangambi' Yangambi Km-5 (AAA) and FHIA-23 (AAAA)	Suckers	MS + BAP (0, 2, 4, 6 mg l^{-1}) and IAA (0.5 and 1.0 mgl^{-1})	MS + BAP (4 mgl^{-1}) + IAA (0.5 mgl^{-1}) resulted in multiple shoot formation in case of GCTCV-215 and Yangambi, while MS + BAP (4 mgl^{-1}) gave better results in FHIA 23	Qamar et al. (2015)
	Musa spp. cvs. 'Amritasagar' and 'Sabri'	Shoot tips	MS + BAP (0.0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0 mgl^{-1}) + IBA (0.0, 1.0, 2.0, 3.0, 4.0, and 5.0 mg l^{-1})	MS + BAP (0.5 mgl^{-1}) showed highest number of single shoot formation	Ferdousa et al. (2015)

(continued)

Table 1 (continued)

Technique	Genotype/variety	Explant	Composition of medium	Remarks	Reference
	Musa acuminata L. (red banana)	Suckers	MS + BAP (1, 2, 3 and 4 mgl^{-1}) + Kin (1, 2, 3 and 4 mgl^{-1}) + IAA (1 mgl^{-1}) for shoot proliferation	Highest number of shoots/explant (8.80) was observed on MS + BAP (4 mgl^{-1}) + IAA (1 mgl^{-1})	Uzaribara et al. (2015)
	Musa spp. cv. 'Virupakshi' (hill banana)	Apical meristem from sucker	MS + BAP (10 mgl^{-1}) + IAA (1 mgl^{-1})	Higher number of shoots (134.3 shoots/explant) within 168 days (24 weeks)	Karule et al. (2016)
	Musa spp. cvs. 'Rasthali' (AAB, Silk), 'Grand Naine' (AAA, Cavendish) and 'Udhayam' (ABB, Pisang Awak)	Shoot meristems (5.0 cm^3 size) from 'sword sucker'	MS + BAP (3 mgl^{-1}) + IAA (1 mgl^{-1}) + different gelling agents (sago, isabgol and agar singly as well as in combinations)	Medium supplemented with sago + isabgol produced the maximum number of shoots (ten per explant) in 'Udhayam' and 'Rasthali', while sago alone produced the maximum number of shoots (six per explant) in 'grand Naine'	Saraswathi et al. (2016)
Somatic embryogenesis	*Musa* spp. cvs. 'Grande Naine', 'Yangambi', 'French Plantain', 'Mysore', 'Silk' and 'Pelipita'	Immature male flowers	MS + biotin (4.09 µM) + 2,4-D (18.1 µM) + IAA (5.7 µM) + NAA (5.37 µM) + sucrose (0.87 µM) + agarose (7 gl^{-1})	After 4–5 months, somatic embryos were isolated and were placed on MS + BAP (0.22 µM) + IAA (1.14 M + sucrose (0.87 M) + gelrite (2 gl^{-1})	Escalant et al. (1994)
	Musa spp. cv. 'Grande Naine'	Immature male flower buds	MS + Biotin (4.1 µM) + 2,4-D (18 µM) + IAA (5.7 µM) + NAA (5.4 µM) + sucrose (87 mM) + agarose (7 gl-1)	Yellow and compact calli were formed, which were used for preparing embryogenic cell suspensions in liquid medium	Cote et al. (1996)

	Musa spp., Cavendish subgroup AAA cv. Grand Naine	Immature male flowers	MS + biotin (4.1 µM) + 2,4-D (18 µM) + IAA (5.7 µM) + NAA (5.4 µM) + sucrose (87 mM) + agarose (7 gl^{-1})	Friable calli were used for preparing cell suspension cultures. After subculturing, these were used for protoplast isolation, which were used for the development of microcalli which developed into somatic embryos at high frequency	Assani et al. (2001)
	Banana hybrid cultivar FHIA-18 (AAAB)	Immature male flowers	MS medium + biotin (1 mgl^{-1}) + glutamine (100 mgl^{-1}) + malt extract (100 mgl^{-1}) + 2,4-D (1 mgl^{-1}) + sucrose (45 mgl^{-1})	Embryogenic tissue was obtained from the explants. Bioreactors were used for secondary multiplication of somatic embryos. 89.3% plant regeneration was obtained on temporary immersion systems	Kosky et al. (2002)
	Musa spp. AAB cv. Dwarf Brazilian	Immature male flower buds	MS + biotin (1 mgl^{-1}) + malt extract (100 mgl^{-1}) + glutamine (100 mgl^{-1}) + 2,4-D (4 mgl^{-1}) + IAA (1 mgl^{-1}) + NAA (1 mgl^{-1}) + sucrose (30 gl^{-1}) + phytagel (2.6 gl^{-1})	Primary somatic embryos were obtained and were transferred on medium supplemented with casein hydrolysate (200 mgl^{-1}) + proline (2 mgl^{-1}). Further transfer on MS + coconut water (10%) resulted in the development of secondary somatic embryos which regenerated into plantlets	Khalil et al. (2002)

(continued)

Table 1 (continued)

Technique	Genotype/variety	Explant	Composition of medium	Remarks	Reference
	Musa spp. cv. CIEN-BTA-03 AAAA	Shoot apices (8 mm × 1.5 mm) derived from in vitro regenerated plantlets	½ MS + 2,4-D (1 mgl^{-1}) + zeatin (0.219 mgl^{-1}) + phytagel (1.8 gl^{-1})	Embryogenic calli were obtained, from which embryogenic cell suspensions were prepared, which lead to the formation of secondary somatic embryos	Ramírez-Villalobos and de Garcia (2008)
	Musa spp. cv. Spambia (AAB)	Scalps from proliferating shoots (meristems)	MS + 2,4-D (4.5 mgl^{-1}) + zeatin (1 mgl^{-1})	Friable calli were obtained which were used for preparing ECS on liquid medium of same composition. Maturation of somatic embryos was increased to 2.6-fold on medium supplemented with 2.5 µM ABA	Sholi et al. (2009)
	Diploid *Musa acuminata* cvs. Matti (AA), Sannachenkadali (AA), Chingan (AB) and Njalipoovan (AB)	Bracts associated with male flowers	MS + TDZ (0.045–9.00 µM) + picloram (0.041–10.35 µM) + 2,4-D (0.045–13.5 µM) + sucrose (30 gl^{-1}) + agar (0.7 gl^{-1})	Production of somatic embryos increased with the addition of biotin (8.18, 12.27 and 16.37 µM) in cv. Matti, Chingan and Njalipoovan, respectively, whereas the glutamine (6.84 µM) increased somatic embryogenesis in cv. Sannachenkadali	Divakaran and Nair (2011)

	Musa spp. (AA) cv. 'Calcutta 4'	Two explants were used, i.e. meristematic domes of axillary-sprouted buds and scalps from cauliflower-like Meristems	½ MS + sucrose (30 gl^{-1}) + ascorbic acid (10 mgl^{-1}) + 2,4-D (1 mgl^{-1}) + zeatin (0.22 mgl^{-1} + malt extract (100 mgl^{-1}) + glutamine (100 mgl^{-1}) + biotin (1 mgl^{-1}) + casein hydrolysate (200 mgl^{-1}) + proline (4 mgl^{-1}) + Gelrite (2 gl^{-1})	Meristematic domes of axillary-sprouted buds and showed best embryogenic response (8%) on culture medium	Torres et al. (2012)
	Musa acuminata cv. 'Burmannica'	Mature and immature zygotic embryos	MS supplemented with different concentrations and combinations of 2,4-D, picloram and IAA	MS + 2,4-D (4.5 µM) was found to be the best medium for embryogenic callus proliferation. Calli formed from immature embryos showed higher regeneration (76.6%) as compared to mature embryos (50.6%)	Uma et al. (2012)
	Musa spp. cv. 'Anupam'	Leaf and stem segments	MS+ 2,4-D (3 mgl^{-1}) + NAA (0.5 mgl^{-1})	Callus formation occurred; rhizogenesis in callus and morphogenic responses could play a role for physiological studies in vitro and served to develop a suitable composition of medium for callus culture	Pervin et al. (2013)
	Musa spp. cv. Rajeli (AAB)	Immature male flower buds	MS + 2,4-D (4 mgl^{-1}) + IAA (1 mgl^{-1}) + NAA (1 mgl^{-1}) + D-biotin (100 mgl^{-1}) + malt extract (100 mgl^{-1}) + glutamine (100 mgl^{-1}) and 30 gl^{-1} sucrose	Liquid medium was used for embryogenic cell suspension cultures. Differentiation of embryos occurred and the embryos developed into plantlets on MS + BAP (0.5 mgl^{-1})	Kulkarni and Bapat (2013)

(continued)

Table 1 (continued)

Technique	Genotype/variety	Explant	Composition of medium	Remarks	Reference
	Musa acuminata cv. Berangan (AAA)	Immature male flower clusters	MS supplemented with different concentrations and combinations of proline and glutamine (0, 100, 200, 300, and 400 mgl^{-1})	Glutamine (400 mgl^{-1}) resulted in optimum somatic embryogenesis with enhanced regeneration frequency	Husin et al. (2014)
	Musa acuminata cv. Grand Naine (AAA)	Shoot tips derived from in vitro shoot cultures (4-week-old)	MS + picloram (0–8.28 µM) + BAP (0.22–4.44 µM)	100% embryo induction was observed on MS + picloram (4.14 µM) + BAP (0.22 µM), plant regeneration from somatic embryos occurred on MS + NAA (0.53–2.68 µM) + BAP (2.22–44.39 µM) + TDZ (4.54 µM) + glutamine (200 mgl^{-1})	Remakanthan et al. (2014)
	Musa acuminata cv. Berangan	Male inflorescences	MS + myo-inositol (100 mgl^{-1}) + biotin (1 mgl^{-1}) + IAA (1 mgl^{-1}) + NAA (1 mgl^{-1}) + 2,4-D (4 mgl^{-1}) + ascorbic acid (10 mgl^{-1}) + sucrose (30 gl^{-1}) + Gelrite (2 gl^{-1})	Friable embryogenic callus was used for preparation of cell suspension cultures. When 51 balloon-type bubble column bioreactors were used, the yield of cell suspension cultures increased from 165% to 210%	Chin et al. (2014)
	Musa acuminata cv. 'Berangan'	Male buds	MS + 2,4-D (23 µM)	Embryogenic cell suspensions were established on MS + biotin (4.1 µM) + ascorbic acid (10 mgl^{-1}) + glutamine (100 mgl^{-1}) + malt extract (100 mgl^{-1}) + 2,4-D (4.5 µM) + zeatin (1.0 µM). 69% of the embryos developed shoots	Jafari et al. (2015)

	Musa spp. cv. 'Grand Naine' (AAA) and 'tropical' (AAAB)	Immature male flowers	MS + IAA (1 mgl^{-1}), 2,4-D (4 mgl^{-1}) + NAA (1 mgl^{-1}) + various concentrations of glutamine (0, 50, 100, 150, and 200 mgl^{-1})	Glutamine-free medium was used for induction of somatic embryos in cv. Grand Naine as compared to cv. 'Tropical' in which somatic embryos were formed in glutamine-supplemented medium. Plant regeneration from somatic embryos occurred on MS + BAP (0.8mgl^{-1}) + IAA (0.7 mgl^{-1}) medium	Morais-Lino et al. (2015)

MS Murashige and Skoog, *BAP* 6-benzylaminopurine, *IAA* indole-3-acetic acid, *Kin* kinetin, *NAA* naphthalene acetic acid, *IBA* indole-3-butyric acid, GA_3 gibberellic acid, *2-iP* 2-isopentenyladenine, *mT* meta-topolin, *TDZ* thidiazuron, *2,4-D* 2,4-dichlorophenoxy acetic acid

that the multiplication rate of shoot-tip explant was higher as compared to floral apices (Cronauer-Mitra and Krikorian 1987). Bhasker et al. (1993) studied three different types of explants, i.e. shoot tip, floral apex and eye bud. They observed that the establishment of shoot tip and eye bud occurred earlier as compared to floral apex explants. Somatic embryogenesis refers to the remarkable ability of non-zygotic plant cells (including haploid cells) to develop through characteristic embryological stages into an embryo capable of developing into a mature plant (Rose 2004). It is one of the potential methods for in vitro regeneration of the plantlets and serves as a key prerequisite for the genetic transformation of plants with desirable traits. Somatic embryogenesis is of two types, direct and indirect somatic embryogenesis. Direct somatic embryogenesis is defined as the formation of somatic embryos directly from the explants without an intervening callus phase, while indirect somatic embryogenesis involves the formation of intermediate callus phase which further regenerates into plantlets. The first observation of in vitro somatic embryogenesis was made in *Daucus carota* (Steward et al. 1958; Reinert 1959). Plant regeneration through somatic embryogenesis in banana has been reported by several studies (Table 1).

Genetic variation may already be present in nature, may be obtained after several years of selection or may be produced through hybridization (for seed-propagated crops). Also, the nature and extent of genetic variability are important for the development of new varieties with beneficial agronomic traits. Mutagenic agents such as radiation and certain chemicals can be used to induce mutations at a higher frequency and generate genetic variation from which the desired mutants may be selected. Generally, banana is cultivated in southern states of India. Recently, Punjab Agricultural University, Ludhiana, has recommended banana cv. 'Grand Naine' for commercial cultivation in central districts of Punjab because frost susceptibility of the commercial banana cultivars of southern India is a major limitation in commercial adoption of banana in Punjab. To realize the real potential of banana in the state, there is demand for the production of frost-tolerant and short-duration varieties, which will result in higher productivity as well as increases net returns to the farmers (Chahil et al. 2008). Induction of genetic variation could be the starting point for the improvement of banana through gamma irradiation under in vitro conditions. Stover and Buddenhagen (1986) proposed mutation techniques as a tool for banana improvement. Also, being a seedless and vegetatively propagated plant, it is very difficult to induce more genetic variability in banana through conventional methods like cross hybridization. In vitro culturing has allowed production of pathogen-free plants and has widened the genetic variability in the existing cultivars as a result of somaclonal variation (Daniels and Smith 1992; Walther et al. 1997). Somaclonal variation, i.e. phenotypical and cytogenetical modification, originates from the exposure of dedifferentiated tissues to tissue culture cycles. However, the degree and expression of variation depend on the genotype and the genetic basis of the species. Variability in a given banana population may be used to isolate variant or mutant lines with interesting characteristics such as resistance to biotic and abiotic stresses.

2 Banana Propagation Through Tissue Culture Technologies

Plant tissue culture refers to in vitro cultivation of plants, seeds and plant parts (tissues, organs, embryos, single cells, protoplasts, etc.) on nutrient media under closely controlled and aseptic conditions. The notion of totipotency given by Gottlieb Haberlandt (1902), which refers to the capability of a plant cell to give rise to a complete plant under suitable experimental conditions, has major implications to manipulate plant cells for rapid multiplication of plants. Therefore, one of the commercially exploited components of tissue culture has been the rapid clonal propagation through micropropagation and somatic embryogenesis of selected genotypes of banana. The scope and application of tissue culture techniques for large-scale propagation of banana and induction of in vitro mutations through gamma irradiation are highlighted as follows.

2.1 Micropropagation of Banana

Mass multiplication of banana through tissue culture approaches has great potential for obtaining true-to-type and disease-free planting material at desired planting time. Within a year, it is possible to achieve 0.2 million plantlets through tissue culture from a single explants (Bhatt and Bhatt 2003). The ability to generate many plantlets from a single explant and consequently to produce quantities of propagules of selected genotypes has played a key role in the banana improvement programmes (Vuylsteke et al. 1997). The micropropagation process has seen tremendous expansion all over the world both in terms of number of production units as well as in the number of plants after its commercial exploitation during 1970s with the micropropagation of orchids. Under Indian Punjab conditions, the recent success of banana cultivation is due to the exploitation of fast-growing habit of banana plantlets generated under in vitro conditions through tissue culture procedures. In our laboratory, the micropropagation of banana was carried out through adventitious shoot bud initiation and multiplication as well as through axillary shoot proliferation (Manchanda et al. 2012a).

2.1.1 Plant Material

Young and healthy suckers from 2-year-old banana plants growing in the field were used as explants for studies on micropropagation. Many studies have been conducted using various other explants such as floral apices (Alloufa et al. 2002), inflorescence apices (Resmi and Nair 2007), male inflorescences (Darvari et al. 2010), etc., for micropropagation of banana.

2.1.2 Culture Medium

The basal nutrient media used in the study were MS (Murashige and Skoog 1962) and Banana Multiplication Medium (Product code: PT079, HiMedia Laboratories Pvt. Limited). The composition of banana multiplication medium (BMM) is basically the same as that of MS as it contains all inorganic salts and vitamins present in MS medium. In addition to MS constituents, BMM contains L-ascorbic acid (20 mgl^{-1}), glucose (2 mgl^{-1}), IAA (0.175 mgl^{-1}), BAP (4.5 mgl^{-1}) and agar (8 gl^{-1}).

2.1.3 Explant Preparation and Surface Sterilization

Shoot tips were prepared from the suckers by removing the older leaves and extraneous corm tissue with a stainless steel knife. Shoot tips containing several sheathing leaf bases enclosing the axillary buds and subjacent corm tissue and measuring about 2.5–3.5 cm in length were isolated. The sterilization of the explant was carried out with carbendazim (Bavistan™) and mercuric chloride ($HgCl_2$) (Manchanda et al. 2012b). The effectiveness of mercuric chloride for sterilization of explants collected from field-grown suckers of banana has been reported by many workers (Vani and Reddy 1996; Ranjan et al. 2001; Josekutty et al. 2003; Poudyal et al. 2004; Arshad et al. 2005; Martin et al. 2006; Shiragi et al. 2008; Dangi et al. 2009; Kacar et al. 2010). To prevent blackening of the suckers in media by the release of phenolic compounds, antioxidant cocktail comprising ascorbic acid and citric acid was used. The antioxidative effect of ascorbic acid and citric acid cocktail was studied. The control suckers with no antioxidative treatment showed a very low percentage of healthy suckers. The per cent healthy explants were maximum (55.88%) for 50 mgl^{-1} ascorbic acid and citric acid tried. With increasing concentrations of antioxidants, no significant reduction in blackening of media and explant was observed. 20–25 ml of antioxidant cocktail was used for washing. Novak et al. (1990) also reported that the treatment of rhizomatous part of shoot tip with 40 mgl^{-1} ascorbic acid and 50 mgl^{-1} citric acid for about 10 min reduced the blackening of cultured explants. Ascorbic acid and citric acid (1.0:1.5) were used for controlling browning in case of banana (Cavendish) by Kalimuthu et al. (2007).

2.1.4 Shoot Multiplication, Subculturing and Root Proliferation

Suckers (Fig. 1a) cultured on BMM medium supplemented with 6 mgl^{-1} of BAP (establishment medium) showed swelling after few days of culturing (Fig. 1b) and started shoot initiation (Fig. 1c). During shoot multiplication, there was nearly ten times multiplication per cycle of 2 weeks throughout the year. Therefore, 26 cycles could be completed in a year, generating thousands of propagules. In this step, because of relatively high levels of cytokinins in the culture medium, the roots do not develop; hence, multiplication is generally in the form of shoot buds or shoots (Gosal and Kang 2012). The cytokinins that have been commonly used for

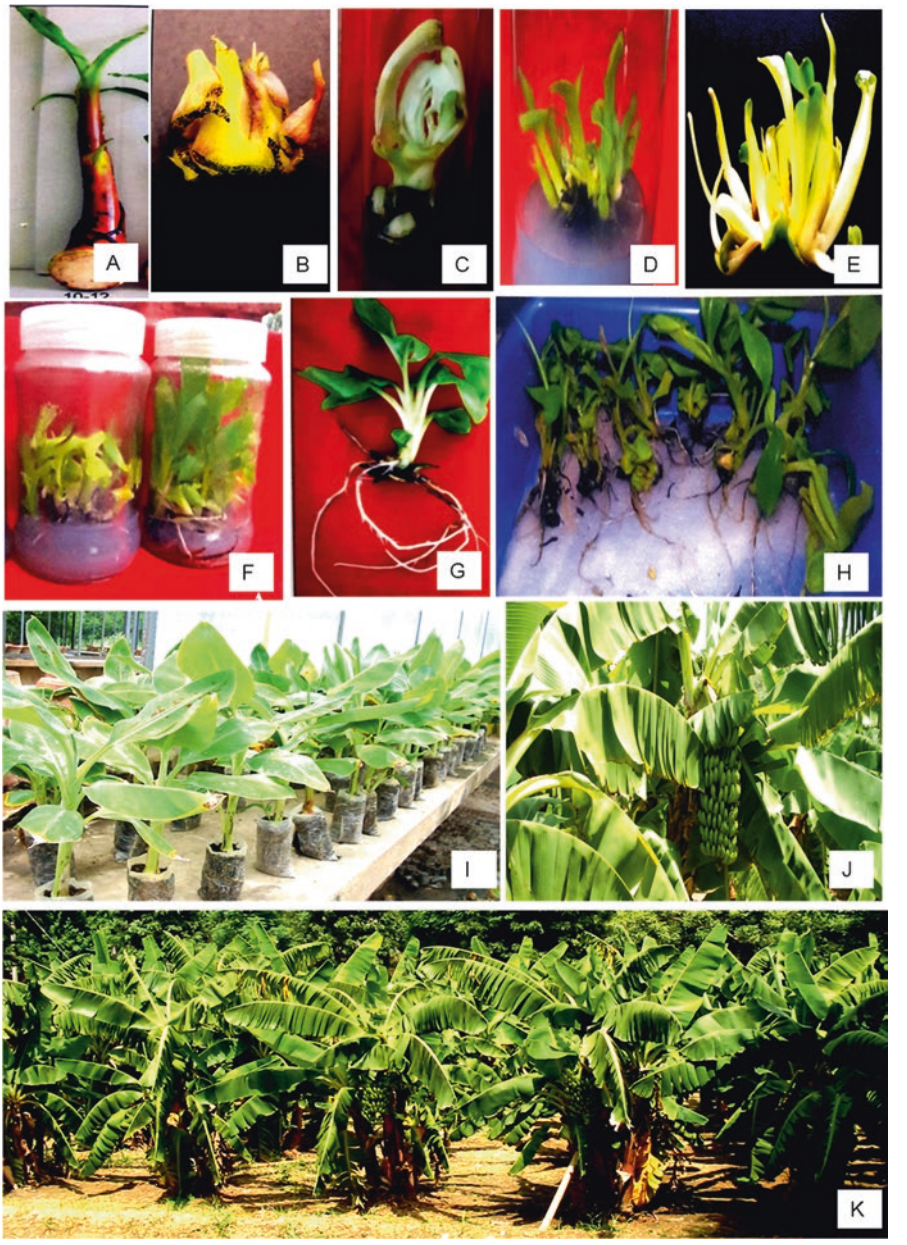

Fig. 1 Figure showing various stages of micropropagation of banana cv. Grand Naine (**a**) Lateral shoot (sucker) taken from the field (explant) (**b**) Shoot tip showing swelling after few days of culturing (**c**) Shoot initiation after 20 days of culturing (**d**, **e**) Shoot proliferation on MS + BAP (3 mgl^{-1}) (**f**) Root induction on BMM medium (**g**) Plantlets with well-developed root and shoot system (**h**) Hardening of shoots on cotton soaked in water (**i**) Plantlets transferred to polythene bags in glasshouse (**j**) Micropropagated banana plant showing fruiting in the field (**k**) In vitro banana regenerants growing in the field

micropropagation of banana in earlier studies are BAP (Cronauer-Mitra and Krikorian 1984; Vuylsteke 1989), isopentenyladenine (2ip) (Doreswamy et al. 1983), kinetin (Cronauer-Mitra and Krikorian 1984) and zeatin (Vuylsteke and De Langhe 1985). With the help of sterilized forceps, the swollen explants on the establishment medium were taken out aseptically, and dead leaves were removed. For subculturing, the explant was cultured upright in a culture jar containing MS medium for shoot multiplication. The addition of antibiotic cefotaxime at 500 mgl^{-1} into the medium controlled the systemic bacterial contamination as well as increased the shoot multiplication rate and elongation with respect to number of shoots, shoot length and plantlet fresh weight (Manchanda et al. 2011). The bud sprouts from established explants were excised and cultured as shoot cluster (four to five shoots per cluster) on MS medium supplemented with cytokinin combinations for further shoot multiplication. Single shoots on subculturing failed to produce multiple shoots. A propagule of four to five shoots showed the best multiplication as compared to smaller propagules. The maximum number of shoots per explant was highest on MS medium supplemented with BAP (3 mgl^{-1}) (Fig. 1d, e). Longitudinally splitting of explants and the microshoots also increased the multiplication rate during subculture. Okole and Schulz (1996) also observed the enhancement effect with the splitting of active shoot tip in the previous reports in banana. Rooting was not a problem in cv. Grand Naine used in the present study. The in vitro multiplied shoots showed root induction simultaneously with shoots on BMM (Fig. 1f).

2.1.5 Acclimatization of In Vitro Plantlets

Tissue culture-generated plants are generally fragile and tender. Therefore, most of them die when transferred directly to field conditions. Therefore, for increasing and improving their survival rate, plantlets with well-developed root and shoot system (Fig. 1g) are hardened. In vitro hardening of the plantlets was carried out on cotton soaked in water for 4–5 days in the incubation room (Fig. 1h). This process acclimatized the plantlets for their subsequent transfer to the soil. The success of micropropagation process depends upon the per cent survival of plantlets. After hardening, the plantlets were transferred to different potting mixtures which exhibited varied survival rate. The maximum mean per cent plantlet survival (98.76) was observed with a combination of garden soil + vermiculite, 1:1 potting mixture which was at par with the combination of garden soil + vermiculite, 2:1 potting mixture (98.27%). All the potting mixtures used in the study exhibited more than 85 per cent plantlet survival. Garden soil when used alone without sand and vermiculite resulted in minimum per cent plantlet survival of 85.7 and when mixed with vermiculite (1:1) resulted in the maximum per cent plantlet survival of 98.76 (Fig. 1i). Due to the presence of vermiculite which has high water-holding capacity, the mortality rate of plants was low when compared to vermiculite-containing mix. When the plants shifted from in vitro to in vivo conditions, some biotic and abiotic stresses caused high mortality. Among the biotic factors, different fungi and bacteria infect the soil-transferred plants. Among the abiotic stresses, temperature, light and humidity are the main reasons. Because of the poor development of cuticles in the leaves, tissue culture-regenerated plants are more vulnerable to transpiration losses. Therefore, maintenance of high humidity

around the plants especially immediately after their transfer to soil is very essential. By improving such factors related to hardening and survival of plants, success has been improved in many plant species (Gosal et al. 1998).

2.2 *Somatic Embryogenesis in Banana*

An orderly developmental process in which the somatic cells are induced and differentiated into zygote-like embryos that give rise to complete plantlets is known as somatic embryogenesis (Hutchinson and Saxena 1996). During the process, the embryogenic competence is achieved by the somatic cells under inductive conditions from which organized embryos are produced after a series of biochemical and morphological changes (Quiroz-Figueroa et al. 2006). The somatic embryos have similar morphological characteristics as zygotic embryos because they also have a bipolar structure (apical and radical meristems). In banana, somatic embryogenesis is carried out to develop high-performance and novel techniques of micropropagation and an efficient method of regeneration useful for genetic transformation studies in banana. Cronauer-Mitra and Krikorian (1983) gave the first report on somatic embryogenesis in banana. He reported the production of somatic embryos from cell suspension cultures using shoot apices as explants. Assani et al. (2001) obtained plant regeneration of the dessert banana cultivar Grand Naine (*Musa* spp., Cavendish subgroup AAA) through somatic embryogenesis. Different behaviour was observed by different genotypes for induction of indirect somatic embryogenesis in banana, and also, the frequency varies from medium to medium. The developmental age of the tissues and organs that serve as a source of explants under in vitro conditions also determines the successful establishment under tissue culture conditions (George et al. 2008). The somatic embryogenic response of two banana cvs. Williams and Grand Naine was determined by studying the effect of interaction between the genotype and developmental age of explants (Youssef et al. 2010). The higher frequency of embryogenic callus formation was observed in 2-week-old explants of cv. 'Williams' (10.01%) as compared to 1-week-old explants (0.78%) which indicates that younger explants have lesser embryogenic potential as compared to mature explants. The process of somatic embryogenesis plays a powerful role during genetic transformation of banana by reducing the risk of chimeric tissues in transgenic plants because of the unicellular origin of embryos (Roux et al. 2004; Escobedo-Gracia Medrano et al. 2014). In our laboratory, we carried out the somatic embryogenesis in banana using explants from in vitro cultures as well as immature male flower buds.

2.2.1 Plant Material

Two banana varieties, viz. Grand Naine and Robusta, were selected for somatic embryogenesis studies. The explants used were in vitro cultures, immature male flowers and suckers in case of cv. Grand Naine, whereas for Robusta, in vitro cultures served as the source of explants for somatic embryogenesis. The culture media used were the same as used in micropropagation studies, i.e. MS and BMM. Different

types of explants have been used to induce embryogenic callus and regenerate plants in banana. These include zygotic embryos (Cronauer-Mitra and Krikorian 1988; Escalant and Teisson 1989; Marroquin et al. 1993; Navarro et al. 1997), rhizome and leaf sheaths (Novak et al. 1989), proliferating meristems and scalps (Cronauer-Mitra and Krikorian 1988; Dheda et al. 1991; Schoofs 1997; Villalobos and Garcia 2008; Shirani et al. 2010) and immature male flowers (Ma 1991; Cote et al. 1996; Grapin et al. 1996; Navarro et al. 1997; Fillipi et al. 2001; Khalil et al. 2002; Jalil et al. 2003; Strosse et al. 2003; Perez and Rossel 2008) and female flowers (Grapin et al. 2000).

In Vitro Cultures

The in vitro cultures were opened in the laminar air flow cabinet and placed in a large sterile Petri dish containing filter paper to absorb excess moisture. The leafy portion was cut from the base leaving a basal clump containing small shoot buds. The basal clump was sliced into two to three pieces. The leafy portion was placed in another sterile Petri dish and leaves, stem and leaf sheaths were separated. Stem portions were cut into pieces of about 1.5–2.0 cm, whereas leaves and leaf sheaths were horizontally cut into two to three pieces, if large and small leaves were left intact. From in vitro cultures, basal clump with shoot buds, stem, leaves and leaf sheaths was used as explant for somatic embryogenesis.

Immature Male Flower Buds

The spathe containing clusters of flowers (Fig. 2a) was excised from the field-grown plants. The outer bracts were removed till the length of the spathe was achieved to 8–10 cm (Fig. 2b). The spathe was taken in laminar air flow cabinet and thoroughly wiped with 70% ethanol. Two to three layers of outer bracts were again removed (Fig. 2c), and flowers (Fig. 2d) were collected from the remaining bracts and placed in a sterile jar.

Suckers

Young suckers of various sizes which emerge around the main mother plant were used as explants. The roots and outer sheaths were removed with the help of a sharp knife. The suckers were cut to a length of about 7–8 cm until the lateral buds were exposed. All the explants were surface sterilized. The culturing of explants was performed under laminar air flow cabinet.

2.2.2 Effect of Different Media Compositions on Explant Establishment

MS medium supplemented with different concentrations of BAP and BMM was used to find out the best medium for achieving maximum culture establishment. The cytokinins play a very important role as they modulate the induction and

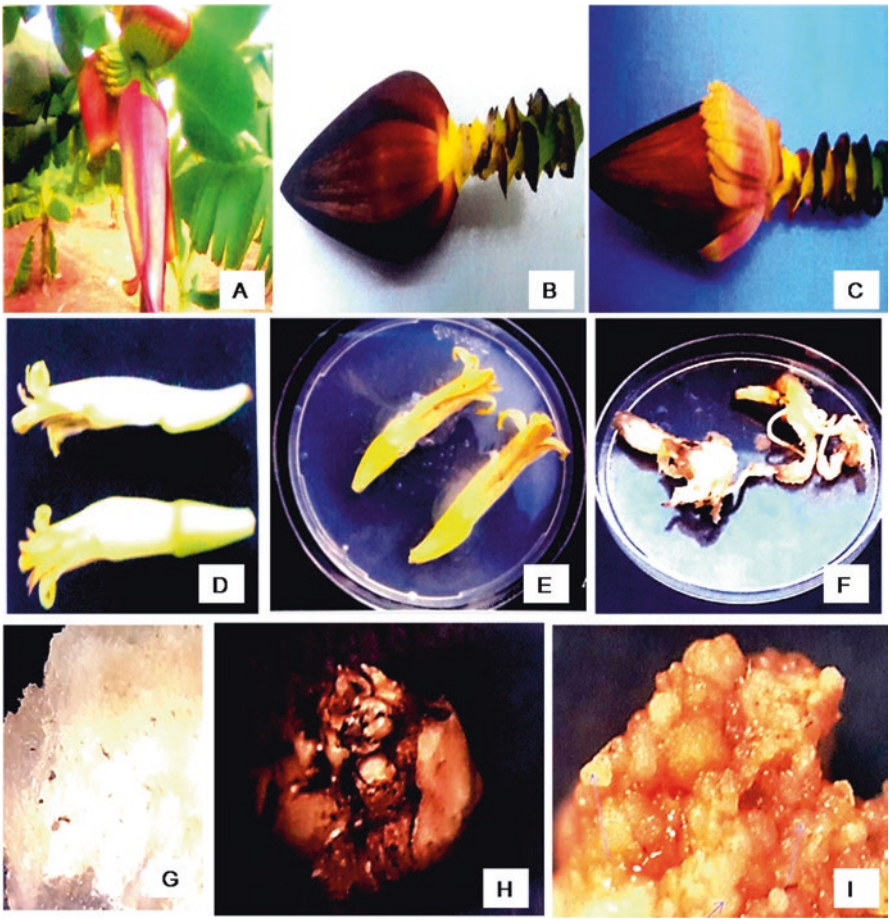

Fig. 2 Callus induction from immature male flowers of banana cv. Grand Naine and from basal clump with shoot buds (scalp) in banana cv. Robusta (**a**) Well-developed banana spathe growing in the field (**b**) Spathe with some of the outer layers removed (**c**) Spathe showing bunches of flowers (**d**) Immature male flowers separated from bunch of flowers from inner layers of spathe (**e**) Greening of the explant (immature male flowers) on callus induction medium [MS + 2,4-D ($4mgl^{-1}$)] (**f**) Callus induction after 4 weeks in complete dark conditions (**g**) Watery, fluffy, soft and non-embryogenic callus formed (**h**) Basal clump containing shoot buds cultured on BMM + 2,4-D ($6\ mgl^{-1}$) (**i**) Sticky and yellow-coloured callus containing globular pro-embryos

development of somatic embryos. The various steps of somatic embryogenesis of banana are controlled by the type and concentration of growth regulators as well as the addition of various supplements like vitamins, amino acids, etc. In case of immature male flower explants, BMM was found to be the best medium with maximum explant establishment of 71.11%, 75.55%, and 35.55% for intact immature male flower (IMF), IMF with tapels removed and half-cut IMF, respectively. Also, IMF with tepals removed was found to show maximum establishment of 75.11% as compared to the other two forms. IMFs began to turn green and showed swelling on BMM after a week (Fig. 2e). Minimum explant establishment was observed on MS

basal medium. Explant establishment was found to increase with an increase in concentration of BAP, but decreased at a concentration of 6 mg l^{-1}. Therefore, concentration of BAP below 6 mg l^{-1} was found to be optimum for explant establishment. Similar pattern of explant establishment was observed in explants from in vitro cultures. Maximum explant establishment of 77.33% and 49.33% was observed on BMM in basal clump with shoot buds and stem sections, respectively. Basal clumps with shoot buds showed significantly higher explant establishment percentage as compared to stem sections. The explant establishment percentage for all media compositions was the same for both the varieties in basal clump with shoot buds as well as for stem sections. Another common observation was that although MS + 2,4-D (4 mgl^{-1}) and BMM have approximately equal concentration of BAP, i.e. 4 mgl^{-1} and 4.5 mgl^{-1}, respectively, explant establishment was little higher in BMM which may be due to the presence of ascorbic acid in BMM which acts as an antioxidant and prevents the secretion of phenolic compounds by the explants and thus, contributes towards better explant establishment. The use of ascorbic acid and other antioxidants in controlling phenolic compounds has been described by many workers (Mante and Tepper 1983; George 1996; Titov et al. 2006). Further, the use of ascorbic acid (10 mgl^{-1}) as an antioxidant in the culture medium has been reported to be used in somatic embryogenesis in banana (Strosse et al. 2003).

2.2.3 Callus Induction

Effect of Different Media Compositions on Callus Induction

The explants in which higher rates of explant establishment were obtained (viz. intact IMF, IMF with tepals removed in case of variety Grand Naine and basal clump with shoot buds of variety Grand Naine were used for callus induction). MS medium was modified with different concentrations of 2,4-D and NAA to study their effect on callus induction in IMF explants of variety Grand Naine. No callus formation was observed on basal MS medium. The medium was supplemented with 2,4-D. 2,4-D at a concentration of 4 mgl^{-1} was found to be the best with callus induction percentage of 53.33 and 57.33 for intact IMF and IMF with tepals removed, respectively. Callus induction percentage was found to be higher for IMF with tepals removed than intact IMF. NAA was also tested at a concentration of 2 mgl^{-1} and 4 mgl^{-1} on MS medium supplemented with 4 mgl^{-1} of 2,4-D for its role in improving callus induction percentage. NAA was found to have no effect on increase in per cent callus induction, and callus induction percentage remained the same as observed on MS medium supplemented with 4 mgl^{-1} of 2,4-D. Days for callus initiation were recorded to be 27.66 (4 weeks) on MS medium supplemented with 4 mgl^{-1} of 2,4-D under complete dark conditions (Fig. 2f). The nature of callus formed was non-embryogenic. The callus appeared watery, soft and fluffy and was whitish in colour (Fig. 2g). The results were in accordance with the previous reports of the use of the 2,4-D at various concentrations either alone or in combination with other growth regulators and additives for callus induction in banana by many workers (Lee et al. 1997; Ganapathi et al. 1999; Namanya et al. 2004).

The 2,4-D at a concentration of 18.10 μM (i.e. 4 mgl^{-1}) has been reported to be used in combination with other growth regulators in callus induction from IMF of banana (Sidha et al. 2007; Youssef et al. 2010). In case of basal clump with shoot buds of varieties Grand Naine and Robusta, 2,4-D at a concentration ranging from 2 to 8 mgl^{-1} was used to study its effect on callus induction (Fig. 2h). MS medium in combination with these varying concentrations of 2,4-D was found to have no effect on callus induction. Therefore, BMM was used. No callus induction was observed on BMM alone. Callus induction was observed on BMM modified with 2,4-D at all concentrations tried (2–8 mgl^{-1}). As the level of 2,4-D was increased, callus induction increased. But 2,4-D at a concentration of 8 mgl^{-1} resulted in decrease in per cent callus induction. Therefore, BMM with a concentration of 6 mgl^{-1} of 2,4-D was found to be the best with callus induction percentage of 28.0 and 44.0 in Grand Naine and Robusta, respectively. Callus induction percentage was found to be higher in Robusta. The results showed that BMM alone which has BAP and IAA at a concentration of 4.5 mgl^{-1} and 0.175 mgl^{-1} was not able to trigger callus induction, but in combination with 2,4-D, good quality callus was formed. Callus formed was sticky and globular, contained pre-embryos and was yellow in colour (Fig. 2i). It took 43.33 days for callus initiation on BMM supplemented with 6 mgl^{-1} of 2,4-D. Also, optimum callus induction from IMF and basal clump with shoot buds was observed under complete dark conditions. Very less degree of callus induction was observed under light conditions. Houllou et al. (2005) and Youssef et al. (2010) also reported the use of complete dark conditions for callus induction in banana.

2.2.4 Direct Somatic Embryogenesis

Induction of Somatic Embryos

As calli obtained could not be used for somatic embryogenesis, a protocol for direct somatic embryogenesis from basal clump with shoot buds was developed. The basal clump was placed on BMM supplemented with NAA (Fig. 3a). BMM was tested at different concentrations of NAA ranging from 0.5 to 10 mgl^{-1} to study its effect on formation of direct somatic embryos in varieties Grand Naine and Robusta. Somatic embryogenesis was observed on BMM medium supplemented with 5 mgl^{-1} NAA (Fig. 3b, c). With the further increase in the concentration of NAA (i.e. 7.5 mgl^{-1} and 10 mgl^{-1}), it resulted in decrease in per cent somatic embryogenesis in variety Grand Naine from 29.33 to 28.0 and 21.33 at 7.5 and 10 mgl^{-1}, respectively. In case of Robusta, BMM medium supplemented with 5 mgl^{-1} was found to be the best for direct somatic embryogenesis. Per cent somatic embryogenesis was found to be higher in variety Robusta as compared to Grand Naine which was in correspondence with the previous report by Sidha et al. (2007). Somatic embryos appeared as white budlike structures emerging from basal clump with shoot buds, when observed under a stereomicroscope (Fig. 3d, e). It took about 10 weeks for the formation of somatic embryos. Nagappa et al. (2009) have also reported the use of NAA in combination with cytokinin for direct somatic embryogenesis in red banana.

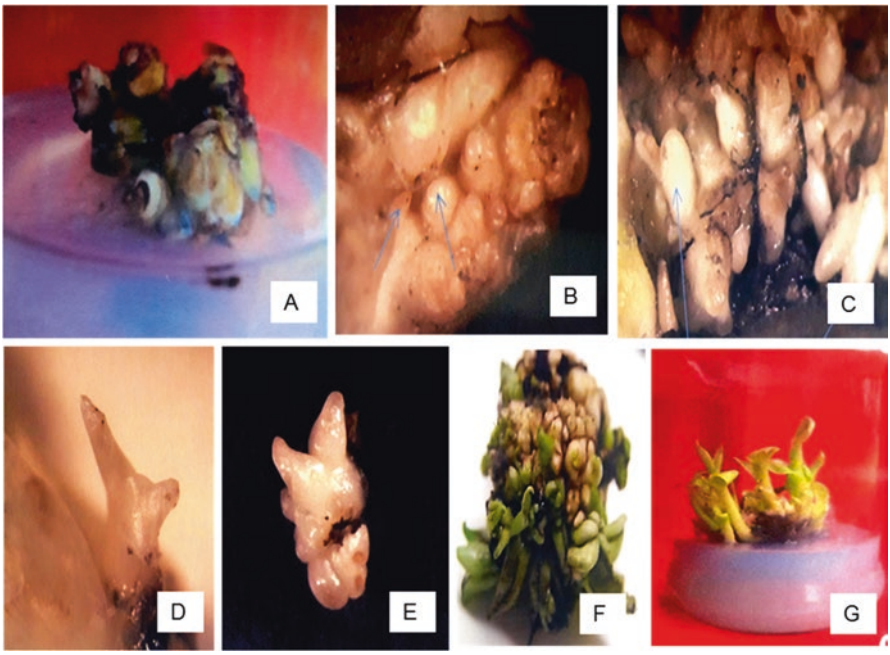

Fig. 3 Direct somatic embryogenesis from basal clump with shoot buds (scalp) in banana cv. Robusta (**a**) Basal clump with shoot buds cultured on BMM + NAA (5 mgl^{-1}) (**b**) Induction of direct somatic embryos (white budlike structures) after 10 weeks in complete dark (**c**) Development and maturation of somatic embryos on same medium under light conditions (16:8 h, light: dark regime) (**d**, **e**) Stereomicroscopic view of an individual somatic embryo (**f**) Regeneration occurred from mature somatic embryos after 3 weeks (**g**) Formation of shoots after 8 weeks

Development and Maturation of Somatic Embryos

Once the somatic embryos were induced, somatic embryos along with explants were subcultured onto the induction medium [BMM + NAA (5 mgl^{-1})] and incubated under light conditions, i.e. 16 h of continuous fluorescent light (5000 lux) followed by a dark period of 8 h for 2 weeks. The presence of light conditions for development and maturation of somatic embryogenesis in banana has been reported earlier by Kulkarni and Ganapathi (2009).

2.2.5 In Vitro Shoot Regeneration, Rooting and Hardening

After the development and maturation of somatic embryos were achieved, the somatic embryos were transferred to regeneration medium. MS basal and BMM were tested for shoot regeneration. BMM was found better for shoot regeneration, with per cent shoot regeneration of 49.33% and 58.0% for Grand Naine and Robusta, respectively, as compared to 30.67% and 44.0% on MS basal medium. Shoot regeneration occurred after 3 weeks (Fig. 3f). These small regenerated shoots

were subcultured on BMM medium for further shoot elongation, and average shoot length was recorded after 4 weeks. Shoot length was recorded to be 4.8 and 6.2 cm in case of Grand Naine and Robusta, respectively. The use of BMM for shoot regeneration has been supported by previous reports of the use of BAP alone or in combination with IAA for regeneration of somatic embryos in banana by many workers (Ganapathi et al. 1999; Kulkarni et al. 2002; Namanya et al. 2004; Kulkarni et al. 2006; Srinivas et al. 2006; Nagappa et al. 2009). After 8 weeks, complete shoots were formed from mature somatic embryos (Fig. 3g). Roots started developing simultaneously along with shoots when small regenerated shoots were subcultured on BMM medium for further shoot elongation. Further subculturing on BMM resulted in good quality roots after 3 weeks. The plantlets were acclimatized under in vitro conditions to prevent the transplantation shock. Hardened plantlets were transferred to glasshouse in polythene bags containing garden soil/vermiculite mixture in 1:1 ratio.

2.3 In Vitro Mutagenesis

As banana is a vegetatively propagated plant, it is essential to generate additional genetic variability to facilitate the selection of desirable traits. The cellular and molecular approaches such as anther/pollen culture, wide hybridization using embryo rescue, somaclonal variation, genetic transformation and molecular markers have been deployed for the improvement of crop plants. The induction of mutation and selection under in vitro conditions for desirable characters offer certain advantages such as mutagenizing of the particular plant parts, uniform mutagen treatment, handling large number of samples in a short time span and facilitating in vitro selection (van Harten 1998). The induction and selection of induced somatic mutations have become more effective through the combination of mutation breeding and in vitro culture, also called in vitro mutagenesis. ‘Somaclonal variation’ is the term used to describe the variation seen in plants that have been produced by plant tissue culture. Therefore, the combined use of mutation induction and in vitro technology is more efficient because it speeds up the production of mutants as a result of increased propagation rate and a greater number of generations per unit time and space (Morpurgo et al. 1997). A somaclonal variant of banana, CIEN BTA-03 (resistant to yellow Sigatoka), from a susceptible banana clone (Williams clone) was obtained, by increasing the production of adventitious buds using BAP at high concentrations. This somaclone exhibited resistance to yellow Sigatoka disease for five consecutive years of asexual reproduction (Vidal and de Gracia 2000). Further, it was found that the incorporation of growth retardants such as ancymidol (ANC) or paclobutrazol (PBZ) in liquid culture media during multiplication stage of micropropagation of bananas decreased the excessive growth of stems and leaves (Albany et al. 2005). The sudden, rare, discrete and heritable changes in genetic material (DNA) of an organism are called mutations, caused by certain agents, known by its generic name, mutagens. Mutagen means ‘change’ and genesis means ‘to give rise to or generate change’; therefore, the mutation can increase its frequency and rate.

These mutations could result in the permanent change in gene expression or can occur spontaneously due to the combined effect of high or low temperatures and presence of chemicals or background radioactivity. The random changes in the nuclear DNA or cytoplasmic organelles are caused by mutagens which results in gene, chromosomal or genomic mutations (Jain and Maluszuynski 2004). Depending upon the nature of mutagens used, the mutagenesis could be of two types:

Chemical mutagenesis Chemical mutagenesis is caused by chemicals for the induction of mutations in cultivated plants. Most of the chemicals belong to a special class of alkylating agents such as ethyl methane sulphonate (EMS), diethyl sulphate (DES), ethylamine (EA), methyl nitroso urethane (ENU), ethyl nitroso urea (ENU) and methyl nitroso urea (MNU). Azides have also been found as effective mutagens for chemical mutagenesis. These chemical mutagens majorly produce point mutations, but these are carcinogenic in nature and penetrate non-uniformly in multicellular tissues.

Physical mutagenesis The physical mutagens such as gamma rays (gamma photons), X-rays, etc., are most commonly used for the induction of mutations. The advantage of using physical mutagens is that they show reasonable reproducibility and have high and uniform penetration of multicellular system in plants, particularly by gamma rays. In banana, in vivo and in vitro cultures were used for the induction of mutations. The in vivo plant material included seeds and suckers of banana. Shoot tips, embryogenic cell suspensions, etc., served as in vitro plant material for the induction of mutations. To produce large number of plants, shoot tips are most widely used because they induce direct shoots by preventing callus formation. Under the database of mutant varieties of FAO/IAEA, two banana accessions were registered as improved banana varieties: 'Novaria' for early flowering and 'Klue Hom Thong KU1' for its bunch size and cylindrical shape from which larger bananas can be selected. Many other desirable variants/putative variants have been identified for release or further confirmation of trials. Examples are shown in Table 2.

Table 2 Various banana putative mutants obtained in different countries

Country	Parent clone	Selected mutant clone	Selected traits	Technique	Place of induction
Cuba	SH-3436	SH-3436-L9	Reduced height	Gamma rays	Cuba
Malaysia	Pisang Rastali	Mutiara	FOC[a] tolerance	Somaclonal variation	Malaysia
	Novaria		FOC[a] tolerance	Somaclonal variation	Malaysia
Philippines	Lakatan	LK-40	Reduced height	Gamma rays	IAEA, Vienna
	Latundan	LT-3	Larger fruit size	Gamma rays	IAEA, Vienna
Sri Lanka	Embul	Embul-35 Gy	Earliness	Gamma rays	Sri Lanka
Austria (IAEA)[b]	Grand Naine	GN35-I to GN35-VIII	Tolerance to toxin from *Mycosphaerella fijiensis*	Gamma rays	IAEA, Vienna

[a]FOC: *Fusarium oxysporum* f. sp. cubense
[b]IAEA: International Atomic Energy Agency

In our laboratory, the in vitro induction of genetic variation through gamma irradiation of banana (*Musa* spp.) was carried out with micropropagated plant material of banana developed through axillary shoot proliferation and through adventitious shoot bud initiation and multiplication. The microshoots obtained through axillary shoot proliferation were separated and transferred to fresh growth medium (BMM) with different concentrations of kinetin (Kin). Cultured shoot tips showed swelling with visible proliferations within 20–25 days. Clusters of buds were developed on the explants. The buds were separated aseptically, and individual buds were cultured onto freshly prepared multiplication medium. It led to the development of clusters of buds again which were subcultured repeatedly by dividing them into groups of one to two buds.

2.3.1 Gamma Irradiation of Cultures

Choice of Explant

Shoot bud cultures (10–15 shoot buds/culture bottle) and microshoot-tip cultures (5–8 shoot tips/culture bottle) were irradiated in Blood Irradiator (^{60}Co source, dose 11 Gy per min) located in the University Orchard. Two-month-old cultures were irradiated at different doses, i.e. 15, 25, 35 and 45 Gy [Gray (Gy) is the SI unit of absorbed radiation dose of ionizing radiation (e.g. gamma rays) and is defined as the absorption of 1 joule of ionizing radiation by 1 kilogram of matter]. Five shoot-tip and bud cultures were irradiated by each dose in a single replication, thereby making 20 cultures in one replication. Cultures immediately after irradiation were termed as M_1V_0. After irradiation, the leafy portion present in either of the cultures was excised. The survival percentage and multiplication ratio of both explants were recorded over a period of three subcultures. Based on the survival response of shoot-bud and shoot-tip cultures, shoot tips were used as a major explant for irradiation. Therefore, 500 shoot-tip cultures were irradiated with four different doses of gamma irradiation, i.e. for each dose, 125 shoot-tip cultures were irradiated.

2.3.2 Effect of Gamma Irradiation on Various Parameters

Response of Shoot Tips After 30 Days of Gamma Irradiation

The percentage survival of shoot tips ranged from 96.0% for the unirradiated control to 46.4% at 45 Gy. Among the irradiated shoot tips, the effect of low levels of irradiation was nearly similar, i.e. 84.80% survival for shoot tips irradiated at 15 Gy and 80.8% for shoot tips irradiated at 25 Gy, but it decreased at higher doses (62.4% for 35 Gy and 46.4% for 45 Gy). All surviving shoot tips did not produce shoots; some developed calloid, while others remained dormant. Similar patterns of survival have been reported by Valerin et al. (1995), Bhagwat and Duncan (1998), Chai et al. (2004) and Mishra et al. (2007). The occurrence of calloid was

10% at unirradiated control and 23.2 at 45 Gy. The calloid occurrence was significantly more in irradiated plants as compared to control, and this increase showed a positive trend across the doses. Maximum calloid occurrence was observed at 45 Gy. The occurrence of calloid in cultures of irradiated shoot tips was first reported by Novak et al. (1990) and was characterized as adventitious in origin. Bhagwat and Duncan (1998) had also reported calloid (with prolific bud proliferation) formation in dissected shoot apices. Some of these buds developed into shoots, while others remain dormant. The number of shoots regenerated was observed to be highest (120) at 15 Gy after 4 weeks of irradiation as compared to unirradiated control (102). With increasing irradiation dose, shoot regeneration decreased significantly. Although no certain explanations for the stimulatory effect of low-dose gamma radiation are available until now, there is a hypothesis that the low-dose irradiation induces the growth stimulation by changing the hormonal signalling network in plant cells or increases the antioxidative capacity of the cells to easily overcome daily stress factors such as fluctuations of light intensity and temperature in the growth condition (Wi et al. 2007). In contrast, the high-dose irradiation that caused growth inhibition has been ascribed to the cell cycle arrest at G_2/M phase during somatic cell division and various damages in the entire genome (Preussa and Britta 2003).

Effect of Gamma Irradiation Dose on Per Cent Root Initiation of Plantlets

Besides the survival and multiplication, the per cent root initiation was also influenced by gamma irradiation. The per cent root initiation in control plants was 71.44%. Among the different gamma irradiation doses, per cent root initiation over a period of 28 days was 58.64% in plantlets arising from 15 Gy-irradiated shoot tips, while plantlets arising from 45 Gy-irradiated shoot tips showed only 35.42% root initiation. Therefore, it was observed in the present study that root initiation declined with increase in irradiation dose indicating an inverse relationship between the two parameters. This clearly indicates that the gamma ray dose had direct influence on appearance of roots in banana shoots and percentage root initiation decreased after mutagenic treatment.

Phenotypic Alterations Observed in M_1V_4 Clones in the Laboratory and Glasshouse After Gamma Irradiation of Shoot-Tip Cultures

A total of 475 plants were obtained in which 47 plants served as control (unirradiated) and 428 plants served as induced variants within 3 months after acclimatization. Considerable phenotypic variation was observed among the plants regenerated from the shoot tips after mutagenic treatment. In early stages of plant development, the irradiation affected emergence and expansion of younger leaves. The variants in M_1V_4 generation comprised altered phenotypes such as changes in plant stature, leaf

morphology, pigmentation and sucker proliferation. The percentage of phenotypic variants ranged from 5.16% to 20% of the M_1V_4 plants at four doses. Maximum variation was observed in plants at 35 Gy (20%) as compared to control (4.4%). Phenotypic alterations such as leaf deformities were found to be maximum (11 plants). The leaves of many plants obtained at 35 Gy were small and ovate. Most of the plant obtained after various doses of irradiation showed thin morphology as compared to the mother plant. One of the variants at 45 Gy showed two pseudostems.

Effect of Different Doses of Gamma Irradiation on Phyllotaxy of Plants Transferred to Glasshouse

The effect of gamma irradiation on arrangement of leaves on the pseudostem, i.e. phyllotaxy, is given in Table 3. Alternate leaf arrangement (leaves attached in alternate direction) along the stem was found to be predominant in both the unirradiated control and irradiated plants. Eighty per cent of unirradiated control showed alternate leaf arrangement. It is evident from the data that gamma irradiation has a strong effect in determining the leaf arrangement on pseudostem. Apart from alternate arrangement, opposite arrangement of leaves was found to a great extent in irradiated plants as compared to control (27.27% at 45 Gy as compared to 16% in unirradiated control). Rosetting of leaves, i.e. negligible spacing in leaves, was observed in 9.99% of the plants at irradiation dose of 15 Gy. Sucker proliferation was observed after 1 or 2 months of transfer at 15, 35, and 45 Gy to the glasshouse in polybags (Fig. 1g, h). The unirradiated control plants showed no sucker proliferation even after 4–5 months in glasshouse.

Table 3 Phenotypic alterations observed in M_1V_4 clones of 'Grand Naine' in the glasshouse after gamma irradiation of shoot-tip cultures

Dose of gamma irradiation (Gy)	Number of plants		Normal: altered phenotypes observed	% variants observed	Types of phenotypic alterations[a]								
	Planted	Survived after 3 months			A	B	C	D	E	F	G	H	I
0	50	47	45:2	4.4	0	0	0	0	0	2	0	0	0
15	206	155	147:8	5.16	1	1	1	0	3	0	0	1	1
25	163	115	107:8	6.9	1	1	2	1	1	1	0	1	0
35	155	95	76:19	20.0	2	2	2	1	4	3	1	2	2
45	140	63	53.10	15.87	1	2	2	0	3	0	1	0	1
Total	714	475	428:47	9.89	5	6	7	2	11	6	2	4	4

Altered phenotypes[a]: A, dwarfism, short intervals among leaves, short petioles and leaves; **B**, long petioles; **C**, long and narrow leaves; **D**, ovate, small leaves; **E**, leaf deformities; **F**, leaf colour changes such as green-yellow stripes, variegation and enhanced pigmentation; **G**, two pseudostems form one corm; **H**, thick leaves; **I**, sucker proliferation

2.3.3 Effect of 3-Day Low-Temperature Treatment on Various Biochemical Parameters in the Leaves of Plants Obtained After Regeneration of Irradiated Shoot Tips

Superoxide Dismutase Activity

Activity (number of units min^{-1} g^{-1} FW) of SOD after 3 days of low-temperature treatment was calculated in all plants. The superoxide dismutase (SOD) activity was observed between 89.74 and 160.50 in the control plants (without irradiation) while plants irradiated at 15, 25, 35, and 45 Gy showed SOD activity of 45.09 to 278.45, 77.84 to 242.98, 74.13 to 240.20 and 69.42 to 200.0, respectively. The plants at all doses showing SOD activity at the maximum of range showed survival to low-temperature treatment. In addition to these surviving plants, one plant at 25 Gy and two plants at 35 Gy showed survival. The plants with lower activity of SOD died after 4–5 days of the low-temperature treatment. The activity of SOD was found to be more in plants surviving the low-temperature treatment. This suggested that low temperature imposes an oxidative stress and oxygen level increases. Increased activity of SOD acts as first line of defence against reactive oxygen species (ROS), dismutating superoxide radical formation from superoxide via the metal-catalysed Haber-Weiss-type reaction. Zhang et al. (2011) reported that SOD activities in the resistant *Musa* cv. Cachaco increased under low-temperature stress. Remarkable changes in SOD activity were also reported in *Musa acuminata* cv. Williams 8818 after 5 °C chill treatment (Kang et al. 2003). After cold stress, an increase in SOD activity has also been reported in *Glycine max* (Posmyk et al. 2005). Therefore, the higher activity of SOD can be used to explain the cold tolerance of surviving plants.

Malondialdehyde (MDA) Content

Malondialdehyde (MDA) content ($nmolg^{-1}$ FW) in leaves after 3 days of low-temperature treatment was determined in all plants. MDA content was observed between 9.87 and 26.76 in the control plants (without irradiation) while plants irradiated at 15, 25, 35, and 45 Gy showed MDA content of 6.94 to 27.22, 5.92 to 29.99, 6.56 to 23.89 and 9.63 to 25.89, respectively. The plants at all doses with least MDA content survived the low-temperature treatment. Also, plants showing survival with higher SOD activity showed lesser MDA content. The plants with high MDA content died after 4–5 days of the low-temperature treatment. Accumulation of ROS in different plant cell compartments occurs under various biotic and abiotic stresses, and one of the indications of ROS injury to cell membranes is the production of MDA. MDA is the end product of lipid peroxidation which increases in chilled tissues. Less amount of MDA detected in the tissues indicate less damage to cell membranes. The surviving plants exhibited less MDA accumulation.

3 Conclusion

Polyploidy and seedlessness traits limit the traditional breeding in banana. Conventional method of vegetative propagation of banana involves the use of suckers (lateral shoots) for its cultivation, which results in the production of four to five banana plantlets per year. Micropropagation, somatic embryogenesis and embryogenic cell suspension cultures are some of the tissue culture techniques that act as powerful tools for rapid multiplication of banana. These techniques could also serve as a source of germplasm conservation of ancestors of dessert banana plants (wild banana). Somatic embryogenesis process is considered a potential technique for transportation of edible banana clones through the production of synthetic seeds. Further, induction of genetic variation through ionizing radiation increases the frequency of mutations, which will, in turn, enhance the production of beneficial genetic variants and their usefulness in genetic improvement of banana.

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Recent Advances in Virus Elimination and Tissue Culture for Quality Potato Seed Production

Prakash S. Naik and Tanuja Buckseth

Abstract High-yielding varieties and quality planting material are two important ingredients for harnessing benefits of modern production technologies in vegetatively propagated crops like potato (*Solanum tuberosum* L.). Low rate of multiplication, high seed rate, progressive accumulation of degenerative viral diseases, perishability and bulkiness are inherent problems associated with potato seed which result in non-availability of adequate quantities of quality planting material at affordable price. Inadequacy of quality planting material at affordable cost and high seed rate are often reflected in 40–50% cost of cultivation on potato seed alone. To circumvent some of these problems, several modifications such as tuber indexing for virus freedom, seed multiplication stages and seed certification standards have been developed and integrated with conventional potato seed production programmes. The advent of tissue culture, in which virus-free plants can be produced through meristem culture, maintained indefinitely under controlled conditions and multiplied in artificial media under sterile conditions in the laboratory throughout the year irrespective of growing season, has revolutionized seed production in potato world over. Recent developments in automation for microtuber and minituber production have further enhanced adaptability of these techniques in potato seed production. In addition to quality assurance through meristem culture, the micropropagation, microtuber and minituber production techniques ensure enhanced multiplication rate in initial stages of seed production. All these techniques with their possible integration in potato seed production are discussed in this chapter.

Keywords Potato · *Solanum tuberosum* L. · Meristem culture · Virus elimination · Tissue culture · Micropropagation · Aeroponics

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1 Introduction

The potato (*Solanum tuberosum* L.) is a major world food crop. In world food production, potato (381.68 million t) was exceeded only by maize (1037.79 million t), rice (741.47 million t) and wheat (729.01 million t) in 2014 (FAO 2017). Potato is a vegetatively propagated, highly heterozygous, tetraploid and semi-perishable crop. It is also susceptible to many diseases and insect pests. The genetic nature, propagation mode and vulnerability to diseases/pests impose several inherent limitations on potato seed production. Potato seed production is characterized by (i) low multiplication rate that ranges from 1:4 to 1:15 depending upon variety, agroclimatic conditions and crop management and (ii) progressive accumulation of degenerative viral diseases during clonal propagations. Therefore, to build up sizable seed stocks, the initial disease-free tuber material needs to be field multiplied for a number of years. With each such multiplication, the viral diseases accumulate progressively causing 'degeneration' or 'running out' of seed stocks (Naik et al. 2000; Naik and Karihaloo 2007). Consequently, non-availability of quality planting material in adequate quantities at right time and affordable price is the major bottleneck in potato cultivation in many countries. This is further aggravated by higher seed rate (3–4 t/ha), making potato cultivation beyond the reach of resource-poor farmers. The cost of seed potatoes alone accounts for about 40–60% of the total production costs in many parts of the world (Sawyer 1979). Being a vegetatively propagated crop that is associated with large number of seed borne diseases, it is imperative to use good quality seed for economically viable potato production. However, availability of quality seed is a major constraint in potato production especially in developing countries (Upadhya 1979), and often the farmers are forced to use locally grown seed known to lower productivity by about 40% (Salazar 1996).

For better health standards, seed potatoes need to be produced in cooler areas where virus transmission and its spread to subsequent generations through tubers are minimal. In tropical and subtropical countries without a long cool winter, the production of seed potatoes is difficult because of high disease pressure throughout the year. As a result, these countries import much of their seed tubers from cooler regions for potato production (Sharma et al. 2011). However, pathogen-free seed tubers are generally expensive and account for about 50% of total potato production cost (Van der Zaag and Horton 1983). Currently, the common method for propagation of commercially important potato cultivars in these countries is repeated multiplication of initial disease-free tubers leading to progressive accumulation of viruses, fungi and bacteria in subsequent generations and thus resulting in significant yield losses and tuber quality over years (Nyende et al. 2005). In a country like India, one major cause of low potato productivity is the use of poor quality seed (Singh 2003), and at present the state and central seed production agencies of the country are able to meet only about 20–25% requirement of quality potato seed (Kumar et al. 2007). The situation is not much different in other developing countries. Therefore, for bridging this wide gap, large-scale integration of conventional and tissue culture techniques like meristem culture and

micropropagation is needed for producing enough quantity of healthy seed tubers in minimum period of time (Pandey 2006).

Potato is perhaps the premier example of a crop plant to which biotechnology has been most extensively applied. Meristem culture was the first biotechnological approach successfully employed to obtain virus-free potato clones (Morel and Martin 1955). During the last few decades, problem-driven use of biotechnology in potato had been instrumental in addressing many inherent problems associated with this crop. Micropropagation of disease-free potato clones combined with conventional multiplication methods has become an integral part of seed production in many countries.

2 Important Potato Viruses

More than 30 viruses and virus-like agents infect potatoes. The yield losses in potato due to viral infections vary from low to very high (Khurana and Singh 1988). Infections of potato virus Y (PVY) and potato leaf roll virus (PLRV) have the potential to reduce the yields up to 60–80%, while mild viruses like potato virus X (PVX), potato virus S (PVS) and potato virus M (PVM) also depress the yields by 10–30% in infected plants. In addition to yield losses, being systemic pathogens, potato viruses and viroids perpetuate through seed tubers and pose a major concern for potato seed production in both developed and developing countries. Details of some of the important potato viruses, their mode of transmission, symptoms and potential yield losses are given in Table 1.

3 Modern Methods of Virus Detection and Diagnosis

In field, one can easily recognize viral disease symptoms, if perceptible. Visual field inspection for symptoms is the oldest and the easiest way to rogue out the diseased plants. But neither all viruses cause severe (clear) symptoms nor it is true that in all varieties same symptoms are expressed for the same virus at all times. Accurate, sensitive and rapid detection of potato viruses is critical for identifying virus-free mother plants for their multiplication in potato seed production programme. A wide array of serological and nucleic acid-based assays is available for accurate detection and diagnosis of potato viruses. Since a comprehensive review on various techniques is beyond the scope of the present chapter, the readers are referred to other reviews by CIP (1980) and Kumar et al. (2012).

Enzyme-linked immunosorbent assay (ELISA), nucleic acid spot hybridization (NASH) and immunosorbent electron microscopy (ISEM) are the most widely used methods for detection of plant viruses (Fig. 1). Serological assays involve trapping virus particles on a supporting surface to which a specific antiserum has been attached. An ELISA in a microtitre plate or dot blots in a nitrocellulose membrane

Table 1 Mode of transmission, symptoms and potential yield losses of some of the important potato viruses

Virus	Mode of transmission	Symptoms and yield losses
Potato virus X (PVX)	Transmission of PVX occurs mainly mechanically, through contact between the plants and due to the passage of machinery	Plants often do not exhibit symptoms, but PVX can cause symptoms like chlorosis, mosaics, decreased leaf size, etc. PVX can interact with PVY and PVA to cause severe symptoms and higher yield losses. The symptoms can be seen more easily during the active growth phase of the crop and in weak sunlight. Potential yield losses range between 10% and 20%
Potato virus Y (PVY)	Potato virus Y is transmitted by aphids (mainly *Myzus persicae*). It is a non-persistent type virus because the aphid can transmit it within a period of 2 h only after acquisition from diseased potato plants or other host plants	The appearance of symptoms on a crop depends on the strain of PVY, variety, weather conditions and the type of infection (primary or secondary). PVY^{0} is common strain that causes mosaic symptoms; PVY^{C} causes stipple streak; and PVY^{N} causes necrosis in leaves. Mixed infections of common and necrotic strains produce hybrid strains (i.e. $PVY^{N:0}$ and PVY^{NTN}). PVY^{NTN} strains cause severe symptoms including tuber necrosis. The secondary infections are more severe than primary infections. PVY strains can interact with PVX and PVA to result in heavier losses. Potential yield losses are 10–80%
Potato virus A (PVA)	Aphids are the agents responsible for transmitting potato virus A. Virus A is a non-persistent type virus because it remains alive for only 1–2 h in the aphid after the latter has become infectious	The appearance of symptoms on a given crop depends on the variety and the weather conditions. However, in primary infection, the symptoms include slight and transient mosaics that are particularly visible in cloudy weather. These mosaics appear as a discoloration of leaf portion not containing veins. In secondary infection, the symptoms are more pronounced, resulting in waffling/embossing of the leaves combined with a glassy appearance. In the case of mixed infection with PVY and/or PVX, the symptoms are severe.PVA symptoms are easier to see in cold and cloudy weather. Potential yield loss is about 15%
Potato virus S (PVS)	PVS is transmitted by contact as well as non-persistent transmission by aphids	PVS generally causes weak (latent) and variable symptoms. The symptoms consist of lightening of foliage, deepening of the veins on the upper side of the leaves, reduction in leaf size and bronzing and necrotic spots on leaves. Potential yield loss is 10–40%

(continued)

Table 1 (continued)

Virus	Mode of transmission	Symptoms and yield losses
Potato virus M (PVM)	PVM is transmitted through contact as well as by aphids in a non-persistent mode	The most common symptom is the appearance of 'spoon-shaped' leaves like soft curling of the leaves. This occurs preferentially on the topmost leaves. Some other symptoms are slight discolouration of the veins of the top leaves, curling of the leaf edges, mosaic, etc. The symptoms are clear in cool and cloudy weather. Potential yield loss is 10–40%
Potato leaf roll virus (PLRV)	PLRV is transmitted by aphids in a persistent manner. PLRV is persistent because it takes longer to be acquired (10–30 min) and transmitted (24–48 h) by the aphids. This period is required for the movement of the virus into the insect's intestine and then back out through the salivary glands	The appearance of the symptoms on a crop depends on stage of infection. *Primary infection (current year):* the leaves at the top of the plant are slightly curled and show yellowing. Purple pigmentation can sometimes be seen on the edge *Secondary infection (previous year):* the leaves at the base are tightly curled and hardened; the plant growth habit is straighter, and the internodes are shorter. The plant is yellowed and sometimes dwarfed. In certain varieties, internal necrosis can appear as network on tubers. Potential yield loss is 50–90%
Potato spindle tuber viroid (PSTVd)	PSTVd is a viroid. It is composed of self-replicating RNA without protein coat. It is mechanically transmitted through contact. In addition to seed tubers, PSTVd is also transmitted through pollen and TPS (true potato seed). It can also be transmitted by aphids but to a lesser extent	PSTVd causes smaller leaves that curl downwards giving a stiff and upright growth habit. Plants can be stunted with more branches. There is a reduction in tuber size combined with tuber deformation giving rise to spindle-shaped tubers with prominent eyebrows. Potential yield loss is 10–40%

Source: Burrows and Zitter (2005) and Naik and Karihaloo (2007)

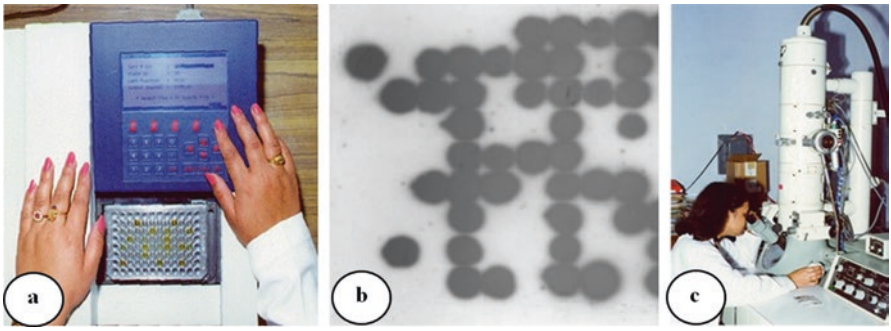

Fig. 1 Some of the sensitive serological and molecular techniques routinely used for virus detection. [ELISA, enzyme-linked immunosorbent assay (**a**); NASH, nucleic acid spot hybridization (**b**); and ISEM, immunosorbent electron microscopy (**c**)]

are used to produce a colour reaction that is dependent on virus concentration. In ISEM, the trapped and negatively stained viruses are directly viewed under the electron microscope. ISEM is used when an available antiserum contains non-specific antigens that reduce ELISA specificity. Protein-A complemented immune electron microscopy (PAC-IEM), a modification of ISEM, makes use of protein-A's high affinity for IgG to enhance trapping and minimize non-specific trapping of virus particles. Over the years various modifications have been introduced in ELISA systems with increasing availability of monoclonal and polyclonal antibodies to reduce host antigen background reactions.

Nucleic acid hybridization is based on specific pairing between the single standard DNA or RNA and a complementary nucleic acid probe to form double-stranded nucleic acid. Thus, either DNA or RNA sequences may be used as probes for detection of plant viruses. Hybridizations are usually carried out on solid filter supports (nitrocellulose or charged nylon) where the target nucleic acids are immobilized and the labelled nucleic acid probe is allowed to hybridize to them. RNA probes specific for potato spindle tuber viroid (PSTVd) have been synthesized from a full length PSTVd cDNA and used successfully for PSTVd detection in potato (Bernardy et al. 1987). Improvements in hybridization assays have been made in recent years using non-radioactive detection systems. Nucleic acid probes can be labelled by incorporation of biotin-11-UTP or digoxigenin-tagged UTP and can be detected by streptavidin or anti-digoxigenin antibody-enzyme conjugate, respectively. Biotin-labelled probes have been reported for PVS, PVX and PSTVd (Querci and Salazar 1998). Polymerase chain reaction (PCR) combined with reverse transcription (RT-PCR) has also been used for detecting picogram quantities of viral nucleic acid in infected tissues. With its relative simplicity and high sensitivity, the PCR-based methods will be more increasingly used in the future to detect and diagnose plant viruses.

4 Conventional Potato Seed Production

Conventional potato seed production involves production of basic seed (also known as breeders' seed in some countries) on special seed farms under scientific supervision. The process of basic seed production starts with the selection of mother plants in the field that are healthy and have a better yield. From each such selected plant (clone), four true-to-type tubers are selected, and all such tubers are clone-wise serially numbered, i.e. 1–4 for plant 1, 5–8 for plant 2, 9–12 for plant 3, etc. From each numbered tuber, a single eye of 1 cm diameter and 1.5 cm length is scooped, and the scooped eyes are serially planted in pots in glass house/net house (Fig. 2a, b). After scooping and cementing scooped cavity, the counterpart tubers are stored for use in the next season. The emerging plants are indexed using ELISA against important potato viruses. In the subsequent season, the counterpart tubers of healthy plants are planted in clone-wise rows in the field at 1 × 1 m distance. Higher planting distance is used to avoid plant-to-plant contact and, thereby, spread of contact viruses, if any. All stages of potato seed crop in tropical and subtropical

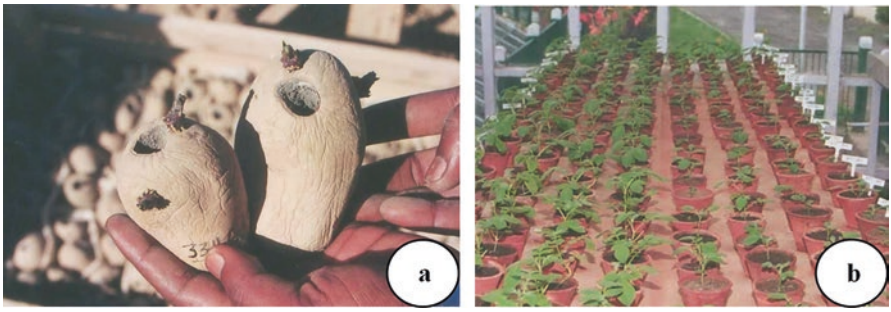

Fig. 2 Selection of virus-free mother tubers for production of basic seed. (**a**) Tubers from apparently healthy plants are scooped and serially numbered. (**b**) Scooped eyes are planted in glasshouse for virus indexing, and based on virus testing reports, healthy counterpart mother tubers are used for basic seed production

regions are grown during low aphid periods to evade spread of potato viruses by aphids. This is known as 'seed plot technique' (Pushkarnath 1967). The field plants are inspected visually for off-types and viruses and randomly tested by ELISA for presence of viruses. The off-type and diseased plants thus detected are rogued out. The healthy clones are further multiplied in three generations under 'seed plot technique' to produce sufficient quantity of basic seed. In each stage, testing and inspection of crop is done to remove the diseased plants and off-types, if any. Most seed production programmes operate a 'flush through' system starting each year with fresh positively selected tubers from healthy plants (true to the type and indexed for freedom from viruses).

The basic seed is further multiplied in three generations by seed agencies and registered seed growers under 'seed plot technique' to produce certified seed. Production of certified seed is supervised and monitored by seed certifying agencies.

Conventional potato seed production system is characterized by low multiplication rate and progressive accumulation of degenerative viral diseases during clonal propagations. Further, in contrast to seed-propagated crops, the multiplication rate in potato is low varying from 1:4 to 1:15 (1 tuber yields 4 to 15 tubers) depending upon variety, agroclimatic conditions and crop management. Therefore, to build up sizeable seed stocks, the initial disease-free tuber material needs to be field multiplied for a number of years. With each such multiplication cycle, viral diseases accumulate progressively causing degeneration or 'running out' of seed stocks. Consequently, non-availability of quality planting material in adequate quantities and at affordable prices is the major bottleneck in potato cultivation in many countries.

Integration of meristem culture along with micropropagation and traditional multiplication can circumvent inherent problems associated with potato seed production. Micropropagation is an asexual reproductive method that allows production of selected genotypes *en masse* for commercial planting (Sarkar and Pandey 2011). The most significant advantages offered by micropropagation are (1) a large number of disease-free propagules can be obtained from a single plant in

short period within a limited space, (2) propagation can be carried out under disease-free conditions throughout the year without intervening seasonal constraints and (3) a constant flow of disease-free plants is possible from the original in vitro stock developed through meristem culture. Micropropagation technology has been successfully used to speed up initial stages of potato seed production programmes by supplementing the traditional seed programmes with disease-free plants obtained from pathogen-free micropropagated shoots or micro-cuttings or microtubers or minitubers (Van der Zaag 1990; Ahloowalia 1994; Sarkar and Naik 1998; Struik and Lommen 1999; Naik and Sarkar 2001; Sarkar and Pandey 2011; Chindi et al. 2014).

5 Meristem Culture for Virus Elimination

Application of tissue culture techniques is now a widespread practice in potato seed production systems in many countries (Farrell et al. 1982; Klein and Livingston 1982; Kwiatkowski et al. 1985; Faccioli and Colombarini 1996). However, development of an efficient virus eradication technique and establishment of virus-free in vitro stocks of commercial cultivars are a crucial prerequisite for production and certification of disease-free seed tubers (Mozafari and Pazhouhandeh 2000). Some potato viruses, like the potato viruses A and Y, cause distinctive symptoms, while others, like the potato viruses M and S, are latent, showing no visible symptoms. Being dependent on the host for RNA/DNA replication and protein synthesis, there is hardly any selective interference on viral multiplication by chemicals without adversely affecting the plant nucleic acid and protein synthesis. Thus, in the absence of chemical control of viral diseases, meristem culture technique is the only option for virus elimination from systemically infected potato clones (Naik and Karihaloo 2007). It is based on the fact that viruses are either absent or present at a very low concentration in rapidly growing meristematic tissues of an infected plant. The meristematic tissues of plants, roots and tuber sprouts may be virus-free, but it is still not clear as to why the apical/axillary shoot meristems contain a little or no viruses. Although not proven unequivocally, there are different hypotheses to explain virus freedom in meristematic region. One hypothesis is rapid chromosomal replications in growing tips and high-auxin content in the meristematic region. These interfere with viral nucleic acid metabolism and, thus, are inhibitory to virus multiplication. Another hypothesis suggests that the virus particles spread through the plant vascular system, and the meristematic region, being a zone of actively dividing cells, is devoid of vascular system.

Meristem culture is a procedure in which apical or axillary growing tips (0.1–0.3 mm) are dissected and allowed to grow into plantlets on artificial nutrient media under controlled conditions. The meristem culture for virus elimination is essentially based on the principle that many viruses are unable to infect the apical/axillary meristem of a growing plant and that a virus-free plant can be produced if a small (0.1–0.3 mm) piece of meristematic tissue is propagated (Morel and Martin 1952). Although, the process is principally used for virus elimination, it often also has the

additional benefits of the simultaneous elimination of other pathogens such as mycoplasmas, bacteria and fungi (Millam and Sharma 2007).

In general, the larger the size of the meristem, the better the chances of its survival in vitro, whereas, the smaller the size of the meristem, the better the chances of it being virus-free (Naik and Karihaloo 2007). For example, meristems of ~ 0.1 mm size produce only about 10% regenerants, but virus freedom in them is about 95%. In addition to the size, the presence of leaf primordia also appears to determine the ability of a meristem to develop into a plantlet. It has been suggested that the excised tip should include the meristematic dome plus one or two leaf primordia (Mellor and Stace-Smith 1987). As the distribution of a virus within a plant is uneven, especially towards the growing tips, meristems of varying sizes are used to regenerate virus-free plants depending on the genotype and virus strain under consideration. Various other factors, such as position/location of the meristem, physiological condition of the donor plant, cultivar and cultural factors, largely affect the success of virus elimination by meristem culture. PLRV can be easily eliminated probably because this virus is limited to the phloem bundle. Similarly, PVA and PVY are easy to eliminate because they cannot invade the meristematic dome. In contrast, contact viruses like PVM, PVX and PVS are in order of increasing difficulty in getting them eliminated from the plant system. It has been found that PVY and PYA can be eliminated from about 70% of plants derived from meristems; for PYX, however, the percentage of success has never been more than 10%. Nevertheless, it is possible to eliminate viruses from potato plants following meristem culture alone, and mericlones once tested negative to the potato viruses continue to remain negative in tissue culture upon subsequent subculturing over a period of 2 years (Hedge et al. 1999).

Plant regeneration from meristems takes 4–8 months depending upon meristem size and genotype of donor plant. Also, depending upon the nature of the virus, the percentage of virus-free plants obtained from regenerated meristems is variable. Therefore, to enhance the proportion of virus-free mericlones, the meristem culture procedure is often combined with thermotherapy and/or chemotherapy. Plant culture at a high temperature (35–40 °C) for varying periods has been found to eliminate viruses from several vegetatively propagated plant species because replication of many plant viruses is significantly reduced at elevated temperatures (Spiegel et al. 1993). At high temperatures, synthesis of viral ssRNA and dsRNA is disrupted, and, on return to normal temperatures, resumption of viral RNA synthesis usually lags behind plant RNA synthesis almost by 4–20 h depending on the virus strain (Mink et al. 1998). Another reason is that the production or activity of virus-encoded movement proteins and coat proteins, which are involved in transcellular movement of viruses through plasmodesmata and long-distance movement through the plant vascular system, is disrupted at elevated temperatures (Martin and Postman 1999). A routine virus elimination programme involves growing whole plants or in vitro cultures at temperatures close to the threshold of normal plant growth. Most potato cultivars can withstand 37 °C for only a few weeks, but the exact temperature and treatment duration vary with the virus and the heat tolerance of the host plant (Khurana and Sane 1998). In potato, meristem culture combined with thermotherapy

is widely used for virus elimination (Lozoya-Saldana and Merlin-Lara 1984; Lozoya-Saldana and Madrigal-Vargas 1985; El-Amin et al. 1994; Millam and Sharma 2007). This combined approach involves incubation of infected source plants in a growth chamber at 35–37 °C under 30–50 μmol m^{-2} s^{-1} light intensity for 2–6 weeks (Mellor and Stace-Smith 1987). After thermotherapy, the meristems are excised and cultured on nutrient medium for growth and proliferation. Since potato spindle tuber viroid (PSTVd) requires high temperatures for replication and accumulation in plant tissues, thermotherapy is ineffective for its elimination, and, as a result, cold therapy (5–10 °C) followed by the excision and culture of meristems is employed for PSTVd elimination (Sarkar and Pandey 2011).

Thermotherapy-meristem culture approach is time-consuming, and its efficacy for virus elimination is low (25–40%). Besides, viruses vary widely in their susceptibility to heat therapy, and heat-stable strains of potato viruses have been reported (Mellor and Stace-Smith 1987). Therefore, an additional tool, viz. chemotherapy, i.e. treatment of plants/explants with anti-viral chemicals such as ribavirin or acyclovir, has also been used to eliminate viruses, either alone or in combination with meristem culture and/or heat treatment (Sanchez et al. 1991). A number of antiviral compounds, which are mostly synthetic nucleotide analogues known for their effectiveness against animal viruses, have been reported to eliminate both DNA and RNA plant viruses. The guanosine analogue ribavirin (virazole: 1-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), uracil analogue 5-dihydroazauracil (DHT) and DHT-derivative diacetyl-5-dihydroazauracil (DA-DHT) are particularly effective for inhibiting different plant viruses (Hansen 1988; Spiegel et al. 1993). Of these three antiviral compounds, chemotherapy of meristematic explants in vitro with ribavirin has an added advantage of eliminating major potato viruses. Though the exact mode of action of ribavirin and other antiviral chemicals on plant viruses is not clear, there are several hypotheses: (1) ribavirin triphosphate, a major derivative of ribavirin, inhibits viral RNA polymerase synthesis; (2) ribavirin-5-phosphate, a derivative of ribavirin, inhibits the activities of IMP-dehydrogenase, thereby decreasing the GTP pool and nucleic acid synthesis and (3) ribavirin interferes with capping at the 5′ end of viral mRNA leading to inefficient translation. Although other antiviral compounds, such as 5-fluorouracil, 2-thiouracil and para-fluorophenylalanine, have been tested for virus elimination in potato, they are not as effective as ribavirin (Cassells 1987). The concentrations of many antiviral compounds required to inhibit virus multiplication are very close to the toxic threshold for the host plant (Sarkar and Pandey 2011). In addition, there is always a possibility of mutations when the plants are exposed to antiviral chemicals that are essentially synthetic nucleotide analogues. The phytotoxic effects of ribavirin on potato were shown to be temporary because the plantlets obtained from ribavirin therapy developed normal shoots when they were taken out (Wambugu et al. 1985). Therefore, in vitro ribavirin therapy at low concentrations combined with thermotherapy has been widely used to eradicate most of the viruses from infected potato cultivars. This combined approach followed by simple culturing of shoot cuttings rather than meristem can even eliminate some slow multiplying viruses like PVA, PVY and

PLRV (Sanchez et al. 1991). Alternatively, new techniques of chemotherapy and electrotherapy have proven to be successful in eliminating potato viruses in recent years. Chemotherapy was employed to eliminate PVS, PVY and PVX (Conrad 1991; Faccioli and Zoffoli 1998), while electrotherapy was reported to be successful in eliminating PVX (Lozoya-Saldana et al. 1996), PVY and PLRV (Pazhouhandeh et al. 2002). Efficiencies of 60–100% were reported for electrotherapy in the production of virus-free plantlets from potato plants with a single infection of PVX (Lozoya-Saldana et al. 1996). Effects of electrotherapy, chemotherapy (ribavirin) and meristem culture were studied comparatively by Pazhouhandeh (2001) indicating that the efficiency of each technique is dependent upon the cultivar virus system and the intensity of the virus eradication treatment.

Yet another technique known as electrotherapy is employed for virus elimination from potato plants. Virus elimination from infected plants by applying electrical shock to the explants is known as electrotherapy. Shoot cuttings of PVX-infected potato plants when exposed to 5, 10 or 15 mA for 5–10 min followed by immediate culturing of the shoot tips in vitro have been shown to produce virus-free plants (Lozoya-Saldana et al. 1996). It has been shown that plant growth is not affected by electric treatment, except for a very high electric field applied for a long time resulting in plant mortality. As for virus elimination, an electric field of 10 mA applied for 5 min resulted in the highest rate of PLRV (46.7%) and PVY (40%) elimination, whereas a filed strength of 5 mA for 5 min resulted in their lowest rate of elimination (Emami et al. 2011). Electrotherapy followed by excision and culture of shoot buds produced the highest rates of PLRV- (67.2%) and PVY-free (62.8%) plantlets when cultured on MS medium supplemented with 30 g L^{-1} sucrose, 20 mg L^{-1} ribavirin and 5–10 M acetylsalicylic acid (ASA). Although, ribavirin alone is highly effective for reducing the virus concentration, but it can foster the highest regeneration rate (72.7%) when supplemented in the medium in combination with ASA. Since this combined approach does not induce any morphological variation, it appears to be the most effective option for virus elimination from systemically infected potato plantlets in vitro. Sarkar and Naik (2000) developed a highly optimized protocol for growth and development of in vitro-derived shoot tips. In this method, phloroglucinol at 0.8 mM in combination with 0.2 M sucrose fostered a high frequency of multiple shoot formation, ensuring a faster rate of potato shoot tip multiplication within a limited time and space. This protocol can be applied together with electro-chemotherapy to obtain virus-free plantlets even from shoot tips (Sarkar and Pandey 2011). Different schemes for virus elimination in potato are depicted in Fig. 3.

The confirmed and pathogen-free mericlones are subjected to rapid micropropagation methods to generate abundant clean cultures for use in potato seed production.

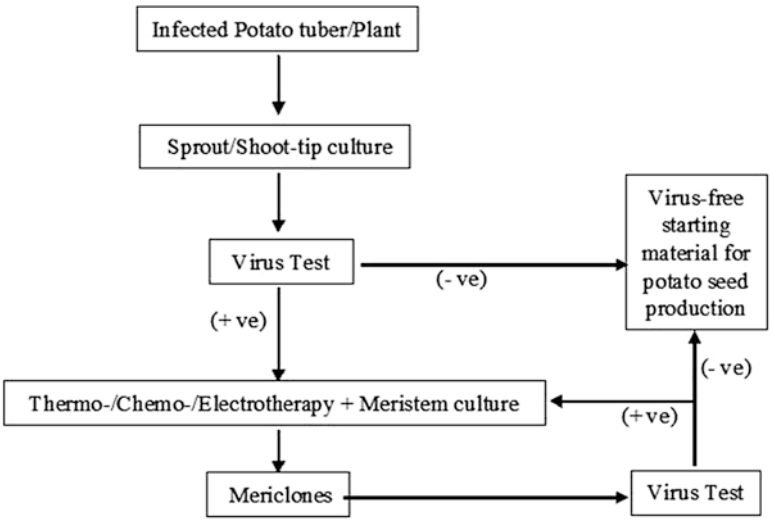

Fig. 3 Schematic presentation of virus elimination in potato

6 Micropropagation

The first establishment of tissue culture from potato tubers was attempted as early as in 1951 (Steward and Caplin 1951), and since then a variety of tissues from different plant organs, such as leaves, petioles, internode segments, ovaries, anthers, stems, roots and shoot tips, have been successfully used (reviewed in Wang and Hu 1985; Bajaj 1987; Wenzel 1994; Naik and Sarkar 2000; Naik et al. 2001). Among all tissue cultural advances, meristem culture and micropropagation are the most potential technologies for production of quality potato seed. Micropropagation refers to the production of a large number of pathogen-free in vitro plants on defined nutrient media under aseptic conditions within a limited time and space. Micropropagation currently underpins many seed potato production systems and specifically provides the nuclear stock material in the form of microplants or microtubers for their subsequent use in a chain of scientific potato seed production programmes (Chindi et al. 2014).

6.1 Micropropagation for Multiplication of Pathogen-Free Mericlones

Pathogen-free in vitro plants obtained through meristem culture are rapidly multiplied on artificial medium under controlled conditions to produce adequate number of plantlets as a starting material for seed production. In order to ensure maximum genetic fidelity of tissue culture-produced propagules, it is particularly important

that the formation of adventitious shoots (through callus or cell masses) be avoided as far as possible (Sarkar and Pandey 2011; Naik and Karihaloo 2007; Chindi et al. 2014). This is because (1) adventitious shoots, in contrast to apical/axillary shoots, frequently derive from single or small groups of cells and are, therefore, more prone to spontaneous or induced mutations (Broertjes and Van Harten 1978), (2) dedifferentiation and redifferentiation in tissue culture are associated with genetic variation and (3) some cultivars having periclinal chimeras could break up into separate genotypes following adventitious shoot formation. Therefore, the basic approach of potato micropropagation is based entirely on shoot proliferation either from apical or axillary buds. Thus, micropropagation in sensu stricto refers to only culture of pathogen-free apical/nodal cuttings on semi-solid (agarified) or liquid culture media, where axillary and terminal buds are induced to grow into new microplants in vitro (Sarkar and Pandey 2011).

Micropropagation allows large-scale multiplication of virus-free potato microplants. Usually, three to four nodal cuttings (size 1.0–1.5 cm) are cut from each meristem derived in vitro plant under aseptic conditions of laminar flow and inoculated in fresh culture tubes (three cuttings/tube) or magenta boxes (ten cuttings/tube) containing MS medium supplemented with 2.0 mgl^{-1} D-calcium pantothenate, 0.1 mgl^{-1} GA_3, 0.01 mgl^{-1} NAA and 30 gl^{-1} sucrose. The culture tubes are closed with cotton plugs and incubated under a 16-h photoperiod (50–60 μ mol m^{-2} s^{-1} light intensity) at 24 °C. Within a period of about 3 weeks, the axillary/apical buds of these cuttings grow into full plants (Fig. 4a). These plants can be further subcultured on fresh medium. Assuming effective generation of minimum three nodal cuttings by an in vitro plant and subculturing interval of 25 days, theoretically 3^{15} (14.3 million) microplants can be obtained from a single virus-free microplant in a year. The multiplication rates can further be increased many folds if liquid culture method is deployed.

Above in vitro-derived microplants are used for potato seed production by diverse means. The simplest procedure is to harden and transplant them under aphid proof net house on nursery beds for production of minitubers (Naik 2005). The in vitro plants can also be used for the production of microtubers in the laboratory (Wang and Hu 1982). Microtubers are miniature tubers developed under tuber-inducing conditions in vitro and are convenient for handling, storage and long-distance transportation. In some countries, where the environmental conditions are highly favourable like in Da Lat in Vietnam (mean maximum and minimum temperatures 23 and 14 °C with a seasonal range of 2 °C and relative humidity above 70%), farmers plant them in small pots prepared from banana leaves and then transplant in fields (Van Uyen and Vander Zaag 1983).

Fig. 4 Integration of tissue culture in potato seed production. (**a**) Rapid micropropagation of virus-free mericlones on semi-solid medium. (**b**) Multiplication of micropropagated plants in liquid medium for production of microtubers. (**c**) Potato microtubers developed under dark in MS medium supplemented with 10 mg l^{-1} BA plus 80 g l^{-1} sucrose. (**d**) Harvested microtubers. (**e**) Pathogen-free high-density potato crop raised either from in vitro plants or microtubers in net house. (**f**) Harvested minitubers from high-density net house crop. (**g**) Healthy field crop raised from minitubers harvested in previous season

6.2 Micropropagation for Microtuber Production

Seed potato production through micropropagation is mostly based on the production of pathogen-free in vitro plantlets followed by microtubers and/or minitubers (Ranalli 1997). Microtubers are miniature tubers and differ from minitubers in that they are smaller in size and are produced under aseptic in vitro conditions. The biological basis for the induction of microtuberization in potato is strongly related to the artificial simulation of the natural tuber-inducing conditions found in the field (Naik and Karihaloo 2007). The specific carbon source and its concentration within the tissue culture medium have great significance, with sucrose being the favoured substrate, largely because of its translocation to the developing microtubers (Khuri and Moorby 1995). The optimum sucrose concentration for microtuber production ranges from 60 to 80 g/l, which is up to two to three times the normal amount used for micropropagation. It has been suggested that sucrose plays a dual role in microtuber development by providing a favourable osmolarity for tuber development in addition to its role as a carbon source (Khuri and Moorby 1995; Momena et al. 2014). Apart from sucrose, other treatment combinations including effect of phytohormone on microtuberization also depend on plant genotype (Romanov et al. 2000; Kefi et al. 2000). Furthermore, cytokinin increases microtuber number because of its positive effect on cell elongation and tuberization (Romanov et al. 2000). Jasmonate is a growth regulator normally produced by plants that have been exposed to stress and has previously been found to be highly effective in the induction of microtubers (Koda et al. 1991; Van den Berg and Ewing 1991). However, although the use of jasmonate has been widely advocated, the response was shown to vary with cultivar (Pruski et al. 2003). MS basal nutrient mixtures are universally used for potato microtuberization. Sucrose is the most effective carbon source, and an increase in its concentration up to 8% induces early tuberization, whereas, at levels above 8% sucrose is inhibitory. Temperature and photoperiod are two important physical factors that affect potato microtuber induction in vitro. The optimum temperature for in vitro tuberization is 20 °C with a constant temperature being more effective than alternating day-night temperatures. Temperatures below 12 °C and above 28 °C have been found to be inhibitory to potato microtuber production. In general, optimum microtuberization occurs under continuous darkness during cytokinin-induced tuberization (Naik and Karihaloo 2007). General microtuber production procedure consists of mass multiplication of above in vitro plants in liquid propagation medium in flasks/magenta boxes. Stem segments (each with three to four nodes) obtained from five to six in vitro plants are cut and inoculated in each container containing 20 ml liquid propagation medium and the containers incubated stationary under normal potato propagation conditions (22–25 °C temperature and 40–50 μmol m^{-2} s^{-1} light intensity for 16 h). Under these culture conditions, a number of plantlets grow from axillary buds and fill the container within 15–20 days (Fig. 4b). For microtuber production, the liquid propagation medium from the container is decanted under laminar flow conditions and replaced by MS medium supplemented with 10 mg l^{-1} BA plus 80 g l^{-1} sucrose, and the cultures are incubated

under complete darkness at 20 °C. Microtubers begin to develop epigeally 1–2 weeks after incubation depending on the genotype and are harvested after 60–75 days of incubation. It has been shown that bigger microtubers can be produced by growing potato plantlets in liquid culture (Leclerc et al. 1994). However, as compared with their aerial counterparts, the microtubers remaining submerged in liquid cultures are considered to store poorly and tend to be very soft, with open lenticels that become the site of entry for pathogenic microorganisms. The factors affecting the success of in vitro tuberization and storage have been reviewed by Paet and Zamora (1991) and Donnelly et al. (2003).

Microtubers have been shown to produce genetically true-to-type potato propagules both under in vitro (Estrada et al. 1986; Tiwari et al. 2013) and ex vitro conditions (Hoverkort and Van der Zaag 1989; Rajapakse et al. 1991; Naik and Karihaloo 2007).

Microtubers can be utilized commercially for minituber production in greenhouses and, less commonly, can be directly field planted. The growth and the yield of potato plants grown from microtubers in field trials have been investigated (Kawakami et al. 2003), and it was found that the microtuber-derived plants had a lower initial increase in root and leaf area index than conventional seed tuber plants. The first tuber formation in microtuber plants was about 7 days later than in conventional seed tuber plants, and tuber bulking occurred about 14 days later in microtuber plants. At harvest, the tuber fresh weight of microtuber plants was 82% that of conventional tuber plants, suggesting the potential for using microtubers for direct field planting, although with certain limitations. Perez-Alonso et al. (2007) demonstrated that the plants that originated from microtubers produced better seed tubers than standard tissue-cultured plantlets under field conditions. One problem with the wider commercial uptake of microtubers is its small size that cannot perform adequately under field conditions. Microtubers having more than 0.5 g weight perform well in field. Although most protocols fail to generate big-sized microtubers that meet these criteria, several reports do state that microtubers of 0.5–1.0 g can be consistently generated (Akita and Takayama 1994; Leclerc et al. 1994). Big microtubers are generally easier to handle, are less subjected to excessive shrinkage in cold storage, have a shorter dormancy period and exhibit greater survival rates when planted out directly in the field (Leclerc et al. 1994). Microtubers are also convenient for handling, storage and transport of germplasm (Estrada et al. 1986) and, in comparison with in vitro plantlets, do not need a hardening period in the greenhouse or field (Ranalli 1997; Coleman et al. 2001). However, a major constraint to the wider uptake of microtubers is that microtubers, after in vitro tuberization, are generally very dormant (Tabori et al. 1999) and will not sprout unless stored for 4 months or more at low temperatures. During storage, heavy losses occur due to shrinkage, rottage and excessive sprouting. Greening of the microtubers for 10–15 days under diffused or artificial light before harvesting is highly beneficial in avoiding these losses (Naik and Sarkar 1997). During greening, the skin of the microtubers hardens, and accumulation of glycoalkaloids on exposure to light provides protection against fungal/bacterial rottage. Harvested green

microtubers are washed, treated with fungicide, dried under dark, packed in perforated polythene bags and stored at 5–6 °C in walking chamber or refrigerator till dormancy release.

Microtuber production technology is evolving rapidly. Fermenters (containers in which plant material continuously or at intervals is in contact with nutrient solution) and bioreactors (vessels containing plants on screens or porous substrate subjected to nutrient mist and aeration cycles of varying duration) have recently been described for commercial scale microtuberization. These include 8–10 L jar fermenter (Akita and Takayama 1994), nutrient mist bioreactor (Hao et al. 1998), double RITA (Teisson and Alvard 1999) and continuous immersion system with or without net (Piao et al. 2003). These fermenters and bioreactors produce more microtubers synchronously. From 100 nodal cuttings, 10 L fermenter of Akita and Takayama (1994) yielded 500–960 microtubers of uniform weight. Microtuber yields could be increased by extending microtuber growth period and refreshing the medium at intervals. With increased microtuberization time, 1653 microtubers with total fresh weight of 1420 g were produced. About 30% of these microtubers were weighing >1 g. Teisson and Alvard (1999) in 1 L RITA system (Temporary Immersion System for Plant Tissue Culture) could produce up to 3 microtubers per original node and 90 microtubers per vessel, 50% of which were above 0.5 g. Two types of low-cost automated bioreactor systems, viz. continuous immersion (with or without net) and temporary immersion using ebb and flood, were developed by Piao et al. (2003) for production of potato microtubers in two steps. These were 10 L capacity bioreactors and contained 1.5 L medium. In the first step, nodal cuttings were inoculated into the systems for growth and multiplication of plantlets. After 4 weeks, the propagation medium was replaced by microtuber induction medium. Inoculation density of 50 nodal cuttings/vessel, inclusion of 6-benzylaminopurine in microtuber induction medium and medium renewal during microtuber growth were observed to produce about 90 microtubers/vessel. These microtubers were larger than 1.1 g. Immersion-type bioreactor with net was observed to be more valuable for large-scale application.

A simple system for mass propagation and microtuber production was developed using a bioreactor without forced aeration (Akita and Ohta 1998). In this system, explants were cultured in 1 L bottles equipped with an air-permeable membrane on the cap, and these bottles were slowly rotated on a bottle roller. Microtubers of potato were induced using a two-step culture method. In the first step, potato plantlets were grown from nodal cuttings under static conditions. After shoot proliferation, the culture medium was replaced with a microtuber induction medium, and the bottles were rotated at 1 rpm. One hundred microtubers were produced per bottle in 200 ml medium, and this number of microtubers was more compared to the static cultures without rotation. These slowly rotating containers appear to be simpler and less expensive than airlift or immersion-type fermenters.

Aseptic production and possibility of reducing cost through automation are important factors that are likely to promote use of microtubers in potato seed production world over (Hoverkort and Van der Zaag 1989). Any breakthrough technology that increases microtuber size will eliminate intermediary step of

minituber production. With this increasing interest and rapid development in the field of commercial microtuber production, there are few patents describing microtuber production technology (Donnelly et al. 2003).

6.3 Minituber Production in Glasshouse or Net House

Minitubers are small seed potato tubers that can be produced in glasshouses or net houses from in vitro propagated plantlets (Wiersema et al. 1987; Naik 2005) or microtubers under high-density planting. These can also be produced in containers using different substrate mixtures (Jones 1994) or even in hydroponic cultures (Muro et al. 1997; Struik and Wiersema 1999). Lommen (1995) presented alternative production techniques for minitubers using very high plant densities and non-destructive, repeated harvesting of minitubers by lifting plants carefully from the soil mixture and replanting them after the harvest. These techniques allowed production of large number of minitubers of ideal size. Many seed programmes prefer to use minitubers, defined as the small tubers (usually 5–25 mm), which can be produced throughout the year under semi-in vitro conditions in glasshouse and screen-houses using in vitro propagated plantlets, planted at high density. This technology package is a fundamental component of many seed potato production programmes. The basis of minituber production is to facilitate the stage between the delivery of virus-free material derived from meristem culture nuclear stock and the production of tubers destined for field planting (Millam and Sharma 2007). Although schemes can vary between countries and markets, the format remains constant. The basic starting point is the nuclear/buffer stock that comprises in vitro pathogen-tested microplants or microtubers to ensure that the starting material is pathogen-free, according to a programme of official testing for indigenous and non-native pathogens. Microplants or microtubers derived from the nuclear stock can then be grown in a pathogen-free medium to produce minitubers or, less commonly, can be grown in the field to produce tubers under protected environments. Production rates vary between systems and the method of harvesting. Some commercial companies quote rates of up to 1000 minitubers per square metre with average tuber weight ranging from 1 to 5 g following a non-destructive harvesting every 40–50 days from a crop derived from a single microplant under optimum glasshouse conditions (http://www.quantumtubers.com/techinfo.htm).

Minituber is an intermediate stage of potato seed production between laboratory micropropagation and field multiplication. The simplest method of producing minitubers from in vitro plants has been described by Naik (2005). In this method, 15–20-day-old cultures are kept in glasshouse or polyhouse for 8–10 days for hardening without removing culture tube plugs or lids of magenta boxes. The hardened in vitro plants are removed from culture vessels with the help of forceps, washed to remove adhering medium and cut into two pieces after cutting out the root zone. The lower portion (about 0.5 cm) of the cuttings is dipped in rooting hormone powder (soft wood grade) and planted in pre-prepared nursery beds (soil/

FYM/sand 1:1:1 v/v) at plant-to-plant and row-to-row distance of 10 cm in a vector-free net house. It is beneficial to drench nursery beds with fungicide solution before planting. Three to four water sprays are given daily with a sprayer to keep the soil moist and maintain humidity for the initial 1 week. If available, mist irrigation is also suitable. Once the plants establish and start growing, normal irrigation with a watering can or any other means be followed. With progressive growth of the plants, additional soil substrate is added on the nursery beds to bury lower nodes (Fig. 4e). This is important to optimize minituber production from buried axillary buds. The crop is allowed to mature on the nursery bed, and minitubers are harvested (Fig. 4f). The minitubers are cold stored and used as planting material in the next crop season (Fig. 4g). In general, 80–90% cuttings establish and produce about 8–12 minitubers per plant of average 10–15 g depending upon variety. Bigger size of the minitubers as compared to microtubers facilitates robust post-harvest handling and ease in field planting (Naik and Karihaloo 2007). Minitubers can also be produced from microtubers under high-density planting on nursery beds as in the case of in vitro plants (Naik 2005). The main advantages of microtubers over in vitro plants are that they are less delicate, easy to handle and transport and require less care during planting and post-planting operations (Hoque et al. 1996; Naik et al. 2000; Wang and Hu 1982). However, it takes 2–3 months longer in laboratory for production of microtubers which also need to be stored for dormancy breaking. Further, yields from microtubers on nursery beds are similar to those of in vitro plants though microtubers tend to produce larger sized minitubers (Ahloowalia 1994).

6.4 Minituber Production Using Hydroponic and Aeroponic Systems

Recently hydroponic/aeroponic systems have been developed for production of minitubers from in vitro plants. In addition to reducing the cost of production, these systems facilitate round the year production and adoption of phytosanitary standards. Hydroponically developed minitubers such as Technitubers^{R} are produced under stringent sanitary conditions in high-density plantings and harvested at intervals from plants growing in nutrient film (Gable et al. 1990). Technitubers are miniature seed potatoes measuring 10–15 mm in diameter. These are ideal for storage, shipment and mechanized planting with the help of vacuum seeder. Agronomic packages have also been developed and field trials conducted over years in several countries, and it has been demonstrated that a healthy and vigorous potato crop can be raised from technitubers. Quantum Tubers™ is another patented process in which minitubers are produced from in vitro plants by M/S Quantum Tubers Corporation, USA. This technology also has the advantage of short propagation time for harvestable minitubers (40–50 days) and high numbers of minitubers per square metre (10,000 minitubers/m^2/annum) in the 1–5 g weight range. These

minitubers can be planted in open field conditions to propagate seed stocks. In one of the experiments conducted at the University of Wisconsin, the yields of cv. Atlantic were 45 t/ha from minitubers of 1–10 g, 35 t/ha from minitubers of 0.5–1 g and 22 t/ha from minitubers of 0.3–0.5 g (http://www.quantumtubers.com/).

Production of minitubers using aeroponics is an alternate soil-less culture technology. This technology consists in enclosing the root system in a dark chamber and spraying a nutrient solution on roots with a mist device. The modified device consists of aeroponic chamber, pump, spraying tube, timer and nutrient solution reservoir (Singh et al. 2011, 2012). Production and utilization of minitubers using aeroponics have been reviewed by Buckseth et al. (2016). A tube with several nozzles passes through the aeroponic chamber and sprays nutrient solution on root zone of plants. The aeroponic chamber has a removable top with holes for holding potato plants (Fig. 5a). Front of the aeroponic chamber is fixed with hinges and can be opened to harvest minitubers of optimum size repeatedly at different time intervals. In vitro plantlets are planted in the holes and fixed by sponge. The nutrient solution is sprayed for 30 s after every 3 min in initial growing stages. After 1 week, root system starts developing inside the growth chamber. The nutrient solution spraying interval is prolonged up to once in 15 min with progressive growth of the plants. Stolon and tuber formation is initiated at different intervals depending upon the variety. Picking of the tubers starts after 60–65 days when some of the tubers attain 15–17 mm dia. Once the first flush is harvested, formation of additional tubers is triggered resulting into more minitubers/plant (Fig. 5b). In this system, harvesting is done after every 2 weeks, and in the total four flushes are taken. On an average 30–35 minitubers can be harvested from a single plant as against 8–10 minitubers under the net house. These harvested minitubers (Fig. 5c) are stored at 2–4 °C and are used for planting in the next season (Fig. 5d, e). There are differential responses of varieties and day lengths on growth of in vitro plants. Therefore, there is a need to work out nutritional and plant management (bower system under long day conditions) requirements for varieties and growing conditions (Singh et al. 2011, 2012; Pandey and Singh 2014; Pandey et al. 2014; Buckseth et al. 2016).

It is essential to maintain pH of the nutrient solution at the desired level throughout the crop period and its replenishment from time to time (Otazu 2010). In aeroponic system, it is possible to produce 2094 minitubers/m^2 as compared to 771 minitubers/m^2 in nursery beds/substrate culture. Therefore, aeroponic technique offers many interesting opportunities for developing enhanced production systems, mainly for minitubers. Although requiring a degree of technical sophistication in design, establishment and run, the benefits offered are sufficient for such systems for their adoption by potato seed production companies worldwide (Millam and Sharma 2007; Buckseth et al. 2016).

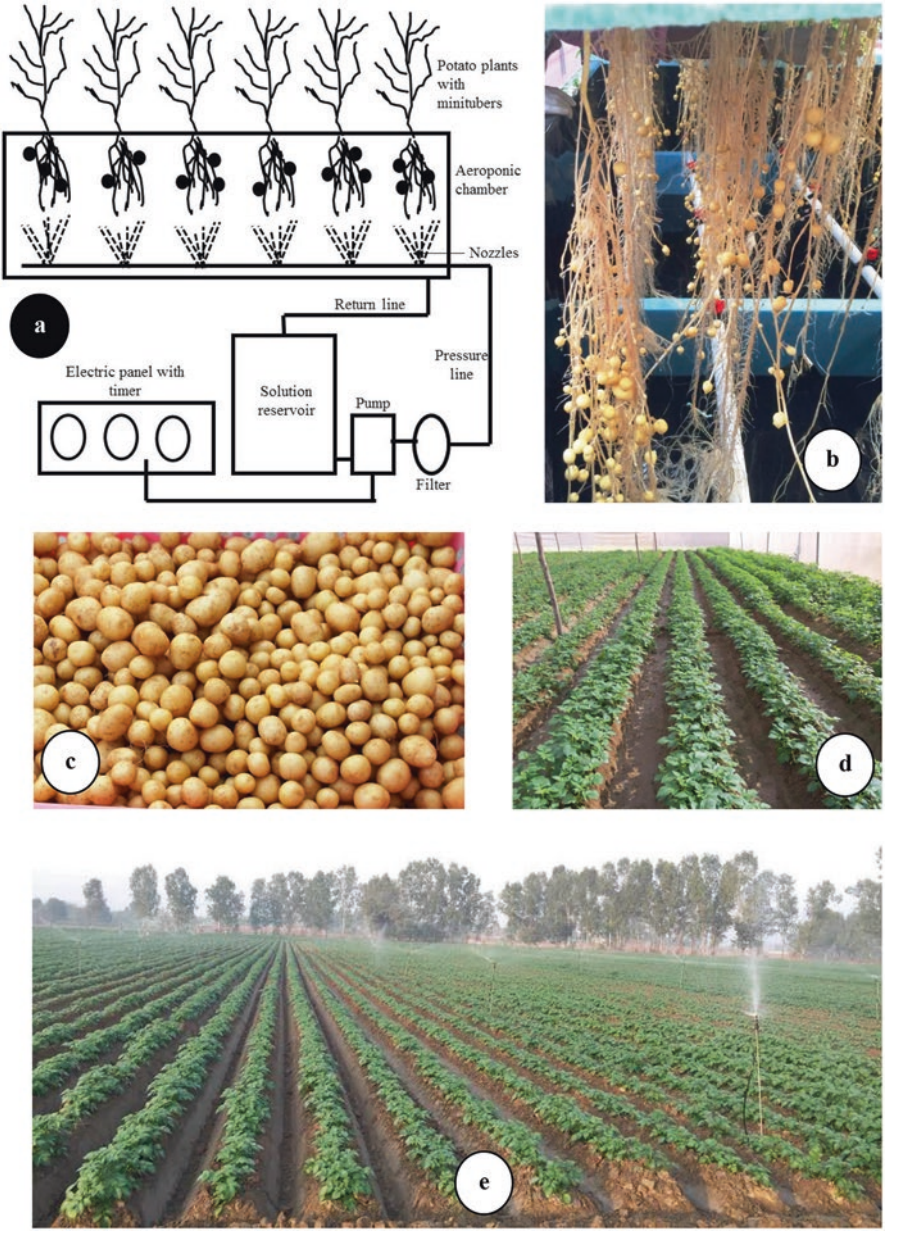

Fig. 5 Aeroponics in potato. (**a**) Diagrammatic presentation of aeroponic system. (**b**) Minitubers developed in aeroponic chamber. (**c**) Harvested minitubers. (**d**) Minituber crop in net house. (**e**) Minituber crop in the field

7 Potato Seed Production Using Minitubers

The most common intermediate stage of potato seed production between laboratory micropropagation and field multiplication is minituber. As described above minitubers can be produced from in vitro plants or from microtubers. Researchers in Taiwan (Wang and Hu 1982) reported production of 36,000 microtubers from 1200 culture flasks incubated on 10 m^2 area in a period of 4 months in stationary cultures, and after 3 field multiplications, these microtubers produced 1800 t potato seed, which was enough for 2000 ha on a schedule of one-third land rotation per year. In India, Naik (2005) reported a possibility of producing 264,500 tubers (13.2 t) after 1 nursery bed and two field multiplications of microtubers obtained from 1 in vitro plant.

The minitubers ('nucleus seed' or G-0 stage), thus, produced are cold stored and used for field planting in next season. The pathogen-free nuclear seed is multiplied two times (G-1 and G-2 stages) under strict sanitary and phytosanitary conditions on research farms to produce 'basic seed' (produce of G-2 stage). This basic seed is further multiplied by registered growers and other seed-producing agencies for 3 more years (G-3, G-4 and G-5 stages) to produce 'certified seed' (produce of G-5 stage) as per minimum seed certification standards. In all these multiplications, limited generation system wherein the planting of each seed class is limited as per the eligibility by compliance with established disease tolerance levels and the number of field multiplications in a particular country is followed. During potato production, the plant is constantly exposed to sources of contamination. The probability of a seed tuber or seed lot becoming infected with pathogens progressively increases every year. To minimize this, seed certification agencies have enacted regulations to basically restrict or limit the number of years the seed lot can be eligible for the seed certification process. A generalized potato seed production system using tissue culture as proposed by Naik and Khurana (2003) is shown in Fig. 6.

8 Future Prospects

According to recent estimates, developing countries are likely to have higher growth rates in production and productivity of potatoes during 1993–2020 than the world average growth rate (Scott et al. 2000). According to these estimates, the developing countries are likely to produce 48% world potatoes by 2020 as against 10.5% in 1961. The increase will be more pronounced in non-traditional potato-producing countries. Shortage of good quality seed is recognized as the single most important factor inhibiting potato production in these countries. Availability of quality planting material of improved potato varieties in adequate quantities, therefore, is the major issue that needs to be attended by respective national agricultural research system in order to attain above production projections.

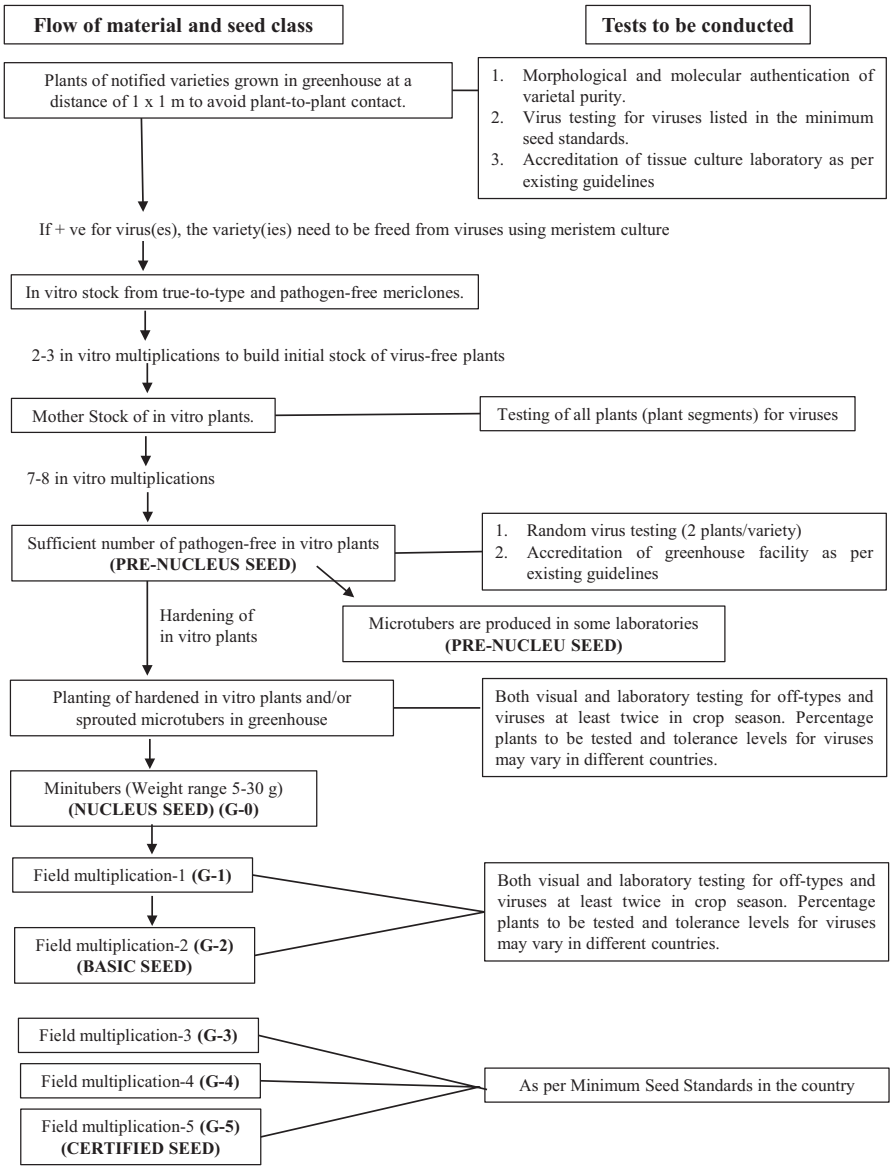

Fig. 6 Integration of tissue culture in quality potato seed production

Strengthening of potato seed production system by integrating micropropagation is a sound technological option. Large-scale micropropagation and microtuber production still continues to be time-consuming and labour intensive. Automation of micropropagation is expected to reduce the production cost as well as increase the production per unit time and space. Ex vitro or semi-in vitro minituber production

from in vitro plants/microtubers is more promising than micropropagation alone. The success of such adoption, however, will rely heavily on simultaneous development of low-cost virus/pathogen diagnostic methods and development of seed certification standards. In terms of greater efficiency of seed potato production and reduced energy input, research into soil-free techniques will continue to be the subject of focus in both established and developing potato-producing areas in the near future. Advances in engineering technology will also assist in the development of more automated and controlled seed propagation systems. However, there are also options for simplifying the seed potato production systems for adaptation to low-technology situations, which has greater scope and relevance towards the increasing trends of potato production in developing countries.

The other important components of seed security that need immediate attention are (i) capacity and infrastructure building, (ii) developing national seed policies (encompassing variety development, evaluation and registration, plant variety protection legislation, seed certification standards, quarantine, etc.) and (iii) harmonizing regional and global seed policies (standards for seed purity and testing, plant protection and quarantine regulations, plant variety protection, uniform seed certification and marketing regulations) to facilitate exchange of seed. Once a strong formal seed system is in place, it needs to be integrated with informal seed system and private sector so that benefits of new varieties and technological advances percolate rapidly to the farming communities.

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In Vitro Approaches for the Improvement of Eucalyptus

Shuchishweta Vinay Kendurkar and Mamatha Rangaswamy

Abstract Eucalyptus is an economically important hardwood tree. The ever-increasing demand for *Eucalyptus* wood for industrial uses has necessitated better and faster methods of propagation of genetically superior trees with better qualities. Micropropagation has been considered as a potential method for the mass propagation of mature, difficult-to-propagate/difficult-to-root trees and natural hybrids resulting in the production of quality propagules for plantation forestry, afforestation programmes, etc. In vitro culture of *Eucalyptus* dates back to the 1960s with successful culture of juvenile tissue and eventually with mature tree-derived explants in the last few decades. Although mass propagation through axillary meristem proliferation has witnessed the success, the full potential of organogenesis and somatic embryogenesis is yet to be realized. These modes will have the wider application like cryopreservation, synseed development, somaclonal variation and genetic transformation. Clonal propagation of *Eucalyptus* is a commercial reality being practiced for large-scale multiplication of identified, superior clones and their field planting. The great potential of micropropagation for large-scale plant multiplication can be tapped by cutting down the cost of production per plant by reducing the unit cost of micropropagule and plant production without compromising the quality. With the ever increased rate in which the forest cover is getting disappeared from the earth, it is an urgent call to restore at least a few percentage of the lost plantation to avoid severe climatic changes.

Keywords Eucalyptus · Clonal propagation · Micropropagation · Organogenesis · Somatic embryogenesis · Hardening · Acclimatization · Field planting

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1 Introduction

Trees are an integral part of forests at social, economic and ecological levels. The contributions of trees to the well-being of humankind are far reaching in combating rural poverty, ensuring food security and providing decent livelihoods. They play a pivotal role in providing clean air, water, recycling of carbon, conservation of biodiversity and mitigation of climate change. India occupies the tenth position with 70,682 hectares of forest covering 24% of land area, which is 2% of the total global forest (FAO 2015). Over the last 25 years, the area of planted forest has increased in all climatic domains, by over 105 m/ha since 1990, and accounts for 7 percent of the world forest (FRA 2015). However, the annualized rate of increase in the area of planted forests is 1.2%, whereas the 2.4% rate suggested is needed to supply all of the world's timber and fibre needs (Payn et al. 2015). In the future, well-managed planted forests will be the source of wood, fuel, paper and other wood-derived products as these tree plantations usually have higher yields of wood with annual growth rates of 10–30 m^3/ha compared to 1–5 m^3/ha for natural unmanaged forests (Evans and Turnbull 2004). Hardwoods represent approximately 40% of the forest plantations in the world which mainly include *Eucalyptus* spp. and *Populus* spp., Conifers like *Pinus* spp., *Picea* spp., and *Pseudotsuga* spp. are the most widely planted timber and pulpwood species, covering over 60% of the planted forests worldwide (FAO 2000). *Eucalyptus* is the most characteristic genus, widely planted in many countries for timber, oils, tannins, etc., and dominates the scenery in parts of some countries like California, East Africa, Sri Lanka, India and Portugal (Expert workshop report: world heritage Eucalypt theme 1999). The term 'eucalypt' refers to taxa in the family Myrtaceae which is remarkable for its rapid growth. Most of the species are popularly called gum trees in Australia, although the exudation from them is not a gum, but it is an astringent, tanniferous substance called KINO (Anonymous, Wealth of India).

1.1 Taxonomic Diversity of Eucalypts

Eucalypts represent a large taxonomic group of woody dominants, which has diverged almost entirely on a single continent (Mabberley 1997). A number of taxonomic classifications have been proposed for eucalyptus at different times. Some have recognized the existence of separate taxon groups within *Eucalyptus* (Bentham 1867; Blakely 1934; Pryor and Johnson 1971; Chippendale 1988). In general, the comprehensive classification by Pryor and Johnson (1971) has been the most widely accepted. The taxon *Eucalyptus* is divided into eight subgenera, the subgenus Symphyomyrtus being the largest and containing the majority of the species (Poke et al. 2005), all with great environmental value; 37 of these species are of interest for the forest industry while only 15 are used for commercial purpose. The species that are distributed among the major eucalypt groups are *Angophora* (11–13 species); *Corymbia* gen.nov (102+ species) and *Eucalyptus* sensu stricto (600+ species) (Ladiges 1997).

1.2 Genetic Diversity

The overall level of genetic diversity of the eucalypts is high (Moran 1992) comparable with gymnosperms and other long-lived woody perennials (Potts and Wiltshire 1997). Widespread eucalyptus species tend to have greater levels of intra- and interpopulation genetic diversity compared with more localized species (Potts and Wiltshire 1997). Eucalypts are diploid plants with a haploid chromosome number of 11. So far there are no known polyploids in the genus (Eldridge et al. 1993; Potts and Wiltshire 1997).

1.3 Eucalyptus Present Scenario

The eucalypts are highly diversified (Pryor and Johnson 1971). The genus includes over 800 species, and more than 200 species have been introduced to other countries (Mabberley 1997). At present, Eucalyptus is grown in over 90 countries of tropical and subtropical areas, covering more than 22 million hectares worldwide, although only 13 million hectares have industrial productivity. Natural populations of eucalypts in Australia form the genetic resource base, for domestication programmes being undertaken in countries around the world (Eldridge et al. 1993).

1.4 Economic Importance

Eucalypts are the most important plantation hardwoods, yielding industrial wood, fuel wood, essential oils, shade and shelter. It is used worldwide as a raw material for pulp and paper industry. Estimated demand of hardwood requirement for the pulp and paper industry is 3.18 million tons per year. Wood is also used for light and heavy construction, railway sleepers, bridges, piles, poles and mining timber. Leaves are rich in essential oils that have many medicinal uses. Leaf extracts have pesticidal properties and can be promoted as a biopesticide. *E. tereticornis* is a major source of pollen in apiculture and produces a medium amber honey of distinctive flavour. The wood and bark of the tree have tannin content of 6–12% and 3–15%, respectively, though not used as a commercial source of tannin (Turnbull 1991). Outstanding diversity, adaptability and growth of eucalypts have made them a global renewable resource of fibre and energy, representing a suitable option to meet the growing wood demands of the world and a great way to prevent deforestation of natural forests. In the afforestation programme, eucalypts are the most widely planted hardwoods in the tropical and subtropical regions of the world (Eldridge et al. 1993). Eucalypts are capable of exceptionally high growth rates when planted outside of their natural Australian habitats, and they have been described as the most important plantation trees worldwide (Mabberley 1997). Ten

eucalypt species contribute to most overseas plantings: *Eucalyptus grandis*, *E. saligna*, *E. globulus*, *E. camaldulensis*, *E. tereticornis*, *E. urophylla*, *E. robusta*, *E. maculata*, *E. paniculata* and *E. viminalis* (Brown and Hillis 1978). An estimated several million hectares of eucalyptus plantations have been established in other countries, particularly in Brazil, Chile, China and South Africa. Their principal uses in other countries include wood production for building, fibre and fuels including charcoal (Eldridge et al. 1993; Clark 1995). They are also used for a wide variety of other purposes such as a source of oils and tannins, including medicinal, industrial chemical products, as ornamentals and for cut foliage (Boland et al. 1991; Mabberley 1997). Potts and Dungey (2004) reported that *E. grandis*, *E. urophylla* and their hybrids are most favoured for pulp and increasingly for solid wood production in tropical and subtropical regions, whereas *E. globulus* is more common in temperate regions (free of severe frosts). In comparison with other *Eucalyptus* species, *E. globulus* has superior fibre morphology and requires fewer chemicals to obtain bleached pulp due to its lower lignin content (Eldridge et al. 1993; Doughty 2000). *E. camaldulensis* is known for its ability to thrive in arid and semiarid regions, *E. urophylla* is highly productive, and *E. nitens* is recognized for its adaptability to colder climates (Teulieres and Marque 2007). *E. tereticornis*, hybrid 'Mysore gum', is the main species planted in India, which is fast growing and produces timber and paper pulp as well as being used in amenity planting (Anonymous Wealth of India; Gupta et al. 1991).

1.5 Eucalyptus in India

Eucalyptus has a long history in India. It was first planted around 1790 by Tippu Sultan in his palace garden on Nandi Hills near Bangalore. According to one version, he received seeds from Australia and introduced about 16 species (Shyam Sundar 1984). Subsequent to it, the next significant planting of *Eucalyptus* was in Nilgiri Hills, Tamil Nadu, in 1843, and regular plantations of *E. globulus* were raised to meet the demands for firewood, from 1856 (Wilson 1973). There were several other attempts to introduce Eucalyptus at various parts of the country. It is reported that during 1954–1955, herbarium specimens of eucalyptus trees grown at Nandi Hills were sent to Australia and identified as *E. camaldulensis*, *E. citriodora*, *E. major*, *E. intermedia*, *E. polyanthemos*, *E. robusta*, *E. tereticornis*, *E. tessellaris*, a hybrid of *E. robusta* x *E. tereticornis* and a hybrid of *E. botryoides* x *E. tereticornis*. The first Forest Department *Eucalyptus* plantation was in 1877 at Malabavi (Devarayanadurga), Tumkur District (Kadambi 1944). Until the end of the nineteenth century, small blocks of plantations were raised, often for experimental purposes (Nanjundappa 1957). The total area of eucalypts planted in India is estimated to be at least 2,500,000 ha (Dewees and Saxena 1995; Midgley et al. 2002). Several pulp and paper companies, forest departments and forest development corporations have substantial areas of plantations either directly under their control from which

timber is procured on a buy-back basis (Verghese et al. 2009). The wood yield from Mysore gum plantations averages around 7 m3/ ha/year (Chandra et al. 1992).

2 Clonal Propagation of Eucalyptus

The ever-increasing demand for *Eucalyptus* wood for industrial uses has necessitated better and faster methods for propagation of genetically superior trees with better qualities, such as straight and clear bole, disease and pest resistance, drought tolerance, fast growing, etc. Due to extensive cross-pollination, seedlings of superior trees do not maintain these characteristics, exhibiting a lot of heterogeneity, poor survival and low productivity among seed-raised populations (McComb 1995). Therefore, in such cases there is a need for the alternative methods of propagation. Macropropagation through conventional route of cuttings is a reality for a large number of amenable genotypes. Prior to progress with mass vegetative propagation, all *Eucalyptus* plantations were derived from seeds with varying degrees of establishment and competitive success. Due to inbreeding depression from self and/or related mating, observed genetic parameters derived from open-pollinated eucalypt populations may be inaccurate (Watt et al. 2003). Clonal propagation represents the most effective way of capturing both additive and nonadditive genetic effects brought about by both traditional tree breeding and biotechnology, thus accelerating exploration of genetic gains in plantations (Mullin and Park 1992). *Eucalyptus* vegetative propagation is mainly done with rooted cuttings, a method explored in several clonal propagation programmes (Watt et al. 2003; de Assis et al. 2004). However, this strategy is limited by the heterogeneous rooting ability response among clones and decreasing rooting potential because of ageing of parent plants (Eldridge et al. 1993; Watt et al. 2003; Mankessi et al. 2010).

3 In Vitro Propagation

Micropropagation, the process of rapid multiplication of selected clones via in vitro techniques, occupies a key position in the tree improvement programmes. It has been considered as a potential method for the mass propagation of mature, difficult-to-propagate/difficult-to-root trees and natural hybrids resulting in the production of quality propagules for plantation forestry, afforestation programmes and clonal banks. It helps to overcome seed dormancy, scarce seed supply, low rates of rooting of cuttings and grafting associated with conventional vegetative propagation. It can contribute in capturing existing genetic gains by mass multiplication of selected genotypes resulting in increased productivity per unit area (Timmis et al. 1987; Kendurkar 2013). The rapid growth and uniformity of the planting material are some major benefits of the micropropagation techniques (Tables 1, 2 and 3).

Table 1 In vitro shoot proliferation in *Eucalyptus*

I	II	III	IV	V	VI	VII	VIII	IX	X	XI
Sl. No	Species	Explant type	Basal medium	Sucrose	Additives	Plant Growth regulator	Culture conditions	Response	Plantlets	Reference (By year)
1	*E. grandis*	Nodal explants	Specific media							de Fossard et al. (1973)
2	*Eucalyptus spp.*	Seedling explants	MS	2%	Ferric citrate 20 mg/l	BAP	Light	Shoot buds		Sita and Vaidyanathan, (1979)
3	*E. citriodora*	Shoot tips from 2–3-year-old tree	MS			BAP, IAA		Shoot buds	Yes	Grewal et al. (1980)
4	*E. citriodora*	Apical tips of 20-year-old tree	MS	2%	Calcium pantothenate (0.1 mg/l), biotin (0.1 mg/l)	Kn, BAP	Initially in light and later shifted to 16 h light/8 h dark	Shoot buds and callus	Yes	Gupta et al. (1981)
5	*E. citriodora*	Buds from mature trees	MS	2%	Calcium pantothenate (0.1 mg/l), biotin (0.1 mg/l)	Kn, BAP	Initially in light for 72 h and later shifted to light	Shoot buds	Yes	Mascarenhas et al. (1982)
6	*E. tereticornis* *E. globulus* *E. citriodora*	Terminal buds from 10–20-year-old tree	MS	2%	Calcium pantothenate (0.1 mg/l), biotin (0.1 mg/l)	Kn, BAP	Initially in light and later shifted light	Shoot buds	Yes	Mascarenhas et al. (1982)

7	*E. nova-anglica*, *E. viminalis*	Cotyledon, shoot tips, hypocotyls and root-tip explants of seedlings	GD			Zn, IBA	Continuous light	Shoot buds	Yes	Mehra-Palta (1982)
8	*E. torelliana and E. camaldulensis*	Nodal segments from 20–30-year-old trees	MS	2%	Calcium pantothenate (0.1 mg/l), biotin (0.1 mg/l)	Kn, BAP	Initially in light for 72 h and later shifted to 18 h light/6 h dark	Shoot buds	Yes	Gupta et al. (1983)
9	*Eucalyptus* spp.	Shoots	Modified MS/2	3%		BAP, NAA	16 h light/8 h dark	Shoot buds and callus	Yes	Boulay (1983)
10	*E. grandis*	Nodal segments of 5-year-old mature tree	MS	nd	Thiamine	BAP, NAA	nd	Multiple shoot buds	Yes	Lakshmi Sita and Rani (1985)
11	*E. grandis*	Nodal segments of juvenile and mature stages	MS	2%	PVP (800 mg/l)	BAP, NAA	16 h light/8 h dark	Shoot buds	Yes	Sankara Rao and Venkateswara (1985)
12	*E. parvifolia*	Apical shoots of 2-year-old tree	Quoirin and Le Poivre (1977) Macro elements de Fossard (1977) vit	3%		GA3		Elongated shoot buds		Texier and Foucher (1986)
13	*E. tereticornis*	Nodal segments	MS		Adenine sulfate (5 mg/l)	BAP, Kn		Shoot buds	Yes	Ilahi and Jabeen (1987)

(continued)

Table 1 (continued)

I	II	III	IV	V	VI	VII	VIII	IX	X	XI
Sl. No	Species	Explant type	Basal medium	Sucrose	Additives	Plant Growth regulator	Culture conditions	Response	Plantlets	Reference (By year)
14	*E. citriodora* *E. camaldulensis* *E. tereticornis* *E. globulus* *E. torelliana*	Nodal segments from mature trees	MS	2%	----------	--------	18 h light/6 h dark			Gupta and Mascaranhens (1987)
15	*E. dunnii*	Nodal segment of greenhouse-grown plants	MS	3%	Adenine sulfate (20 mg/l)	BAP, IBA	16 h light/8 h dark	Shoot buds	Yes	Graca and Mendes (1989)
16	*E. dunnii x Eucalyptus* spp.	Nodal segment of greenhouse-grown plants	MS+ GW vitamins	3%	Activated charcoal	BAP, IBA, Kn	16 h light/8 h dark	Shoot buds	Yes	Fantini and Graca (1989)
17	*E. urophylla*	3-week-old seedlings	MS			BAP, NAA or IBA		Shoot buds		Umboh et al. (1989)
18	*E. tereticornis*	Nodal segments from 8–10-year-old trees	MS			BAP, NAA		Shoot buds	Yes	Das and Mitra (1990)
19	*E. grandis*	Nodes from 3-month-old seedlings	MS	3%		BAP, NAA				MacRae and van Staden (1990)
20	*E. tereticornis*	Shoots from aseptic seedlings	B5 and others			BAP, IBA			Yes	Subbaiah and Minocha (1990)

21	*E. globulus*	Epicormic or coppice shoots from 12-year-old tree	De Fossard (1974)			BAP/Kn, IBA			Yes	Trindade et al. (1990)
22	*E. grandis* hybrids	Nodal segments from selected clones	Modified MS		Glutamine 200 mg/l, Coconut water 10%	NAA, BAP, IBA	Initially dark and later 16 h light/8 h dark	Shoot buds	Yes	Warrag et al. (1990)
23	*E. camaldulensis*	In vitro shoots	MS	3%	Activated charcoal 2 g/l	IBA, GA3,	Dark, later 12 h light/12 h dark	Nodular meristematic clusters	Yes	Boxus et al. (1991)
24	*E. regnans*	Nodal segments from in vitro seedlings	MS, WP, Hoagland's	2%	Activated charcoal	Zeatin, NAA, IBA	12 h light/12 h dark	Shoot buds	Yes	Blomstedt et al. (1991)
25	E. dalrympleana	Nodal segments from sterile seedling shoots	MS, ½ Knop (1865)	3%	Activated charcoal 10 g/l	BAP, NAA, IBA		Shoot buds	Yes	Kuzminsky and Lubrano. (1991)
26	*E. grandis*	Shoots from aseptic seedlings	MS, de Fossard (1977) vitamins, ½ Knops (1865)			BAP, NAA	Darkness 3 weeks	Shoot buds	Yes	Lubrano. (1991)
27	*E. macarthurii*, *E. smiithii*, *E. macarthurii x E. grandis*, *E. saligna*	Nodal explants from field-grown seedlings	MS	2%	PVP (1.0 g/l)	BAP	Initially dark and later 16 h light/8 h dark	Shoot buds	Yes	Roux and Staden (1991)

(continued)

Table 1 (continued)

I	II	III	IV	V	VI	VII	VIII	IX	X	XI
Sl. No	Species	Explant type	Basal medium	Sucrose	Additives	Plant Growth regulator	Culture conditions	Response	Plantlets	Reference (By year)
28	*E. marginata*	Nodes from mature tree	MS, ¼ MS major and minor minerals	2%	¼ $CaCl_2$	BAP, NAA, IBA			Yes	Bennett et al. (1986, 1992, 1993)
29	*E. grandis*	Segments from sterile seedlings	MS	3%		NAA, BAP		Shoot buds		Watt et al. (1991)
30	*E. radiata*	Nodal segments from mature trees and coppice	MS, ½ MS	3%		NAA, BAP, GA3		Shoot buds	Yes	Chang et al. (1992)
31	*E. sideroxylon*	Seedling shoots or nodes	½ MS, B5 vitamins	2–6%		IBA, NAA				Chang et al. (1992)
32	*E. torelliana X E. citriodora*	Nodal segments from 8-year-old tree	MS	3%		BAP, NAA, IBA		Shoot buds	Yes	Kapoor and Chauhan (1992)
33	*E. regnans*	Nodal segments Seeds germinated aseptically	MS			Zeatin, NAA, IBA	12 h light/12 h dark	Shoot buds	Yes	Willyams et al. (1992b)
34	*E. globulus*	Nodal segments Seeds germinated aseptically	MS, WPM			Zeatin, IAA, IBA	12 h light/12 h dark	Shoot buds	Yes	Willyams et al. (1992a)

35	*E. globulus*	Nodal explants of seedlings, coppice from the stumps of 4–5 years old	MS	2%		BAP, NAA, Kn	16 h light/8 h dark	Shoot buds	Yes	Bennett et al. (1994)
36	*E. grandis x E. urophylla*	Nodes from hedged cuttings	½ MS	2%	Activated charcoal 10 g/l	BAP, NAA			Yes	Jones and van Staden (1994)
37	*E. grandis*	Nodes from coppice shoots	Laine and David (1994)	2%		BAP, NAA, Zn				Laine and David (1994)
38	*E. marginata*	Nodes from 1 ½-year-old plants	MS, ¼ MS minerals, full micro, ½ Fe	2%		BAP or TDZ, NAA, IBA		Shoot buds	Yes	McComb et al. (1990, 1994)
39	*E. microcorys*	Juvenile material	MS/2			BAP, IBA		Shoot buds	Yes	Niccol et al. (1994)
40	*E. nitens*, *E. urophylla*	Nodes from 1 ½-year-old plants	MS, ½ MS	2%		NAA, TDZ, IBA		Shoot buds	Yes	Tibok et al. (1994)
41	*E. grandis x E. urophylla*	5-month-old epicormic shoots	Gonçalves (1980) modified media	3%		BAP	16 h light/8 h dark	Shoot buds		Belt et al. (1995)
42	*E. grandis*, *E. urophylla*	In vitro germinated seeds	MS/2			IBA,	14 h light/10 h dark	Growth	Yes	Grattapaglia et al. (1995)
43	*E. calycogona*	Nodal explants from 1–2-year-old tree	MS	3%		BAP, Kn, NAA,IBA		Shoot buds	Yes	McComb et al. (1996)

(continued)

Table 1 (continued)

I	II	III	IV	V	VI	VII	VIII	IX	X	XI
Sl. No	Species	Explant type	Basal medium	Sucrose	Additives	Plant Growth regulator	Culture conditions	Response	Plantlets	Reference (By year)
44	*E. halophila*, *E. radiate*, *E. spathulata*, *E. wandoo*, *E. yilgarnesis*	Nodal explants from 1–2-year-old tree	MS, ½ MS	3%		BAP, NAA, IBA		Shoot buds	Yes	McComb et al. (1996)
45	*E. kondininensis*, *E. kumarlensis*, *E. myriadenu* *E. occidentalis*, *E. platycorys*	Nodal explants from 1–2-year-old tree	MS, ½ MS	3%		BAP, NAA, IBA		Shoot buds	Yes	McComb et al. (1996)
46	*E. globulus*	Nodal segments from epicormic shoots of 10–30-year-old tree	de Fossard (1974)	Glucose 3%		BAP, IBA	16 h light/8 h dark	Shoot buds	Yes	Trindade and Pias (1997)
47	*E. grandis*	Nodal explants from orthotropic shoots of 25-year-old tree	MS	3%	nd	BAP, IBA, NAA	Initially dark and later 16 h light/8 h dark	Shoot buds	Yes	Wachira (1997)
48	*E. tereticornis*	Nodal segments from adult trees	MS		Calcium pantothenate	BAP, Kn, biotin		Shoot buds	Yes	Patil and Kuruvinashetti (1998)

49	*E. nitens* *E. globulus*	Cotyledon and hypocotyledon explants from in vitro germinated open-pollinated seeds	MS	3%		NAA, BAP	16 h light/8 h dark	Indirect shoot regeneration, shoot buds	Yes	Bandyopadhyay et al. (1999)
50	*E. tereticornis*	Nodal segments from actively growing branches of elite 4-year-old tree	Nutrient media by Das and Mitra (1990)	3%		NAA, BAP	16 h light/8 h dark	Shoot buds	Yes	Sharma and Ramamurthy (2000)
51	*E. camaldulensis*	Nodal segments with two unfolded leaves from elite 30-year-old tree	Modified MS	Not used	CO_2, florialite		16 h light/8 h dark		Yes	Zobayed et al. (2000)
52	*E. gunnii*	Leaves, internodes, nodes from in vitro cultures	MS	3%		Picloram, BAP	Initially dark and later 16 h light/8 h dark	Indirect shoot organogenesis		Herve et al. (2001)
53	*E. grandis* Hill ex Maiden	Nodal segments from elite trees	MS/2			BAP, IBA		Shoot buds	Yes	Castro and Gonzalez (2002)
54	*E.* tereti*cornis*	Nodal segments	MS	3%	CO_2	BAP	16 h light/8 h dark	Shoot buds		Sha Valli Khan et al. (2002)
55	*E. pellita*	Nodal segments from greenhouse stock material	DKW	2%	Activated charcoal	BAP, NAA		Shoot buds	Yes	Moon et al. (2003)

(continued)

Table 1 (continued)

I	II	III	IV	V	VI	VII	VIII	IX	X	XI
Sl. No	Species	Explant type	Basal medium	Sucrose	Additives	Plant Growth regulator	Culture conditions	Response	Plantlets	Reference (By year)
56	*E. citriodora*	Shoot apex and nodal segments from aseptically grown seedlings	MS, WP, SH			BAP, NAA		Shoot buds	Yes	Koriesh et al. (2003)
57	*E. camaldulensis*	Nodal explants from 2–3-year-old plant	MS	5%	Adenine sulfate (10 mg/l)	BAP, Kn	16 h light/8 h dark	Adventitious shoot buds through indirect regeneration	Yes	Rahim et al. (2003)
58	*E. nitens*	Nodal segments from 8-week-old seedlings	MS/2	2%		NAA, BAP	16 h light/8 h dark	Shoot buds	Yes	Gomes and Canhoto (2003)
59	*E. grandis x E. urophylla*	In vitro leaf explants	MS salts and White vitamins	3%	PVP (800 mg /L0	TDZ, BAP and NAA	16 h light/8 h dark	Adventitious shoot buds through indirect regeneration		Alves et al. (2004)
60	*E. phylacis*	Nodal explants	Half MS	nd	Activated charcoal (1%); potassium citrate:citrate (10:1 ratio)	Zeatin, TDZ, GA3	Initially dark and later 16 h light/8 h dark	Adventitious shoot buds through indirect regeneration	Yes	Bunn et al. (2005)

61	*E. camaldulensis*	Cotyledonary explants	MS	3%		NAA, BAP	16 h light/8 h dark	Adventitious shoot buds through indirect regeneration	Yes	Dibax et al. (2005)
62	*E. urograndis*	Nodal segments from in vitro cultures	MS liquid	Not used	CO_2	IBA	16 h light/8 h dark	Shoot growth	Yes	Tanaka et al. (2005)
63	*E. grandis* Hill ex Maiden	Nodal segments from plus trees	MW			BAP, NAA	10 h light/14 h dark	Shoot buds	Yes	Chen et al. (2006)
64	*E. erythronema*× *E. stricklandii*	In vitro germinated seedlings	WPM	2%		BAP, NAA, GA3	16 h light/8 h dark	Shoot buds	Yes	Glocke et al. (2006)
65	*E. erythronema*, *E. stricklandii* and their hybrids	Apex and leaf explants of 18-month-old plant from open-pollinated seeds	MS, WPM	3%		BAP	Initially dark and later 16 h light/8 h dark	Shoot buds		Glocke et al. (2006)
66	*E. grandis x E urophylla clones*	Nodal segments from 5-month-old vegetatively propagated plants	MS	3%	Biotin (0.1 mg/l) and calcium pantothenate (0.1 mg/l)	IAA, BAP	16 h light/8 h dark	Indirect shoot regeneration	Yes	Hajari et al. (2006)
67	*E. marginata*	In vitro cultures from 12–18-month-old seedlings	MS	2.5%	2-(N-morpholino) ethanesulfonic acid (MES)	NAA, BAP	16 h light/8 h dark	Shoot buds	Yes	Woodward et al. (2006)

(continued)

Table 1 (continued)

I	II	III	IV	V	VI	VII	VIII	IX	X	XI
Sl. No	Species	Explant type	Basal medium	Sucrose	Additives	Plant Growth regulator	Culture conditions	Response	Plantlets	Reference (By year)
68	*E. globulus*	Nodal segments from in vitro germinated seeds	MS			NAA, BAP		Adventitious buds		Gomez et al. (2007)
69	*Eucalyptus globulus Labill. ssp. maidenii*	Shoot tips and nodal pieces of young epicormic shoots from 3–8-year-old elite trees	MS, Quoirin and Lepoivre (1977)	3.0%		BAP, NAA, IBA	16 h Light/8 h dark	Shoot buds	Yes	Sotelo and Monza (2007)
70	*E. polybractea*	Nodal segments from glasshouse-grown seedlings, field-grown saplings and field-grown coppice arising from commercially harvested, mature lignotubers	MS, WP	3%		NAA, BAP	16 h light/8 h dark	Shoot buds	Yes	Goodger et al. (2008)

71	*E. camaldulensis*		MS		Calcium pantothenate (0.1 mg/l)	Kn, BAP, IAA			Yes	Shekhawat and Dixit (2008)
72	*FRI-5 (E. camaldulensis x E. tereticornis) and FRI-14(E. torelliana x E. citriodora*	Nodal segments from the mature trees	MS	2%		BAP, NAA	16 h light/8 h dark	Shoot buds	Yes	Arya et al. (2009)
73	*E. microtheca*	1-year-old seedlings	Modified MS	nd		Kn, NAA	16 h light/8 h dark	Shoot buds		Mamaghani et al. (2009)
74	*E. camaldulensis*	Cotyledonary explants	MS, WPM	2%		NAA, BAP	Initially dark and later 16 h light/8 h dark	Adventitious shoot buds through indirect regeneration	Yes	Dibax et al. (2010)
75	*Eucalyptus globules* hybrids	Nodal segments from in vitro cultures	MS, JADS	3%	800 mg/l PVP	BAP, NAA	Initially dark and later 16 h light/8 h dark	Shoot buds		Borges et al. (2011)
76	*E. benthamii x E. dunnii*	Nodal segments from 1-year-old plant	½ MS	1.5–3%	250 mg/l PVP	NAA, BAP	16 h light/8 h dark	Shoot buds	Yes	Brondani et al. (2011)
77	*E. ,globulus*	Germinated seeds	MS	3%	PVP	NAA, BAP	16 h light/8 h dark	Shoot buds		González et al. (2011)

(continued)

Table 1 (continued)

I	II	III	IV	V	VI	VII	VIII	IX	X	XI
Sl. No	Species	Explant type	Basal medium	Sucrose	Additives	Plant Growth regulator	Culture conditions	Response	Plantlets	Reference (By year)
78	*E. tereticornis*	Nodal segments from freshly coppiced shoots of 10-year-old trees	MS	20%		NAA, BAP	16 h light/8 h dark	Shoot buds	Yes	Aggarwal et al. (2012)
79	*E. grandis*	Nodal segments from seedlings	MS	3%	Calcium (1.10 mg/l) and boron (119.950 mg/l)	NAA, BAP	12 h light/12 h dark	Adventitious shoot buds through indirect regeneration	Nd	Brondani et al. (2012)
80	*E. benthamii*	Nodal segments from mini stumps of cuttings cultivated in semi-hydrophonic system	MS, WP, JADS	3%		BAP, NAA, GA3, IBA	12 h light/12 h dark	Shoot buds	Yes	Brondini et al. (2012)
81	*E. camaldulensis x E. tereticornis*	Nodes, internodes and leaf discs	MS	nd	PVP (10gm), coconut water (50 mg/l)	BAP	Light and dark	Adventitious shoot buds through indirect regeneration	Nd	Mahajan et al. (2012)

82	*E. urophylla x E. grandis*		WPM, JADS, QL	3%	PVP-40 (500 mg/l)	TDZ, NAA	16 h light/8 h dark	Adventitious shoot buds through indirect regeneration		Buttencourt et al. (2012)
83	*E. camaldulensis*	Nodal segments of 18-month-old plant	MS	3%		BAP, NAA	16 h light and 8 h dark	Shoot buds	Yes	Girijashankar (2012)
84	*E. urophylla x E. grandis*	Stem segments from seedlings	MS	3%	Vitamin C	BAP, NAA	16 h light and 8 h dark	Adventitious shoot buds through indirect regeneration	Yes	Ouyang et al. (2012)
85	*E. grandis, E. grandis* × *E. nitens hybrid, E. grandis* × *nitens*	Nodal segments	MS	2%	Biotin, calcium pantothenate (0.04 mg/l)	BAP, NAA, Kn	16 h light and 8 h dark	Shoot buds	Yes	Nakhooda et al. (2012)
86	*E. grandis*	Mini-cuttings or nodal segments from 1-year-old tree	MS	2%	Biotin, calcium pantothenate (0.04 mg/l)	BAP, NAA, Kn, IAA, IBA, TIBA	16 h light and 8 h dark	Soot buds	Yes	Nakhooda et al. (2013)
87	*E. globulus*	Nodal segments	WP M			BAP, NAA	16 h light and 8 h dark	Shoot buds		Cordeiro et al. (2014)
88	*E benthamii X E. dunnii*	In vitro explants	Modified MS, WPM and JADS	3%	PVP 250 mg/L	NAA, TDZ	Initially dark and later 16 h light/8 h dark	Adventitious shoot buds through indirect regeneration		Cauduro et al. (2014)

(continued)

Table 1 (continued)

I	II	III	IV	V	VI	VII	VIII	IX	X	XI
Sl. No	Species	Explant type	Basal medium	Sucrose	Additives	Plant Growth regulator	Culture conditions	Response	Plantlets	Reference (By year)
89	*E. camaldulensis*	Shoot tips and nodal segments from meristematic region	MS			BAP, NAA		Shoot buds		Shukor (2014)
90	*Eucalyptus grandis* × *nitens and E. grandis* × *urophylla*	Axillary buds	MS	2%	Biotin (0.1 mg /L) calcium pantothenate (0.1 mg /L)	BAP, Kn	16 h light and 8 h dark	Shoot buds	Yes	Watt, (2014)
91	*E. tereticornis 103*	Nodal buds	MS, Nitsch and WP	2%		IAA	16 h light and 8 h dark	Shoot buds	Yes	Logeswari and Kanagavalli (2014)
92	*E. camaldulensis*	Nodal segments from adult trees	MS, WPM	2–3%		NAA, BAP, IBA	16 h light and 8 h dark	Shoot buds	Yes	Shanthi et al. (2014)
93	*E benthamii*									Baccarin et al. (2015)
94	*E. saligna*	Cotyledonary nodal segment from seedlings	MS	3%	Coconut water (10%)	NAA, BAP	Initially dark and later 16 h light/8 h dark	Shoot buds	Yes	Da Silva et al. (2015)

95	*E. dunnii*									Oberschelp and Goncalves (2015)
96	*E. cloeziana*	Hypocotyl, cotyledon and root	WP	3%		NAA, BAP		Indirect organogenesis	Yes	Zichner (2015)
97	*E. urophylla*	Hypocotyls from seedlings	MS, SDM	3%	N-phenyl-N′-[6-(2-chlorobenzothiazol) -yl] urea	BAP, NAA	Initially dark and later 16 h light/8 h dark	Shoot buds through indirect regeneration	Yes	Li et al. (2015)
98	*E. cloeziana*	Epicormic shoots from adult shoots	MS	3%		BAP, NAA	12 h light/12 h dark	Shoot buds	Yes	Oliveira et al. (2015)
99	Clones of *Eucalyptus grandis x Eucalyptus globulus* and three clones of *Eucalyptus urophylla x Eucalyptus globulus*	Sprouts from multiplication phase	MS	3%	PVP 30 mg/l	BAP, NAA	16 h light and 8 h dark	Shoot buds	Yes	Oliveira et al. (2016)
100	*E. marginata*	Nodal segments from seedlings	½ MS	3%						Willyams (2016) (Conference papers)

Summary of in vitro media and techniques applied to *Eucalyptus* species. Species (Column II) indicates the *Eucalyptus* spp. Explant type (Column III) describes the starting material. Basal medium (Column IV) represents the type of the medium used during the culturing. Sucrose (Column V) denotes the concentration of the sucrose used. Additives (Column VI) represents the type of the additives. Column VII summarizes the combinations of growth substances that gave the most favourable results for in vitro shoot proliferation. Column VIII indicates the incubation conditions for the cultures. Column IX gives morphogenetic response obtained for experiment. Column X represents the formation of the plantlets. References are listed (last column no. XI) by year.

Table 2 In vitro morphogenesis in *Eucalyptus*

I	II	III	IV	V	VI	VII	VIII	IX	X	XI
Sl. No	Species	Explant type	Basal medium	Sucrose	Additives	Plant growth regulator	Culture conditions	Response	Plantlets	Reference (by year)
1	***E. leichow***	In vitro seedlings	nd	nd	nd	nd	Light	Embryoids	Yes	Ouyang et al.
2	***E. citriodora***	Leaves and stem segments from seedlings and mature trees	MS	nd	Calcium pantothenate (0.1 mg/l), biotin (0.1 mg/l)	Kn, BAP, NAA,	nd	Callus initiation, differentiation and shoot formation	Yes	Gupta et al. (1983)
3	***E. grandis***	Internodal segments (5-year-old plants)	MS	2%	nd	NAA, BAP	16 h light/8 h dark	Proembryos	No	Lakshmi Sita et al. (1986)[a]
4	***E. gunnii***	Hypocotyl and internodes	MS	nd	nd	NAA, BAP	nd	Embryogenic callus	No	Bouley (1987a, b)[a]
5	***E. perriniana***	Young stems	MS	3%	Coconut milk 7%	2,4-D, BAP		Callus		Fuyura et al. (1987)
6	***E. citriodora***	Zygotic embryos	B5	nd	nd	NAA	Dark	Embryogenic callus, somatic embryos	Yes	Muralidharan and Mascaranhas (1987)
7	***E. gunnii***	Leaf, hypocotyls, seedlings internodes	MS	nd	nd	NAA, BAP/Kn	nd	Somatic embryos	No	Franclet and Boulay (1989)[a]
8	***E. citriodora***	Embryo explants	Modified B5	5%	Glutamine 500mgl and casein hydrolysate 500 mg/l	NAA	Dark and later shifted to light for germination	Somatic embryos	Yes	Muralidharan et al. (1989)

9	***E. gunnii***	Leaves from glasshouse plants	MS			2,4-D, BAP		Callus		Teuliers et al. (1989a)
10	***E. citriodora***	Mature zygotic embryos	B5	5%	nd	NAA	Dark	Embryogenic callus, somatic embryos	Yes	Muralidharan et al. (1989)
11	***E. botryoides*****,** ***E. dunnii*****,** ***E. grandis*****,** ***E. rudis***	Cotyledons, hypocotyls, seedlings	Modified MS and RV vitamins	5%	nd	2,4-D	nd	Embryo-like structures	No	Chang –Le and Kirby (1990)
12	*E. globulus*	2-week-old cotyledons	MS			BAP, NAA, IBA		Shoots from callus	Yes	Chrique et al. (1991)
13	*E. gunnii*	Hypocotyl from 1–2-week-old seedlings	MS			BAP, NAA, TDZ		Shoots from callus		Chrique et al. (1991)
14	***E. grandis hybrids***	Hypocotyl explants and different types of explants from 5-year-old tree	MS, B5 vitamins	4.5%	Glutamine 200 mg/l, coconut milk 10%	Kn, NAA		Callus and shoot organogenesis		Warrang et al. (1991)
15	***E. grandis***	Young leaves from in vitro shoots	MS	3%	–	2,4-D	Dark	Somatic embryos	Yes	Watt et al. (1991)
16	***E. grandis***	Nodes from coppice shoots	Laine and David (1994)	2%		BAP, NAA, Zn, IBA		Callus and organogenesis	Yes	Laine and David (1994)
17	***E. citriodora***	Seeds stored at 4 °C for 2 years	MS, B5	3%		NAA		Somatic embryos		Muralidharan and Mascaranhas (1995)

(continued)

Table 2 (continued)

I	II	III	IV	V	VI	VII	VIII	IX	X	XI
Sl. No	Species	Explant type	Basal medium	Sucrose	Additives	Plant growth regulator	Culture conditions	Response	Plantlets	Reference (by year)
18	***E. globulus***	Zygotic embryos	WPM	3%	Coconut milk (10%)	BA	Dark	Somatic embryos	No	Trindade (1996)
19	***E. dunnii***	3-day-old seedlings	B5	2%	Activated charcoal	NAA	Dark	Somatic embryos	No	Termignoni et al. (1996)
20	***E. nitens***	Mature zygotic embryos	nd	nd	nd	nd	nd	Embryo-like structures	nd	Ruaud et al. (1997)[b]
21	***E. nitens*** ***E. globulus***	2–3-week-old seedlings	MS	3%		NAA, BAP	16 h light/8 h dark	Embryo-like structures	No	Bandyopadhyay et al. (1999)
22	***E. nitens***	2–3-week-old seedlings	MS	3%		NAA, BAP	16 h light/8 h dark	Embryo-like structures	No	Bandyopadhyay and Hamill (2000)
23	***E. globulus***	Cotyledons/ hypocotyl	MS	3%		IBA	16 h light/8 h dark	Somatic embryo	No	Nugent et al. (2001b)
24	***E. globulus***	Mature zygotic embryos	MS			NAA	Dark	Somatic embryos and emblings	Yes	Pinto et al. (2002)
25	***E. globulus***	Leaf of epicormic shoots	nd	nd	nd	IBA	nd	Embryogenic callus	No	Oller et al. (2004)
26	***E. tereticornis***	Cotyledon explants from 7–28-day-old seedlings	MS			BAP, NAA			Yes	Prakash and Gorumurthi (2005)
27	***E. erythronema*****, *E. stricklandii*** ***E. erythronema X E. stricklandii***	Apex and cotyledon explants	MS			NAA, 2,4-D		Somatic embryos and direct shoot formation	Yes	Glocke et al. (2006)

28	***E. grandis x E. urophylla***	In vitro shoots with axillary buds removed	MS	3%		IAA, BAP	Dark	Shoots with small quantity of callus	Yes	Hajari et al. (2006)
29	***E. globulus***	Zygotic embryos	MS	3%		NAA	dark	Somatic embryos	Yes	Pinto et al. (2007)
30	***E .grandis***	cotyledons	MS	3%		Picloram	nd	Somatic embryos	Yes	Titon et al. (2007)
31	***E. globulus***	Mature zygotic embryos	Several media	3%		NAA	Dark	Somatic embryos	Yes	Pinto et al. (2008a)
32	***E. globulus***	Mature zygotic embryos	MS	3%		NAA	Dark	Somatic embryos	Yes	Pinto et al. (2008c)
33	***E. microtheca***	Leaf, stalks and twigs from seedlings	MS			TDZ	16 h light/8 dark	Callus, somatic embryos	Yes	Mamaghani et al. (2009)
34	***E. camaldulensis***	Mature zygotic embryos and cotyledon explants	MS	1–4%		BAP, NAA				Prakash and Gurumurthi (2010)
35	***E. tereticornis***	Nodal and leaf explants	MS			2,4-D, IAA, BAP		Callus, indirect organogenesis		Nair and Vijayalakshmi (2010)
36	***E. globulus***	Mature zygotic embryos	MS	3%		NAA	Dark	Somatic embryos	Yes	Pinto et al. (2010)
37	***E. globulus***	Zygotic embryos, cotyledons, hypocotyls	MS	3%		NAA	Dark	Callus, somatic embryos	Yes	Andrade et al. (2011) (poster presentation – BMC proceedings)
38	***E. globulus***	Zygotic embryos	B5	3%		NAA, BAP, 2,4-D	Dark	Somatic embryos	Yes	Gómez et al. (2013)

(continued)

Table 2 (continued)

I	II	III	IV	V	VI	VII	VIII	IX	X	XI
Sl. No	Species	Explant type	Basal medium	Sucrose	Additives	Plant growth regulator	Culture conditions	Response	Plantlets	Reference (by year)
39	***E. globulus and E. saligna x E. maidenii***	In vitro cultures	MS	3%	Ascorbic acid, citric acid, folic acid, casein hydrolysate	NAA, picloram	Dark	Somatic embryos	Yes	Corredoira et al. (2015)
40	***E. erythronema*, *E. stricklandii*, *E. erythronema X E. stricklandii***	Nodal explants from in vitro seedlings	MS	nd		NAA, 2,4-D	Dark	Callus and rrganogenesis	Yes	Sedgley et al.
41	***E. grandis***	Leaves from in vitro cultures	MS	3%		IAA, IBA, trans-zeatin	Dark	Callus and SE induction	Yes	Nakhooda and Mandiri (2016)
42	***E. globulus***	Zygotic embryos	MS	3%		NAA	Dark	Organogenesis		Dobrowolska et al. (2017)

[a]According to Le Roux and Van Staden (1991a)
[b]According to Watt et al. (2003)
Summary of media and techniques applied to *Eucalyptus* species. Species (Column II) indicates the *Eucalyptus* spp. Explant type (Column III) describes the starting material. Basal medium (Column IV) represents the type of the medium used during the culturing. Sucrose (Column V) denotes the concentration of the sucrose used. Additives (Column VI) represents the type of the additives. Column VII summarizes the combinations of growth substances that gave the most favourable results for in vitro shoot proliferation. Column VIII indicates the incubation conditions for the cultures. Column IX gives morphogenetic response obtained for experiment. Column X represents the formation of the plantlets. References are listed (last column no. XI) by year.

Table 3 Repetitive somatic embryogenesis in *Eucalyptus*

I	II	III	IV	V	VI	VII	VIII	IX	X	XI
Sl. No	Species	Explant type	Basal medium	Sucrose	Additives	Plant growth regulator	Culture conditions	Response	Emblings	Reference (by year)
1	***E. gunnii***	Embryogenic callus	MS	nd	nd	Various combinations	nd	nd	nd	Boulay (1987a)[a]
2	***E. citriodora***	Somatic embryos	B5	5%	Activated charcoal, glutamine	NAA	Dark	Secondary somatic embryos	Yes	Muralidharan et al. (1989)
3	***E. citriodora***	Somatic embryos	B5	3%		NAA		Somatic embryos	Yes	Muralidharan and Mascarenhasl (1995)
4	***E. globulus***	Somatic embryos	MS			NAA	Dark	Secondary somatic embryos	Yes	Pinto et al. (2004)
5	***E. globulus***	Somatic embryos	MS			NAA	Dark	Secondary somatic embryos	Yes	Pinto et al. (2008a)
6	***E. globulus***	Somatic embryos	MS, B5			Several	Dark and light	Somatic embryo	nd	Pinto et al. (2008b)

[a]According to Le Roux and Van Staden (1991a)

Summary of media and techniques applied to *Eucalyptus* species. Species (Column II) indicates the *Eucalyptus* spp. Explant type (Column III) describes the starting material. Basal medium (Column IV) represents the type of the medium used during the culturing. Sucrose (Column V) denotes the concentration of the sucrose used. Additives (Column VI) represents the type of the additives. Column VII summarizes the combinations of growth substances that gave the most favourable results for in vitro shoot proliferation. Column VIII indicates the incubation conditions for the cultures. Column IX gives morphogenetic response obtained for experiment. Column X represents the formation of the plantlets. References are listed (last column no. XI) by year.

3.1 Modes of Micropropagation

Micropropagation can be achieved by in vitro shoot proliferation, organogenesis and somatic embryogenesis (George and Sherrington 1984). In vitro shoot proliferation (apical and axillary meristem proliferation) has been a method of choice since in shoots originating from preformed meristem genetic integrity is very high, which is very important for maintaining clonal fidelity (McCown 1984). Organogenesis which is shoot and root formation directly on explants or through intervening callus is a preferred method for genetic transformation studies. Somatic embryogenesis occurring directly or indirectly was considered to have potential for large-scale propagation (Blakeway et al. 1993; George et al. 2008) and for synthetic seeds. In vitro propagation of desired clones of *Eucalyptus* has the potential to provide very high multiplication rates of selected tree genotypes. One of the objectives of micropropagation is to clone species or hybrids that have higher growth rate, tolerance to low temperature, salinity and resistance to pests/diseases (McComb and Bennett 1986). The rejuvenation or reinvigoration of adult tissues can be obtained through micropropagation by successive subcultures in vitro (Gonçalves 1982; Bonga and von Aderkas 1992; Grattapaglia and Machado 1998). Assis and Mafia (2007) reported that the repeated subculture of adult tissues improves the rooting. They also complement physiological homogenization of the tissues, whether at rooting potential or quality of the root system. The increase in the number of subcultures in *Eucalyptus* in vitro restores the youthful features as the greatest rooting potential (Chaperon 1987; Assis 1996; Xavier and Comério 1996). The rejuvenation or restoration of the rooting competence is obtained, generally after 10–12 subcultures. Coppicing of the selected trees and use of explants from coppice shoots are also the desired methods (Alfenas et al. 2004). The importance of in vitro micropropagation in *Eucalyptus* is evident from the amount of work carried out on the genus (Mullins et al. 1997; Barrueto Cid et al. 1999; Sharma and Ramamurthy 2000). Micropropagation of several *Eucalyptus* species has been very well covered in several reviews (McComb and Bennett 1986; Le Roux and Van Staden 1991b; Lakshmi Sita 1993; McComb et al. 1996; Nakhooda and Jain 2016; Pinto et al. 2016). *Eucalyptus* species and hybrids are well known by their recalcitrance to micropropagation and genetic manipulation (Girijashankar 2011). According to Chaperon (1987), there are three situations in which the micropropagation of *Eucalyptus* has been recommended: (1) when other propagation techniques like vegetative propagation do not present satisfactory results in species; (2) when the selected tree cannot be rejuvenated through the promotion of basal shoots; and (3) when it is desired to increase the propagation rate and shorten the time for its use commercially.

In vitro culture of eucalyptus dates back to the 1960s; the early reports came from France (Jaquiot 1964a, b); America (Sussex 1965) and India (Aneja and Atal 1969). Regeneration was reported from juvenile tissues or embryos and not from tissues of mature trees as explants collected from field-grown plants posed problems of contamination, phenolics and maturity of trees (Cresswell and Nitsch 1975).

Early reports of regeneration of *E. citriodora* were from lignotubers (Aneja and Atal 1969), cotyledon callus (Lakshmi Sita 1979), hypocotyls and apical meristems (Lakshmi Sita and Vaidyanathan 1979). In the early 1970s, Dr. Ron de Fossard and his co-workers reported on callus culture, organogenesis, media for shoot culture and techniques for rooting (de Fossard 1974; Lee & de Fossard 1974; Cresswell and de Fossard 1974; Cresswell and Nitsch 1975; Barker et al. 1977; Gorst et al. 1983). He discussed the utility of seedling explants over mature ones and significance of rejuvenation. The need for systematic evaluation of media ingredients (de Fossard et al. 1974) and methods for low-cost tissue culture (de Fossard et al. 1978) were described. Franclet and Boulay (1982) explored their previous expertise in rejuvenation of eucalypts for provision of suitable shoots for cuttings (Franclet 1956). By the 1980s, successful micropropagation of elite eucalypts using mature tree-derived explants became a reality (Gupta et al. 1981, 1983; Bennett and McComb 1982; Lakshmi Sita 1982; Gupta and Mascarenhas 1987). This was followed by mass multiplication of plus trees and field planting of tissue culture-raised propagules. Simultaneously work was carried out on suspension cultures (Teulieres et al. 1989b) and somatic embryogenesis (Muralidharan and Mascarenhas 1987; Watt et al. 1999). Research on species of interest to India has been reviewed by Lakshmi Sita (1993).

Rajbhandary (1990) reported direct rooting of micropropagated shoots in soil. Paton et al. (1970) indicated that ontogenic ageing of *E. grandis* seedlings involves a direct and quantitative association between decreased rooting ability of stem cuttings and increased levels of a rooting inhibitor in the tissue forming the base of the cutting, and the inhibitor is present only in adult tissue, which rarely forms roots from stem cuttings. Several species of *Eucalyptus* (over 70 spp.) and interspecific hybrids (over 10) have been studied in vitro, such as *E. alba*, *E. badjensis*, *E. bancroftii*, *E. benthamii*, *E. botryoides*, *E. bridgesiana*, *E. alophylla*, *E. camaldulensis*, *E. cladocalyx*, *E. cloeziana*, *E. coccifera*, *E. citriodora*, *E. curtisii*, *E. dalrympleana*, *E. deglupta*, *E. delegatensis*, *E. diversicolor*, *E. dunnii*, *E. erythronema*, *E. ficifolia*, *E. gomphocephala*, *E. globulus*, *E. grandis*, *E. gunnii*, *E. henryi*, *E. impensa*, *E. laevopinea*, *E. leichow*, *E. macarthurii*, *E. macrorhyncha*, *E. maculata*, *E. marginata*, *E. megacarpa*, *E. melliodora*, *E. microcorys*, *E. microtheca*, *E. nicholii*, *E. nitens*, *new E. anglica*, *E. obtusiflora*, *E. occidentalis*, *E. oreades*, *E. pauciflora (= E. niphophila)*, *E. pellitta*, *E. phylacis*, *E. polybractea*, *E. radiata*, *E. regnans*, *E. resinifera*, *E. robust*, *E. rubida*, *E. rudis*, *E. saligna*, *E. sargentii*, *E. sideroxylon*, *E. smithii*, *E. stricklandii*, *E. stuartiana (= E. ovata)*, *E. tereticornis*, *E. torelliana*, *E. urnigera*, *E. urophylla*, *E. viminalis*, *E. viridis*, *E. wandoo*, *E. youmanii*, *Eucalyptus x trabutti*, *E. camaldulensis x E. tereticornis*, *E. dunnii x E. sp.*, *E. erythronema x E. stricklandii*, *E. grandis x E. camaldulensis*, *E. grandis x E. nitens*, *E. grandis x E. robusta*, *E. grandis x E. tereticornis*, *E. grandis x E. urophylla*, *E. gunnii*, *E. dalrympleana*, *E. gunnii x E. globulus*, *E. macarthurii x E. grandis*, *E. tereticornis x E. camaldulensis*, *E. tereticornis x E. grandis*, *E. torelliana x E. citriodora*, *E. urophylla x E. grandis*.

3.2 In Vitro Shoot Culture

The stages of micropropagation are, namely, collection of explants from the selected plants/trees in the field, cleaning and disinfestation prior to inoculation in the culture, followed by establishment in vitro, multiplication, elongation, rooting in vitro/ex vitro and finally acclimatization (Murashige 1974). The critical factors determining the success of micropropagation are choice of explant, physiological status of the mother plant, season of collection, culture media and physical parameters. Age of plant material and season were important factors determining success in establishing aseptic cultures (Sharma and Ramamurthy 2000; Grewal et al. 1980).

3.2.1 Explants

The best sources of explants for the culture establishment were generally juvenile or rejuvenated tissues (George 1986; Jones and Van Staden 1997). Although various explants like shoot tips (Gomes and Canhoto 2003), axillary buds (Jones and van Staden 1994; Mokotedi et al. 2000), nodes (Gomes and Canhoto 2003; Arya et al. 2009; Hung and Trueman 2011), cotyledons (Bandyopadhyay et al. 1999; Nugent et al. 2001a) from field-grown seedlings and hypocotyls, leaf fragments from in vitro raised seedlings have been used (Subbaiah and Minocha 1990; Termignoni et al. 1996; Sharma and Ramamurthy 2000; Prakash and Gurumuthi 2005), plantlets obtained have the disadvantage of the genotype different from the parent plant (Lakshmi Sita 1979). Other explants like coppiced shoots (Burger 1987; Aggarwal et al. 2012), scion shoots (Gonçalves 1980; Franclet and Boulay 1982), epicormic shoots (Ikemori 1987) and young, vigorously growing shoots from mature trees (de Fossard and Bourne 1977; Sankara Rao 1988; Aggarwal et al. 2010, 2012) have also been used. Successful micropropagation has also been reported using nodal explants from mature trees of 5 years old (Lakshmi Sita et al. 1986), 20 years old (Gupta et al. 1981) and 32 years old (Arya et al. 2009) leading to a true-to-type progeny.

3.2.2 Disinfestation

In vitro establishment of germ-free cultures is a crucial step in micropropagation, especially if the explants are derived from plants in the field because of high contamination rates (Alfenas et al. 2004). Disinfestation procedures may vary with the season of collection, physiological status of the mother plant and type and source of explants. Numerous procedures and products are used for the disinfestation of explants. The most commonly used are 70% ethanol and sodium hypochlorite in various concentrations. For the nodal explants derived from the shoots of 17-year-old trees *E. benthamii*, Hansel et al. (2005) recommended immersion in 70% alcohol (1 min) and 2% sodium hypochlorite (10 min) and in 2% sodium hypochlorite (5 min) for shoot apices. Active chlorine (0.5%) was used in *E. benthamii* x

E. dunnii (Alfenas et al. 2004; Brondani 2008; Brondani et al. 2009). In *E. grandis*, Holden and Paton (1981) disinfested explants with saturated calcium hypochlorite for 75 min, followed by 4 h under ultraviolet irradiation. Hajari et al. (2006) used calcium hypochlorite (10 g/L) for 2 min in *E. grandis* x *E. urophylla*. Similarly, mercuric chloride ($HgCl_2$) was tested at 0.05% for 15 min in *E. citriodora* (Gupta et al. 1981), 1.0 g/L for 10 min in *E. tereticornis* (Sharma and Ramamurthy 2000) and 0.2 g/L for 2 min in *E. grandis* (Watt et al. 2003). Mercury chloride is highly toxic and used in concentrations lower than hypochlorites, while benzalkonium chloride is poorly toxic to tissues and can be used in similar concentrations to hypochlorites (Grattapaglia and Machado 1998). For seeds, the size and thickness of the shell coating determine the disinfestation procedure (McComb and Bennett 1986). Termignoni et al. (1996) used sodium hypochlorite (0.12%) for 15 min in *E. dunnii*, whereas for 1 h in *E. marginata*, as the seeds have larger and thicker coatings (Cahill et al. 1992). Usually, drops of detergent or Tween 20 (0.01–0.05%) are added in order to increase the sterilizing agents' contact with the tissues.

3.2.3 In Vitro Establishment of Cultures

After disinfestation, the explants were inoculated in nutrient medium and incubated for 20–30 days for the initiation of culture. However, the occurrence of oxidations and/or contaminations may be noticeable in the first week of inoculation (Fig. 1). In order to avoid widespread contamination, the explants are inoculated into test tubes where they are individualized, rather than cultured jars. At this stage, the composition of the culture medium may vary according to the nutritional needs of species. In order to avoid or reduce the oxidative process, antioxidants, such as polyvinyl, incubation of cultures in the absence of light or low luminous intensity for 7–14 days, cold pretreatment (Gupta and Mascarenhas 1987), frequent shifting to fresh culture medium, the use of Gelrite ® or TM as Phytagel or the addition of activated charcoal can prevent the phenolic oxidation. Fungicides and bactericides also can be added to the culture medium to control contamination (Dutra et al. 2009).

3.2.4 Culture Media

Culture media such as MS (Murashige and Skoog 1962), JADS (Correia 1993), WPM (woody plant medium) (Lloyd and McCown 1980) and MS medium with half of the normal concentrations of its salts (MS1/2) have been commonly used for in vitro initiation of cultures. Addition of growth regulators is optional. Glocke et al. (2006) reported micropropagation of ornamental *Eucalyptus* hybrid (*E. erythronema* var. *erythronema* x *E. stricklandii* cv. '*Urrbrae Gem*') through enhanced axillary shoot proliferation. Multiplication was higher on WPM and QL (Quoirin and Lepoivre 1977) media supplemented with BA, NAA and GA3 as compared to MS, B5, AP (Almehdli and Parfitt 1986) and TK (Tabachnik and Kester 1977) media with the same composition of plant growth regulators (PGRs). In this study, WPM and QL were shown to provide better shoot growth than MS, AP, B5 and TK media.

Fig. 1 Micropropagation of *Eucalyptus*. (**a**) Collection of material. (**b**) Establishment of shoot cultures. (**c**) Multiplication of shoots. (**d**) Hardened plants in greenhouse. (**e**) Tissue culture-raised plants in field

3.2.5 Shoot Multiplication and/or Elongation

The elongated shoots originating from the establishment phase are further segmented and inoculated in culture media containing combinations of auxins and cytokinins, which varies with species. Cytokinin, BAP (0.006 μM–8.8 μM), was effective to induce the proliferation of *Eucalyptus* (Del Ponte et al. 2001). Other cytokinins such as kinetin (Kin) (0.23 μM–2.5 μM), thidiazuron (TDZ) (0.045 μM–0.05 μM) and 2iP (2.5 MM) have also been used. Cytokinins (viz., BAP, Kin) have also been used either alone and/or in combination with auxins (naphthaleneacetic acid) (NAA) (0.01 μM–5.3 μM), indolebutyric acid (IBA) (0.05 μM) and 2,4-D (0.2 μM). Gupta et al. (1981, 1983) and Gupta and Mascarenhas (1987) have reported multiple shoot induction and proliferation of *E. citriodora*, *E. torelliana* and *E. camaldulensis* from nodal segments collected from mature trees (10–20 years) on MS medium with different concentrations of Kn, BAP, calcium pantothenate and biotin. Das and Mitra (1990) achieved shoot multiplication on MS medium with 0.5 μM NAA and 4.4 μM BA using shoot-tip explants collected from coppiced mature trees of *E. tereticornis* wherein the addition of charcoal and gibberellic acid (GA3) was found beneficial. Sharma and Ramamurthy (2000) achieved successful shoot induction on MS medium with 4.4 μM each of BA and NAA nodal segments using nodal explants

from 4-year-old elite clones of *E. tereticornis.* March to April period was shown to be the best periods for culture establishment. The main objective of elongation stage is the preparation of the shoots for rooting. Depending on the genotype, in some cases the multiplication and elongation of shoots occur at the same time, whereas for others an elongation phase may be necessary where various combinations of BAP, NAA, IBA and GA3 are effective. Reducing the concentration of cytokinins used in the multiplication phase may also help in the elongation. Multiplication followed by elongation of shoots was obtained with combinations of 1.0 mg/L BAP with 0.04 mg/L GA3 in *E. tereticornis* x *E. grandis* (Joshi et al. 2003); 1.0 and 2.0 mg/L BAP with 0.01 and 1.0 mg/L NAA in *E. tereticornis* x *E. camaldulensis* (Bisht et al. 1999); 0.1 mg/L of IBA with 0.1 mg/L BAP in *E. urophylla* (Santos et al. 2004); and 0.1 mg/L GA3 in *E. saligna* (Fantini Junior and Graça 1990). Elongation of shoots of *E. benthamii* x *E. dunnii* clones was obtained by adding 0.10 or 0.20 mg/L GA3 combined with 0.10 mg/L BAP and 0.5 mg/L NAA. Phytagel was reported as a better gelling agent than agar for shoot elongation, but it sometimes caused hyperhydricity, which was controlled by doubling the concentration of calcium chloride (Brondani 2008). Aggarwal et al. (2012) in *E. tereticornis* reported BAP to be the most effective cytokinin for shoot multiplication and elongation. The initial size of the shoot clump (inoculum) also influenced shoot multiplication and elongation. The number of shoots proliferated and elongated was higher in cultures incubated under photosynthetically active radiation (PAR) compared to those incubated under cool fluorescent lights (CFLs). Osmotic potential of the sap and chlorophyll content of cultures incubated under PAR were also higher than those incubated under CFL (Xavier et al. 2007).

3.2.6 Rooting

After multiplication and elongation, the shoots are rooted, either in vitro or ex vitro. For rooting of *Eucalyptus* microshoots, auxins have been widely used (Gupta et al. 1983; Bennett et al. 1994; Sharma and Ramamurthy 2000; Aggarwal et al. 2012). Out of the various auxins tested, the efficacy of IBA for induction of roots in microshoots of various plant species was higher than other auxins like NAA and IAA (Kato 1985). The concentration and mode of application of IBA vary with different plant species (Kato 1985; Jha and Sen 1992). Gupta et al. (1983) were able to induce rooting in microshoots of *E. torelliana* by treatment with α-naphthalene acetic acid (NAA), whereas treatment with a mixture of indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), indole-3-propionic acid (IPA) and NAA in the dark for different time intervals was essential for the induction of roots in microshoots of *E. camaldulensis*. After auxin treatment, transfer of shoots to a charcoal-containing medium and incubation under the light were beneficial for root induction. Rooting occurred within 15–20 days in 70% of the shoots of *E. torelliana* treated with NAA for 48 h. On the other hand, 50% of *E. camaldulensis* microshoots treated in the dark for 72 h with a mixture of auxins containing IBA, IPA, IAA and NAA rooted within 20–25 days. Rooted plantlets thus obtained were successfully transferred to pots

and field. Further, lowering of nutrient salt concentration was also reported to be beneficial for rooting of microshoots in *Eucalyptus* (Bennett et al. 1994; Aggarwal et al. 2012). Similarly, Das and Mitra (1990) reported that incubating cultures in dark for a short period was the key factor for root induction in *E. tereticornis*, and genotypically different populations responded differently during culture, in spite of optimal growth conditions. Subbaiah and Minocha (1990) achieved 100% rooting on mWPM supplemented with 2.4 μM IBA in the case of *E. tereticornis*. Further, Aggarwal et al. (2012) have reported that PAR light was more effective for efficient rooting than CFL in *E. tereticornis*. There was increase in rooting efficiency under PAR light, which may be due to the involvement of blue light responding cryptochromes and red/far-red light responding phytochromes as reported by Lin (2002). Light quality has been shown to promote rooting efficiency in some plant species (Rossi et al. 1993). Xavier and Comério (1996) stated that rooting in vitro may be waived, when elongated in vitro shoots are rooted under greenhouse conditions. It was advantageous to avoid the manipulation of plants, reduce the period of formation, increase the production capacity and reduce a developmental phase (rooting in vitro). The ex vitro rooting provides reduction in labour costs and space-saving growth, electrical energy and culture medium. Quality ex vitro rooting tends to produce a more complete and functional root system, with higher number of secondary roots (Grattapaglia and Machado 1998). The ex vitro rooting method has been adapted extensively for the large-scale production of tissue culture-raised propagules *E. tereticornis* and *E. camaldulensis* at NCL under NABARD Project and TCPP Project of DBT (Personal communication).

3.2.7 Acclimatization

A major limitation in large-scale application of plant tissue culture technology is high mortality experienced by tissue culture-raised plants during or after laboratory to land transfer, mainly due to the extreme differences between the in vitro and ex vitro environment. Plants produced under in vitro conditions are reported to develop poor photosynthetic apparatus (Brainerd and Fuchigami 1982; Kozai 1991; Sharma et al. 1999). Several methods have been tried for the acclimatization of tissue culture-raised plants for successful field establishment of *Eucalyptus* (Macrae and Van Staden 1993; Machado et al. 1997; Aggarwal et al. 2012; Girijashankar 2012). Aggarwal et al. (2012) reported that plants produced under PAR showed higher survival rates and subsequently more vigorous growth following transfer to soil, which commensurate with higher chlorophyll contents. Girijashankar (2012) reported the use of different soil compositions for successful acclimatization of the micropropagated plantlets of *E. camaldulensis*. Further, in order to enhance the survival rate, covering of micropropagated plantlets with a transparent polythene cover proved beneficial. At NCL, polytunnels were successfully used for simultaneous rooting and hardening of a large number of microshoots (Personal communication). Macrae and Van Staden (1993) and Machando et al. (1997) highlighted the role of

A. rhizogenes strains in inducing rooting in difficult-to-root plants. The hardened plants are shifted to bigger polybags before they are transported to the fields.

3.3 Field Performance

The successful field planting of 150 TC-raised plants from seedling explants, 40 plants from 3-year-old tree and 22 plants from 20-year-old tree of *E. citriodora* was reported (Gupta et al. (1981)). Similarly Gupta et al. (1983) effectively transferred plantlets of *E. citriodora*, *E. tereticornis* and *E. globulus* to fields. Khuspe et al. (1987) reported an overall increase in tissue culture-raised plants of *E. tereticornis* (36.9%) and *E. torelliana* (49.5%) as compared to seed-raised controls. Mascarnhas et al. (1987) reported field evaluation data of *E. torelliana* and *E. tereticornis* regarding diameter, biomass, oil analysis, specific gravity and cost-benefit analysis. Gupta et al. (1991) revealed uniformity, increased biomass and no morphological variation in micropropagated plants of *Eucalyptus* species derived from superior clones over seedling derived from the same plant. Nadgauda et al. (1999) reported extensive multilocational field trials conducted on tissue culture-raised propagules of *E. tereticornis* and *E. camaldulensis* undertaken in collaboration with forest departments/corporations, agricultural universities and private sector organizations. They reported above 93% survival and high uniformity and an increased biomass leading to early rotation. Site-specific suitable clones were identified, and higher benefit-to-cost ratio (3:1) of tissue culture-raised plants was demonstrated. Arya et al. (2009) reported field planting and superior growth of axillary meristem-derived plants of *Eucalyptus* hybrid (*E. camaldulensis* Dehn x *E. tereticornis* Sm and *E. torelliana* F. V. Muell x *E. citriodora* Hook).

4 Organogenesis

In vitro propagation of *Eucalypts* through organogenesis has proved difficult (Watt et al. 2003), but success has been achieved with the cytokinins 6-benzylamino purine (BAP), zeatin and kinetin using either hypocotyl, cotyledon or primary leaf explants for a few species such as *E. grandis* (Warrag et al. 1991), *E. grandis* x *E. urophylla* (Barrueto Cid et al. 1999), *E. nitens* (Bandyopadhyay et al. 1999), *E. globulus* (Nugent et al. 2001b; Trindade and Pais 2003) and *E. camaldulensis* (Muralidharan and Mascarenhas 1987). Also from leaf, stem callus of *E. tereticornis* (Subbaiah and Minocha 1990), shoot apex explants of *E. microcorys* (Niccol et al. 1994) and nodal explants from mature plants (Aggarwal et al. 2010). Liu and Pijut (2008) documented that shoot organogenesis or somatic embryogenesis is more difficult from mature plants than from juvenile tissues (Liu and Pijut 2008). Many factors, such as age of explant, plant growth regulators (PGRs) and antibiotics, among others, have been reported to influence shoot regeneration (Subbaiah and Minocha 1990; Martin et al. 2003).

4.1 Culture Conditions

Subbaiah and Minocha (1990) were the first to report shoot organogenesis in *E. tereticornis*. They reported the regeneration of adventitious shoots from seedling leaf and stem callus on B5 medium with 0.44 μM BAP and 15–20 μM NAA in the dark. Multiple shoots were also regenerated directly from hypocotyl segments of 4–6-week-old seedlings on B5 medium with 2.2 μM BAP. Mullins et al. (1997) reported shoot regeneration protocol for *E. camaldulensis* using leaf explants taken from sterile seedlings on WP medium containing 1.0 g/l casein hydrolysate, with 16.1 μM NAA and 0.45 μM BAP. Out of 24 clones used in the study, only 13 clones regenerated shoots. Subsequently, the same protocol was used for the successful shoot regeneration of other species like *E. microtheca*, *E. ochrophloia*, *E. grandis* and *E. marginata*. However, the frequency of regeneration varied from species to species and clone to clone within the same species which emphasized the need to develop clone-specific protocols. Ho et al. (1998) regenerated shoots from hypocotyl explants taken from aseptically grown 1-month-old seedlings of *E. camaldulensis* on B5 medium with 100 ml/l coconut milk, 200 mg/l glutamine and 100 mg/l casein hydrolysate, 4.4 μM BAP and 15 μM NAA via callus phase. Barrueto Cid et al. (1999) have achieved shoot organogenesis from *E. grandis* x *E. urophylla* using hypocotyls, cotyledons, cotyledonary nodes and primary leaves from 14- to 50-day-old seedlings on modified MS medium with 2.0 μM thidiazuron (TDZ). Shoots were induced from these calli at a high frequency on medium with 5.0 μM BAP and 0.5 μM NAA, which were elongated on medium with 1.0 μM BAP, 0.5 μM NAA and 2.0 μM GA3. In vitro rooting was induced in 50 mm-long microshoots on medium containing 2.5 μM IBA and later transferred to the basal medium for 30 days. Plantlets were then successfully transplanted to the greenhouse conditions. Dibax et al. (2005) also reported shoot organogenesis from cotyledonary leaves of *E. camaldulensis* on MS medium with 2.7 μM NAA and 4.44 μM BAP. Incubation of explants in the dark during the first 30 days increased percentage explants forming callus and reduced explant necrosis. Regeneration frequency from callus further increased when transferred to half-strength basal medium. Later, Dibax et al. (2010) compared MS, WPM and JADS media with 2.7 μM NAA and 4.44 μM BAP for the shoot regeneration from cotyledonary leaves of *E. camaldulensis*. They have also reported the anatomy of the tissue during various stages of shoot regeneration. Oberschelp and Goncalves (2015) reported the effect of the hypocotyl segment's position (distal and proximal) on bud and callus regeneration of *E. dunnii* from cotyledons and hypocotyl explants using combinations of BAP and NAA or IAA. Histological analyses suggested that pluripotent cells give rise to buds and shoots. It has been documented that shoot regeneration or somatic embryogenesis is difficult from mature plants as compared to juvenile tissue (Liu and Pijut 2008). Aggarwal et al. (2010) reported shoot organogenesis from leaf segments of in vitro grown microshoots derived from mature plants of selected elite clones of *E. tereticornis* cultured on MS medium with 5.0 μM BAP and 1.0 μM 2,4-D. The addition of cefotaxime to the medium promoted shoot differentiation, whereas carbenicillin

and cephalexin inhibited shoot differentiation. Leaf maturity was found to influence shoot regeneration; the fifth leaf (14–16 days old) from the top of microshoot was found to be the best for shoot organogenesis. Similar results have been obtained in our laboratory where leaf explants excised from in vitro grown microshoots established from plus trees of *E. tereticornis* on MS medium with BA (Kendurkar, unpublished). This system was further used for genetic transformation studies. However, it is important to develop direct regeneration protocol, which will help in taking up genetic manipulation work. Many *Eucalyptus* species are still considered recalcitrant to tissue culture and genetic engineering. Further, reports exist on the variable regeneration ability of the different species and also among clones of the same species (Mullins et al. 1997). Therefore, there is a need to develop clone-specific micropropagation and regeneration protocols.

5 Somatic Embryogenesis

Researchers and forest industries are beginning to focus their efforts on developing in vitro propagation via somatic embryogenesis (SE), an approach that is recognized as having several advantages over other in vitro shoot propagation systems (Bonga et al. 2010). It is a process where somatic cells acquire embryonic competence that can give rise to embryos (Ikeuchi et al. 2015). Distinguishing between direct and indirect somatic embryogenesis can be difficult as both processes have been observed to occur simultaneously in the same tissue culture conditions (Turgut et al. 1998; Gaj 2004). In contrast to primary somatic embryogenesis induced from explant cells, secondary somatic embryogenesis is the phenomenon whereby new somatic embryos are induced through existing somatic embryos (Raemakers et al. 1995). It is widely recognized that somatic cells can acquire embryogenic potential as a result of different external chemical and physical stimuli, often associated with stress conditions (Gaj 2004). In vitro culture conditions expose explants/cells to significant stresses, as they are removed from the environment of the original tissue and placed on synthetic media in artificial conditions. Wounding itself may be a significant signal for the induction and dedifferentiation (Fehér et al. 2003). Embryogenic competence of in vitro cultured somatic cells can be stimulated by various factors, such as high-auxin level, osmotic pressure, pH, low or high temperature, starvation or mechanical wounding of explants. The molecular mechanisms involved in this stimulatory/regulatory effect of the stress treatment on cell differentiation and morphogenesis remain unclear (Fehér et al. 2003; Gaj 2004). In myrtaceous species in general and *Eucalyptus* in particular, there is a notorious paucity of cytological, histological and ultrastructural information on the different aspects associated with the induction and development of somatic embryos from explant tissues. In *Eucalyptus*, most reports that describe somatic embryos showed morphological resemblances with zygotic embryos at various developmental stages, although in some of them, a clear definition of the different developmental phases was lacking (Muralidharan et al. 1989; Watt et al. 1999). Somatic embryogenesis

can be induced in cultures from various explant types: seedlings and their fragments, petioles, leaves, roots, anther filament, shoot meristems, seeds, cotyledons and immature and mature zygotic embryos (Dunstan et al. 1995; Gaj 2004). Although adult material is desirable as explant source, in most of the SE induction experiments in *Eucalyptus*, juvenile material was preferred may be due to higher response. The effect of the genotype has been considered as a crucial factor in the induction of SE in *Eucalyptus* (Canhoto et al. 1999). In *E. grandis*, Watt et al. (1991) used leaves from in vitro propagated shoots, while in *E. dunnii* 3-day-old seedlings were used (Termignoni et al. 1996). Concerning *E. globulus*, Trindade (1996) tested explants in different phases of development and found that partially germinated seeds were more suitable for SE than cotyledons or leaves from micropropagated plants. Bandyopathyay et al. (1999) observed organized structures resembling somatic embryos obtained from mature seeds, but their evolution was not followed. Later on, Nugent et al. (2001b) were able to use, with considerable success, cotyledons from mature seeds as source material for inducing SE. In these studies complete somatic embryogenesis structures, i.e. having well-defined shoot and root poles, were not obtained. A considerable progress was achieved, when Pinto et al. (2002) tested several types of explants (mature zygotic embryos, isolated cotyledons, hypocotyls, leaves and stems) to induce SE and were able to develop a successful protocol for somatic embryogenesis plant regeneration from mature zygotic embryos in *E. globulus* Labill Pinto et al. 2002). In *E. citriodora* (Muralidharan and Mascarenhas 1995) and *E. tereticornis* (Prakash and Gurumurthi 2005), decoated seeds were used as explant, which included the embryo and cotyledon. Furthermore, according to Watt et al. (2003), a successful protocol of SE with explants of mature trees was already obtained although no details are given.

5.1 Culture Conditions

The most extensively used medium for the induction of SE in several species of *Eucalyptus* is the nitrogen-rich MS medium (Le Roux and Van Staden 1991a; Dunstan et al. 1995; Watt et al. 2003). Pinto et al. (2008a) analysed the effectiveness of several media (MS, ½ MS, B5, WPM, DKW and JADS) during SE induction and expression in *E. globulus*. The results showed that MS and B5 were the best media for SE induction and for embling regeneration. The effect of carbohydrates on induction, maintenance and maturation of somatic embryos has been investigated (Lipavska and Dova 2004). Glucose, fructose, maltose, lactose, cellobiose, mannitol, sorbitol and myoinositol were tested in different species (Canhoto et al. 1999; Pinto et al. 2002; Lipavska and Dova 2004). Sucrose has been commonly employed to induce SE in different plant species. In *Eucalyptus*, the recommended concentrations of sucrose for SE induction are in the range 2–5%. Addition of mannitol to the induction medium inhibited the formation of callus in the surface of the explants during SE induction of *E. globulus* (Pinto et al. 2002). Incorporation of plant growth regulators to the culture medium is preferable to induce morphogenetic responses

in vitro (Gaj 2004; Jiménez 2005). Auxins, such as 2,4-D, IAA, IBA and NAA, are frequently used to reactivate the cell cycle and initiate embryo formation (Merkle 1995). Usually after removing the auxin, the somatic embryos switch to a programme of development, maturation and germination leading to termination of repetitive embryogenesis. Cytokinins (viz., Kin, BAP) in combination with an auxin (viz., IAA, IBA, NAA) in the induction medium have been used for the initiation of somatic embryo formation (Dunstan et al. 1995). Abscisic acid (ABA), ethylene and gibberellic acid (GA3) played a regulatory role during development and maturation (Jiménez 2005).

5.2 Primary Somatic Embryogenesis

Primary somatic embryogenesis and plant regeneration were reported for the first time from the callus of seedlings of "*E.* × Liechow" (Ouyang et al. 1980, 1981, according to Le Roux and Van Staden 1991b). Boulay in 1987b (according to Le Roux and Van Staden 1991a) achieved SE from hypocotyl and internode calli derived from seedlings of *E. gunnii* using two different media and various PGR concentrations. Also, Chang-Le and Kirby (1990) (according to Le Roux and Van Staden 1991a and b) were able to induce embryo-like structures in cultures of hypocotyls, cotyledons and leaves of young seedlings of *E. botryoides*, *E. dunnii*, *E. grandis* and *E. rudis*, as well as from young leaves of cultured shoots of superior adult *E. grandis* clones. The authors used a sequential culture technique in a medium containing MS salts, RV vitamins (reference not given) and amino acids. Slow-growing green protuberances developed from cut surfaces of explants after 2 weeks in culture on a medium containing 1.1 mg/L 2,4-D. These developed into adventitious shoots and embryo-like structures when transferred to a medium with 1.l mg/L BAP. Somatic embryogenesis has been reported from callus derived using shoots of 4–5-year-old trees of E. *grandis* on MS medium with 0.1 mg /L NAA and 5 mg/L Kin (Lakshmi Sita et al. 1986, according to Le Roux and Van Staden 1991a, and b). Somatic embryos were obtained by culturing friable callus in liquid medium containing 1 mg/L of BAP, Kin, NAA and 2,4-D each. In *E. citriodora*, somatic embryos were obtained from zygotic embryos grown on B5 medium with 3 mg/L NAA and 5% sucrose (Muralidharan and Mascarenhas 1987; Muralidharan et al. 1989). In *E. globulus*, a protocol for inducing SE was first reported by Trindade (1996). Later, Bandyopadhyay et al. (1999) and Nugent et al. (2001b) also reported SE induction and embryo formation with low reproducibility. Pinto et al. (2002) reported the regeneration of emblings and studied the effect of the explant, PGRs and time of exposure on the induction process of SE. SE was induced from cotyledon and mature zygotic embryo-derived callus in the presence of NAA. Oller et al. (2004) obtained embryogenic callus from leaf using IBA, but further progress is reported. Prakash and Gurumurthi (2005) reported SE and plant regeneration in *E. tereticornis* from embryogenic calli using mature zygotic embryos. When calli were transferred to the respective induction medium (MS or B5 with 2,4-D or NAA) containing

BAP, somatic embryos developed in 1–2 weeks. Somatic embryos were successfully germinated and converted in MS PGR-free medium, and rooted plants were effectively acclimatized. Later on, Pinto et al. (2008a) investigated the importance of different culture media in the induction phase and found that MS and B5 were the best media. The addition of anti-browning compounds (ascorbic acid, charcoal, dithioerythritol, dithiothreitol, poly vinyl poly pyrrolidone and silver nitrate) to the induction and expression media (MS) to control tissue oxidation in E. globulus SE process was also studied. There was a decrease in accumulation of phenolics and reduced SE potential when these anti-browning agents were added to induction and expression medium. The continuous exposure to antioxidants completely inhibited the SE response (Pinto et al. 2008a).

5.3 Secondary Somatic Embryogenesis

In contrast to primary SE induced from explant cells, repetitive, secondary or recurrent SE also occurs from somatic embryos in culture, either directly or through callus (Merlke et al. 1995; Raemakers et al. 1995). This phenomenon is of potential importance both for mass clonal propagation and for the gene transfer technology (Thorpe 2000). In many cases, the cultures are able to retain their competence for secondary embryogenesis for many years and thus constitute a very useful material for an array of different studies (Martinelli et al. 2001). Similarly to what was described for the induction stage, the proliferation of embryogenic cells may take a number of forms and is influenced by a variety of factors. The embryogenic callus is maintained on a medium similar to that used for induction. In general, liquid medium was preferred for large-scale propagation (von Arnold et al. 2002). In *E. citriodora*, Muralidharan et al. 1989 and Muralidharan and Mascarenhas (1995) were able to develop a highly efficient protocol of secondary SE. According to Muralidharan et al. 1989, the embryogenic potential was maintained for a period over 3 years in dark, on B5 medium containing 5 mg/L NAA, casein hydrolysate (500 mg/L), glutamine (500 mg/L) and 30 g/L of sucrose. The addition of inositol was found crucial for the maintenance of long-term embryogenic competence (up to 9 years) of the same cultures, when grown in liquid medium. Eventually, embryo development occurred on fresh B5 medium without PGRs and in light (Muralidharan and Mascarenhas 1995). In *E. globulus*, the occurrence of repetitive SE was first reported in 2004 by Pinto et al. (2004). The authors developed a protocol where somatic embryos were cultured on MS medium with 3 mg/L NAA and 30 g/L of sucrose and maintained at 24 °C in dark. In order to evaluate the genetic stability and the true-to-type propagation of *E. globulus* via repetitive SE, 8-month-old somatic embryos were investigated using flow cytometry, and no major ploidy changes were detected between somatic embryos and mother plants (Pinto et al. 2004).

5.4 Maturation, Germination and Conversion Somatic Embryos

Only the embryos that have accumulated enough storage materials and have acquired desiccation tolerance at the end of the maturation stage develop into normal plants (Merkle 1995). The addition of PGRs like ABA to the culture medium, as well as the increase of osmotic pressure (e.g. by adding polyethylene glycol), desiccation and low-temperature exposure, may allow the latter phases of SE by stimulating maturation and inhibiting precocious germination (Merkle 1995; Watt et al. 1999; Jiménez 2005). In general, even when large quantities of somatic embryos are obtained, a common bottleneck for large-scale propagation is the conversion of these somatic embryos in plants. On a culture medium lacking PGRs, somatic embryos usually develop into small emblings comparable to seedlings. But in some cases, auxin and cytokinin may be important to stimulate germination/conversion. Furthermore, a significant change in the composition of the basal medium is often required. In some species, it is required to include extra compounds like glutamine and casein hydrolysate (von Arnold et al. 2002). In *Eucalyptus*, mature somatic embryos usually do not develop in the presence of auxin. Plant regeneration (emblings) has been usually achieved in auxin-free medium or, occasionally, in media containing cytokinins and/or gibberellic acid (GA3). In *E. citriodora*, mature embryos germinated easily when transferred to an auxin-free medium. After isolation in a liquid medium, individual mature somatic embryos were transferred to germination medium (B5 medium with 20 g/L sucrose) leading to 52% of the embryo germination with healthy shoot and root systems. The addition of ABA had a negative effect on the growth of embryogenic masses with embryos getting moribund with increasing concentrations (Muralidharan and Mascarenhas 1995). In *E. dunnii* and in *E. grandis*, embryo maturation and subsequent germination were also achieved, despite at low success rates (Watt et al. 1995). In *E. grandis*, the addition of ABA and PEG (alone or in combination) and 3 h of desiccation did not show any success, with the rate of embling regeneration being low or absent (Watt et al. 1995). It is consensual that the regeneration of viable emblings is still a problem in many species (Merlke 1995), and *Eucalyptus* is no exception. In *E. globulus*, the influence of the culture medium (MS and B5), PGRs (auxins and cytokinins) and light on secondary SE was tested (Pinto et al. 2008b). They reported that hormone-free MS medium was more efficient for cotyledonary embryo formation and germination than B5 medium. Besides, reducing the levels of auxin (NAA) increased the proliferation of globular somatic embryos and allowed the maintenance of SE competence maintained on medium-free of PGRs. The addition of two cytokinins (BAP and Kin) to the MS medium did not improve proliferation of globular secondary embryos but was crucial during the later stages of the SE process (germination and conversion). Depending on the SE stage, light also plays an important role, influencing the quality of the process (Pinto et al. 2008b).

5.5 *Acclimatization of the Emblings*

The final application of SE to micropropagate selected individuals can be realized with the successful acclimatization of a large number of plants to field conditions. Also, as the in vitro propagated plants should be true to type, i.e. with the plus characteristics (high yield, uniform quality, shorter rotation period) of the donor genotypes, somaclonal variation is regarded as undesirable, at least at this stage. Therefore it is essential to verify and follow the clonal fidelity and field performance of somatic embryo-derived plants (Tremblay et al. 1999; Kaeppler et al. 2000). Acclimatization of emblings was reported for *E. grandis* (Watt et al. 1991), *E. citriodora* (Muralidharan et al. 1989; Muralidharan and Mascarenhas 1995) and *E. tereticornis* (Prakash and Gurumurthi 2005). In all these species, they have shown that the basic acclimatization procedure included a gradual reduction of the environmental relative humidity and the transfer to soil substrates (peat, perlite or sand). However, in those works, performance of emblings was measured only as survival rates. Up to this moment, no studies are known that have explored important aspects, as histocytology, physiology or genetics, during the embling acclimatization in *Eucalyptus* genus. In conclusion, this chapter reviews the most relevant recent advances on the SE process in *Eucalyptus*, from the somatic embryo induction to the plant acclimatization. Nevertheless, despite of the large amount of research conducted during the last years, there is still a gap on the knowledge of the mechanisms involved in the regulation of SE. Besides, additional research is also needed to identify and eventually overcome some of the current bottlenecks and, so, devise a successful strategy in this economically important forest species to efficiently establish a SE system at the industrial level. In general, the in vitro culture procedures as well as the environmental conditions, genotype and age of explants in culture are often associated with the occurrence of somaclonal variation (Rani and Raina 2000). Morphological markers, chromosome analysis, breeding behaviour, isoenzymes or DNA markers may be used to detect somaclonal variation. The early assessment of genetic fidelity at various culture stages may help to identify which culture condition(s) is inducing the observed variation (Rani and Raina 2000). Flow cytometry (FCM), a high-throughput and reliable method, has been increasingly chosen for analysing major ploidy changes during genetic variation assays. However, up to this moment, very few reports have used this technique to assay somaclonal variation in woody plants (Santos et al. 2007). Also, the preservation of somatic embryos, for example, through encapsulation procedures, has been reported for *E. citriodora* (Muralidharan and Mascarenhas 1995). The application of this preservation method together with cryopreservation is still in its infancy in this genus, but its success strongly depends on the development of reliable SE protocols. The propagation of plants through in vitro culture may result in the formation of plantlets of abnormal morphology, anatomy and physiology. After ex vitro transfer, these plantlets may easily be impaired by sudden changes in environmental conditions, and so a period of acclimatization is needed (Pospísísilová et al. 1999; Hazarika 2006).

6 Conclusions

Clonal forestry is a reality in the propagation of economically important trees. Macropropagation through rooting of cutting is a major route for mass multiplication of superior trees of *Eucalyptus*. Micropropagation in combination with macropropagation is also practiced by many companies. The tissue culture work over the last three decades has proved the benefits of micropropagation and found practical application. With the advent of newer technologies, the scientific knowledge on plant development and the factors controlling different aspects of development has increased. This has opened up new avenues to improve the existing protocols of plant propagation through in vitro methods. Tissue culture experiment holds great promise to the nature lovers, as it supplies a continuous unlimited number of plantlets for the afforestation programmes. Many of the government agencies are funding institutions focused on tree propagation including in vitro methods. With the ever-increased rate in which the forest cover is getting disappeared from the earth, it is an urgent call to restore at least a few percentage of the lost plantation to avoid severe climatic changes, which may become detrimental to the existence of living beings (Satheeshkumar 2012). Successful micropropagation of plants is now a commercial reality. Many commercial laboratories and national institutes worldwide use in vitro culture system for rapid plant multiplication, germplasm conservation, elimination of pathogens, genetic manipulations and secondary metabolite production. Millions of plants are routinely produced in vitro annually. The great potential of micropropagation for large-scale plant multiplication can be tapped by cutting down the cost of production per plant by reducing the unit cost of micropropagule and plant production without compromising the quality. Somatic embryogenesis facilitates cryopreservation, synseed development, somaclonal variation and genetic transformation. Recent progress in genetic manipulation of plant cells has opened new possibilities for improvement of plants which is totally dependent on tissue culture (Kumar and Reddy 2011). However, before going for large-scale propagation, prior field testing of clones is needed. Native species in planted forests are often very seriously damaged by native pests, but they are also subject to increasing problems due to accidental introductions of non-native organisms. Plantations of non-native, or introduced, species such as *Eucalyptus*, *Pinus* and *Acacia* have typically been relatively free of pest problems during the early years of establishment due to a separation from their natural enemies (Wingfield et al. 2008, 2010). The large number of site-specific and field-tested clones in the diverse genetic base is preferred than the monoclonal narrow genetic base to avoid the pests. Given the dramatic increases in the movement of pests globally, largely driven by anthropogenic factors (Liebhold et al. 2012, Santini et al. 2013), forest health will be an increasingly important constraint to forest productivity although the application of modern breeding and other technologies can offset losses (Wingfield et al. 2013).

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In Vitro Propagation of Important Rootstocks of Apple for Rapid Cloning and Improvement

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Abstract Micropropagation of apple rootstocks has played an indispensable role in cloning and multiplication of cultivars possessing desired characters such as early maturity, shortened height, etc. It has further added to a dimension of research in apple by providing season-independent multiplication of disease-free rootstocks. Subsequently many studies were conducted to optimize in vitro propagation of selected apple rootstocks with the aim of rapid cloning of disease-free planting material for grafting and also to undertake trait-specific modifications of selected rootstocks to enhance their potential. Work has also been conducted for development of regeneration protocol through shoot organogenesis and somatic embryogenesis. Some studies on development of genetic transformation protocols of selected rootstocks and modification of some important traits has also been reviewed in this article.

Keywords Micropropagation · Shoot organogenesis · Somatic embryogenesis · Genetic transformation

1 Introduction

Apple (Family: Rosaceae), is an important pomaceous fruit. It is grown in more than 313,040 hectares in Indian Himalayan region (FAOSTAT 2014). India enjoys the position of sixth largest producer of apple in the world (FAOSTAT 2013). The fruit crop is vegetatively propagated through grafting, budding or layering (Westwood 1993). Conventionally, to get the desired characters, scion of an appropriate apple cultivar is grafted over the selected rootstock (Westwood 1993). Juvenile rootstocks and scion counterparts are used for grafting as the mature tissues are difficult to graft (Hackett 1985; Greenwood and Hutchison 1993). Although,

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Table 1 Characteristics of few apple rootstocks

Rootstock	Height	Fruit-bearing age	Habitat	Uses
Dwarf				
M.27	4–6 ft	2 years	Pennsylvania	Dwarf pyramids
M.9	6–8 ft	2–3 years	United Kingdom and Western Europe	Small orchards and gardens
Ottawa 3	8 ft	2–3 years	Canada	Orchards
M.26	8–10 ft	2–3 years	Northern America	Bushes, pyramids and containers
Semidwarf				
G.935	8–12 ft	3 years	Geneva	Bushes, large cordons
Mm.106	10–13 ft	3–4 years	Europe and northern America	Orchards, small gardens
Semi-standard				
Bud.118	10–16 ft	3–4 years	United States	Traditional large trees
M.111	13–15 ft	4–5 years	United Kingdom	High-density orchards
Standard				
M.25	>15 ft	5–6 years	United Kingdom	Traditional orchards, poor ground
Antonovka	14–20 ft	5 years	Russia	Enlarged trees

overall characteristics are governed by the scions, but the characteristics such as tree height, fruit-bearing capacity, quality and maturity age are reported to be effected by the rootstock used (Wheaton et al. 1995). Based on these characteristics, rootstocks are classified into four categories (i) dwarf, (ii) semidwarf, (iii) semi-standard and (iv) standard (Webster 1997). Dwarf rootstocks are generally preferred for commercial plantations due to numerous economic advantages (Fallahi et al. 2002). Characteristics of few important rootstocks are explained in Table 1.

Although, screening has led to the development of various rootstocks with improved characters, various issues such as stress resistance, late fruiting, slow multiplication, etc. are still posing problems. A solution to these problems of traditional propagation lies in the micropropagation technique which ensures rapid multiplication of disease-free plants (Akin-Idowu et al. 2009). Year 1960 was marked with the first reports on in vitro propagation of apple, which remarkably enhance the apple production (Jones 1967; Walkey 1972). Since then, numerous studies on micropropagation of apple and its rootstocks using various explants such as axillary buds, shoot tips, nodal cuttings, etc. have been reported (Bommineni et al. 2001; Chakrabarty et al. 2003; Kaushal et al. 2005; Dalal et al. 2006; Yaseen et al. 2009; Boudabous et al. 2010; Dobranski and da Silva 2010; Amiri and Elahinia 2011; Soni et al. 2011; Bhatt et al. 2012; Keresa et al. 2012; Mehta et al. 2014; Ghanbari 2014). Most of the reports cited above reported a genotype-dependent behaviour of apple cultivars and rootstocks.

In addition to rapid multiplication, incorporation of desired characters such as early fruiting, disease resistance, etc. in rootstocks are seen as important aspects for commercial plantation of apple. For this purpose, genetic transformation of apple rootstocks is often seen as an alternative (Zhu et al. 2001; Zhu et al. 2003). Although various studies on cloning of important genes required for transformation of rootstocks have been reported (Belfanti et al. 2003; Zhang et al. 2006; Pagliarani et al. 2009; Polanco et al. 2010; Flachowsky et al. 2012), successful reports on field trials of transformed apple trees are limited (Smolka et al. 2010).

In order to undertake successful crop improvement programmes based on genetic transformation, development of effective regeneration protocol through shoot organogenesis and/or somatic embryogenesis is a prerequisite. In apple, a huge number of reports are available on regeneration through shoot organogenesis and somatic embryogenesis (Mehra and Saroj 1979; Saad 1965; Hyae et al. 1996; Dobranski et al. 2002; Nhut et al. 2002; Damiano et al. 2003; Modgil et al. 2005; Magyar-Tabori et al. 2011). Present study will focus on the micropropagation of important rootstocks, and shoot organogenesis/genetic transformation of apple for cloning and improvement will also be discussed.

1.1 Importance of In Vitro Propagation of Apple Rootstocks

- Production of plantlets throughout the year
- Production of disease-free planting material
- Easy maintenance and faster propagation of rootstocks
- Plant improvement through trait-specific genetic manipulations (Dobranski and da Silva 2010)
- Facilitates the international exchange of apple cultivars and germplasm as they are free from insects and phytopathogens

2 Micropropagation of Apple Rootstocks

In apple, micropropagation is largely reported from various explants such as axillary buds (Sharma et al. 2000; Kaushal et al. 2005; Soni et al. 2011), nodal segments (Mehta et al. 2014), shoot tips (Dunstan et al. 1985; Dalal et al. 2006), etc. (Table 2). Most of these studies reported genotype-specific response of apple to micropropagation (Abdul-Kader et al. 1991; Karhu and Zimmerman 1993). Therefore, optimizing the medium composition and physical factors for every cultivar is a prerequisite (Modgil et al. 1999). Generally, micropropagation is divided into various stages such as (i) establishment of in vitro cultures, (ii) shoot multiplication, (iii) rooting of microshoots and (iv) acclimatization for field transfer (George and Debergh 2008).

Table 2 Micropropagation in important apple rootstocks

S. No.	Rootstock	Explant	Medium	Response	Reference
1	M26	Shoots (1.5 cm, 15 cm long)	Knop's solution +minor elements of MS medium + BAP (0.1, 1.0[a], 10, 50 mg/l)	Increase in leaf production, fresh weight of the leaves and length of the stem	Jones (1967)
	M.Viii	Shoots (15 cm long)			
2	M.27 M.9 M.26 MM.111 Macspur	Shoots (1 cm long)	MS + 1.0, 5.0[a], 10.0 µM BA	Shoot production	Lane and McDougald (1982)
			MS + 0.1, 0.33, 1.0[a], 3.3[a], 10, 33 µM NAA	Rooting except (M9)	
3	Ottawa 3	Meristem tips	MS + (0.1, 0.5[a], 1.0, 5.0) mg/l NAA + (0.1, 0.5[a], 1.0, 5.0) mg/l BA+ (0.1, 0.5[a], 1.0, 5.0[a]) mg/l GA_3	Shoot proliferation	Pua et al. (1983)
		Microshoots (2–3 cm long)	MS+ (0, 0.05, 0.25, 1.25 6.25[a]) mg/l IBA	100% rooting	
4	M26	Shoot tips (1–2 cm)	Modified MS (0.5, 0.25 strength) + (1[a], 3)% sucrose	90% in vitro rooting	Simmonds (1983)
5	M4	Shoots (1–2 mm dia, 1.5–2.0 cm length, 7–9 nodes)	MS + (1.125, 1.150[a], 1.425) mg/l BA + (0.15[a], 0.20) mg/l IBA	Maximum shoot production	Dunstan et al. (1985)
6	M9	Apical shoots	MS + (0.1, 1.0, 4.0, 12.0[a] or 15.0) µM IBA	80% rooting	Alvarez et al. (1989)
	M26		MS+(0.1, 1.0, 4.0[a], 12.0 or 15.0) µM IBA	100% rooting	
7	M.9	Nodal explants	MS medium+3% sucrose+1 mg/l BA +0.2 mg/l IBA	Reduction in hyperhydric shoots using bioreactor technology	Chakrabarty et al. (2003)
8	MM 111	Axillary buds (0.2– 2.0 cm long)	MS medium + BA (0.5, 1.0[a], 1.5, 2,0 mg/l) + GA_3 (0.5[a] mg/l);	High multiplication of shoots	Kaushal et al. (2005)
		Microshoots (3.0–4.0 cm)	Auxin-free medium followed by transferring on 1/2 MS liquid medium containing 0.5 mg/l IBA	80–85% rooting	
9	M9	Shoot tips (1.0–2.0 cm in length)	MS medium + (1.11, 2.22[a] µM) BAP + (0, 0.49[a], 0.98) µM + (0, 2.32[a]) µM KIN	Multiple shoot formation	Dalal et al. (2006)

10	MM 106	Axillary buds	MS+ (0.1, 0.5[a], 1.0) mg/l BAP+ (0.5, 1.0[a], 5.0) mg/l GA_3+ (0.1[a], 0.5, 1.0 mg/l) IBA + 100[a] mg/l PG	Maximum no. of shoots;	Sharma et al. (2000)
		Microshoots (1.5–2.0 cm long)	Dipped in MS + (30[a], 50, 70, 100) mg/l IBA and then transferred to auxin-free medium	80% rooting	
11	M9	Buds having single or multiple nodes	MS + 0, 2.2, 4.4[a], 8.8 µM BA +1.14, 2.27[a], 4.54, 9.08 µM TDZ	Shoot multiplication (4.9 number/month)	Amiri and Elahinia (2011)
			MS + (0, 2.2, 4.4, 8.8[a]) µM BA + (1.14[a], 2.27, 4.54, 9.08) µM TDZ and (0, 1.40 2.8[a]) µM GA_3	Shoot elongation (17.8 mm)	
			½ MS + (0, 2.2, 4.4[a], 8.8) µM BA + (1.14, 2.27[a], 4.54, 9.08) µM TDZ	42% rooting	
12	M27	Buds having single or multiple nodes	MS + (0, 2.2, 4.4[a], 8.8) µM BA + (1.14, 2.27[a], 4.54, 9.08) µM TDZ	Shoot multiplication (5.7 number/month)	Amiri and Elahinia (2011)
			MS + (0, 2.2, 4.4, 8.8[a]) µM BA + (1.14[a], 2.27, 4.54, 9.08) µM TDZ and (0, 1.40, 2.8[a]) µM GA_3	Shoot elongation (19.0 mm)	
			½ MS + (0, 2.2, 4.4[a], 8.8) µM BA + (1.14, 2.27[a], 4.54, 9.08) µM TDZ	51% rooting	
13	MM106	Buds having single or multiple nodes	MS + (0, 2.2, 4.4, 8.8[a]) µM BA + (1.14[a], 2.27, 4.54, 9.08) µM TDZ	Shoot multiplication (3.9 number/month)	Amiri and Elahinia (2011)
			MS + (0, 2.2, 4.4, 8.8[a]) µM BA + (1.14[a], 2.27, 4.54, 9.08) µM TDZ and (0, 1.40, 2.8[a]) µM GA_3	Shoot elongation (28.0 mm)	
			½ MS + (0, 2.2, 4.4[a], 8.8) µM BA + (1.14, 2.27[a], 4.54, 9.08) µM TDZ	64% rooting	
14	Merton I. 793	Terminal and axillary buds (0.5–2.0 cm long)	MS + (0.5, 1.0[a], 1.5, 2.0) mg/l BA + (0.01, 0.05, 0.1[a]) mg/l IBA	Maximum bud break	Soni et al. (2011)
			MS+ (0.5[a], 1.0, 1.5, 2.0) mg/l BA+ (0.01[a], 0.05, 0.1) mg/l IBA	Eightfold increase in shoot multiplication	
			MS + (0.1[a], 0.3, 0.5) mg/l NAA	66.78% rooting response	

(continued)

Table 2 (continued)

S. No.	Rootstock	Explant	Medium	Response	Reference
15	MM 106	Shoots (1.0–1.5 cm long)	MS medium + 0.5 µM IBA,+4.43 µM BA+90 mM sorbitol+ (0, 20[a], 40, 60, 80, 100 and 120) mM NaCl	Increase in shoot length, fresh weight and root length	Bahmani et al. (2012)
16	M9	Axillary shoot tips (0.5–1.5 cm long)	MS medium+ (0, 0.5, 1.0, 2.0, 3.0[a], 4.0) mg/l KIN+ (0, 1.0, 1.5, 2.0[a]) mg/l BA+ (0, 1.0, 1.5 2.0[a]) mg/l IBA + 3.0% sucrose	Multiple shoot proliferation	Bhatt et al. (2012)
			MS + (0, 1, 2, 3[a]) mg/l IBA + (0, 1[a], 2) mg/l KIN	68.3% rooting response	
17	MM 106; B9	Nodal segments from actively growing shoots	Full strength Half strength Quarter strength-modified MS media	Highest growth on full strength medium; 98% rooting response in MM 106; 96% (B9)	Mehta et al. (2014)
18	M9	Single nodes;	MS medium+ (0.5, 1.0 1.5[a]) mg/l BA;	Maximum shoot formation;	Ghanbari (2014)
		Microshoots	MS + (0.5, 1.0, 1.5[a]) mg/l IBA	60% rooting response	
19	AZxM9	Axillary or apical buds (1–2.5 cm long)	MS+ (0.4[a], 0.8, 1.2) mg/l BAP + 0.1[a] mg/l NAA	Increase in shoot number;	Tabalvandani et al. (2014)
			LS, MS[a]+ (0, 0.8, 1.6, 2.4[a]) g/l thiamine + (0.1, 0.15[a], 0.2) g/l FeEDDHA	Increase in root number	

[a]Denotes concentration of plant growth regulator incorporated in medium on which maximum shoot multiplication or rooting was observed

2.1 Establishment of In Vitro Cultures

2.1.1 Choice of Explant

Depending upon micropropagation route to be adopted, the choice of explant is important for success (Abbott and Whiteley 1976). In majority of studies, nodal segments have been used as explants for culture initiation (Wang et al. 1994; Modgil et al. 1999; Chakrabarty et al. 2003), but apical meristems or shoot tips are also widely used to obtain virus-free plants (Abbott and Whiteley 1976; Kaushal et al. 2005; Dobranski and da Silva 2010). The use of these explants frequently resulted in tissue and media browning leading to death of the explant. The use of axillary buds has been preferred over the other explants, because of lower problem of browning observed in other explants (Kausal et al. 2005; Huth 1978; Sharma et al. 2000). It has been reported that size and position of axillary buds on the mother plant are also determining factors for the extent of browning of medium and thus influence survival (Kaushal et al. 2005). Smaller (0.2–0.6 cm) axillary buds are known to show minimum tissue browning and higher survival rates as compared with larger (0.6–2.0 cm) explants (Kaushal et al. 2005) which contain more phenolics, the main cause of browning. Further, the use of dormant buds present at distal position on mother plant is also reported to be beneficial for reducing problem of browning (Kaushal et al. 2005). The use of internodal segments from the dormant shoots of young plants was observed to give better response with respect to sprouting percentage and reduced browning (Fig. 1a).

Apart from the conventionally used explants, recently the use of thin cell layers (TCL) has also been reported in two important apple cultivars ‘Royal Gala’ and ‘Freedom’ for culture establishment (Dobranszki and da Silva 2011). The response of TCL was also reported to be genotype dependent (Dobranszki and da Silva 2011), and further investigations are required in this direction.

2.1.2 Disinfection

The procedure for explant disinfection holds an eminent position in successful establishment of culture. In apple, disinfection of explants is a complicated process (Modgil et al. 1999). It has been reported in many plant species that the contamination levels depend upon the season of explant collection (Hutchinson 1984; Kumar 1996). Higher contamination levels are reported in apple in autumn and winter in comparison to summer and spring (Modgil et al. 1999), whereas in *Rosa damascena* (another species of family Rosaceae), higher contamination levels were recorded during rainy season (Kumar 1996). Furthermore, in apple the contamination is also cultivar specific (Hutchinson 1984). Thus disinfection protocols are also required to be optimized according to conditions and cultivar. Various protocols have been reported for surface disinfection of apple rootstock explants (Webster and Jones 1989; Wang et al. 1994; Yepes and Aldwinckle 1994; Sharma et al. 2000; Dobranszki et al. 2000). A commonly used procedure of surface disinfection involves 30–60 s

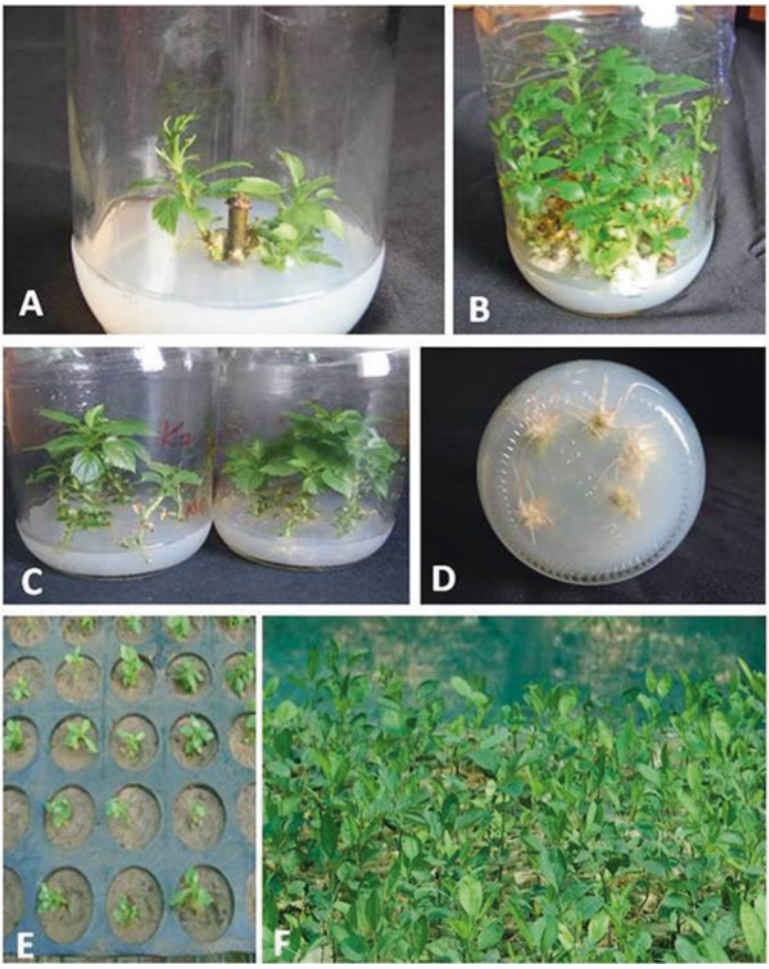

Fig. 1 Micropropagation procedure for important rootstocks of apple. (**a**) Sprouting of shoots from dormant nodal explants; (**b**) Shoot multiplication on MS medium supplemented with BA and NAA; (**c**) Rooting of microshoots on MS medium supplemented with IBA, note early root emergence; (**d**) Advanced stage of rooting with elongated root system; (**e**) Rooted plants transferred to trays for acclimatization; (**f**) Actively growing acclimatized plants of apple rootstocks

dip in 70% (v/v) ethanol followed by 10 min disinfection with 1% (w/v) sodium/calcium hypochlorite or 0.1% (w/v) mercuric chloride and finally rinsing with sterile distilled water (Dalal et al. 2006; Soni et al. 2011; Mehta et al. 2014). Comparisons have also been made between mercuric chloride and sodium hypochlorite for efficient disinfestation. It has been observed that 15% (v/v) sodium hypochlorite solution (4% active chlorine) is equally effective for disinfection of explants and as combination of 100 mg/ml mercuric chloride and 12% domestos (Webster and Jones 1989; Webster and Jones 1991). This disinfection protocol has however been

altered as per requirements of the mother plant. For instance, different sodium hypochlorite concentrations were found to be effective for disinfection of different cultivars [1% (v/v) sodium hypochlorite: 'MM.111' rootstocks, 10% (v/v): 'M9' rootstocks, 15% (v/v): 'B.9', 'Ottawa 3', 'P.2', 'P.22' rootstocks] (Kaushal et al. 2005; Grant and Hammatt 1999; Webster and Jones 1989; Webster and Jones 1991). Sometimes, contamination is not eliminated completely from the explants, despite using effective disinfection procedures; thus a procedure for elimination of such contaminations was reported by Dobranszki et al. (2000), which includes washing of explants under running tap water, followed by treatment with 0.05% (w/v) Tween 20 for 1 h in an incubator shaker under 150 rpm and 26 °C. This was followed by washing and surface disinfection using 70% (v/v) ethanol for about 3 min and then treatment with 0.1% (w/v) $HgCl_2$ solution for 5 min. Before culturing on the medium, treated explants were washed many times with sterile distilled water to remove disinfectants. Further, to avoid extremely high contamination, disinfection was once again carried out next day with 25% (w/v) Clorax (Dobranszki et al. 2000).

2.1.3 Browning

Browning of tissue and medium is one of the major problems associated with woody plant species (Sharma and Singh 2002; Leng et al. 2009). Wounding of the tissues during micropropagation leads to release of various enzymes such as polyphenol oxidase (PPO), superoxide dismutase and peroxidase (Pan and Van Staden 1998). These enzymes come to rescue of the plant by catalysing various reactions to eliminate reactive oxygen species (Sharma et al. 2012). One such reaction results in production of melanin, a dark pigment, resulting in browning of medium and also explants (Onay and Jeffree 2000). This tissue browning may lead to tissue death at early stages of culture establishment (Dobranski and da Silva 2010). The activity of these enzymes is reportedly dependent on physical factors such as light and temperature (Werner and Boe 1980; Modgil et al. 1999). Light and high temperature are known to increase the extent of browning in woody plant species as they result in release of more phenolic compounds in comparison to dark and low temperatures (Hildebrandt and Harney 1988). It has been reported that release of phenols and PPO is highest during active growth season, which can cause browning of medium and explants (Biedermann 1987; Dobranszki et al. 2000). Therefore, for culture establishment, explants should be collected during dormancy period (Wang et al. 1994).

To circumvent the problem of tissue browning, various methods have been reported which include treatment of explants or mother plants (Block and Lankes 1996) or supplementing medium with certain additives such as activated charcoal (Thomas 2008), cytokinins (Dobranszki et al. 2000), adsorbents and antioxidants (Sharma et al. 2000). Among all the additives, activated charcoal is a promising material which provides dark environment on one hand and adsorbs/inactivates phenols/polyphenol oxidase on the other (Tisserat 1979; Thomas 2008). It has also been reported that addition of activated charcoal increase organogenesis frequency in plants by absorbing inhibitory compounds and sustained release of minerals/plant

growth regulators from the medium (Jaiswal and Amin 1987; M'Kada et al. 1991). The extent of browning and its effective control are also genotype dependent, thus different combinations of methods are tested to inhibit browning in every apple cultivar (Block and Lankes 1996).

2.2 Shoot Multiplication

Micropropagation refers to rapid in vitro multiplication of plants; the shoot multiplication is the most important aspect of micropropagation. The efficiency of the shoot multiplication is influenced by many factors such as genotype, medium composition, growth regulators, physical factors, genotype, etc.

2.2.1 Genotype

Shoot multiplication in apple is also a genotype-dependent phenomenon (Lane and Looney 1982; Karhu 1995; Kovalchuk et al. 2009). The use of 6-benzyladenine (BA) has been widely reported for active shoot multiplication in apple (Lane and McDougald 1982). Difference in organogenic response was attributed to variation in endogenous levels and transport of plant growth regulators and metabolic efficiency of the apple cultivars (Lane and McDougald 1982). Webster and Jones (1991) reported rapid multiplication of shoots of apple cultivars 'P.22' and 'Ottawa.3' on medium containing 4.44 μM BA, whereas in rootstocks 'P.2' and 'B.9', shoot multiplication was achieved on medium containing higher levels of BA along with additional requirement of phloroglucinol. Similar genotype-dependent responses of apple cultivars to plant growth regulators have been reported by many other researchers (Dobranszki et al. 2000; George and Debergh 2008; Ciccoti et al. 2008; Ciccotti et al. 2009; Amiri and Elahinia 2011). In addition to plant growth regulators, genotype-based variation in organogenic response has also been reported in the presence of different carbohydrates (Karhu 1995). Sorbitol is reported to result in highest shoot number, whereas sucrose/glucose resulted in maximum shoot length.

2.2.2 Medium

Murashige and Skoog (1962) medium is one of the most commonly adopted media for the micropropagation of apple rootstocks, but the use of other media formulations such as Linsmayer and Skoog (1965), Cheng (1978) and Quoirin and Lepoivre (1977) have also been reported for shoot multiplication of apple rootstocks. However, these media formulations were found to be less effective than MS medium (Welander 1985). The response of apple rootstock to medium composition is also genotype dependent (Ciccoti et al. 2008; Ciccotti et al. 2009). Comparative analysis

between shoot proliferations from different apple rootstocks on various medium combinations revealed different nutrient requirements of rootstocks (Ciccoti et al. 2008; Ciccotti et al. 2009). For instance, *Malus sieboldii* and its hybrid genotype 'C1907' showed maximum shoot multiplication on MS medium, whereas other hybrid genotypes of *M. sieboldii*, 'D2212' and 'H0801', showed best shoot proliferation on QL medium (Ciccoti et al. 2008; Ciccotti et al. 2009). Similar results were observed from rootstocks 'MM.106' and 'M9' and an important apple cultivar 'Gala'. Results of other studies revealed that LS medium is optimum for shoot proliferation in cv. 'Gala'; apple rootstocks proliferate to their best on DKW medium (Driver and Kuniyuki 1984; Van Nieuwkerk et al. 1986; Muleo and Morini 2006).

Inorganic and Organic Compounds

Concentration of inorganic compounds in the basal medium is also reported to influence shoot multiplication in apple (Amiri and Elahinia 2011). Webster and Jones (1991) reported that the replacement of disodium EDTA with EDTA ferric-sodium salt lead to better shoot growth. The study conducted by Mouhtaridou et al. (2004) in apple rootstock 'MM.106' reported the requirement of optimum boron concentration for shoot proliferation, chlorophyll content and shoot length of microshoots decreased with increasing boron concentration (Mouhtaridou et al. 2004). Furthermore, boron was also found to effect uptake of other mineral elements such as potassium, zinc and iron by the plant (Mouhtaridou et al. 2004). The optimum requirement of various vitamins and amino acids for in vitro growth of apple has also been studied intensively (Liu et al. 2009; Nabeela et al. 2009). It has been reported that every cultivar of apple have different requirements for shoot multiplication. Karhu (1997) reported that MS medium devoid of glycine but with increased concentration of thiamine HCl was beneficial for microshoot development in important apple cvs. 'Gala', 'Make' and 'McIntosh'. In a series of experiments by different workers, the use of MS medium with altered vitamin composition such as replacement of MS vitamins with LS or B5 vitamins has been reported to be useful for shoot multiplication (Baraldi et al. 1991; Liu et al. 2009; Nabeela et al. 2009). Further, the effect of D-arginine was also evaluated in cultivar 'Orin' (Liu et al. 2009), and it was observed that increased concentrations of D-arginine (1–5 μM) result in shoot retardation which can be recovered by addition of putrescine in the medium (Liu et al. 2009). Putrescine was also reported as an important molecule for in vitro apple shoot multiplication (Arias et al. 2005). Zanandrea et al. (2006) showed that nitrogen content in the growth medium has direct effect on photosynthesis in apple.

Carbohydrates

Carbohydrates are the most important parts of growth medium as they act as energy sources and also act as osmotic agents for supporting healthy plant growth and multiplication (Wang and Stutte 1992). Murashige and Skoog (1962) have reported the

beneficial effects of 3% (w/v) sucrose over other concentrations. However, in case of apple, sorbitol and sucrose are the major products of photosynthesis; thus the role of both as carbohydrate source has been intensively studied for in vitro growth of apple cultures (Noiraud et al. 2001). The response of apple microshoots to carbohydrate sources was also found to be genotype dependent (Karhu 1995). It was confirmed in case of two important rootstocks 'M9' and 'MM.106', using different concentrations of sucrose and sorbitol (Sotiropoulos et al. 2006). It showed that sucrose concentration was a determining factor for shoot length, whereas sorbitol determined both shoot proliferation as well as growth. Both rootstocks were found to have different sorbitol requirements. Although 'M9' was able to yield maximum growth on 164 mM sorbitol, but the requirement for 'MM.106' was almost double (Sotiropoulos et al. 2006). Sorbitol was also found to effect branching in apple shoots in genotype-dependent manner (Karhu 1995). Axillary branching was found to increase in 'McIntosh', 'Macspur' and 'Jaspi' cultivars in the presence of high sorbitol concentration, whereas no response was observed in cultivar 'Gala' (Pua and Chong 1984; Karhu 1995). Similar results were observed in rootstock 'MM.106', where sorbitol resulted in maximum organogenesis and shoot proliferation in comparison to maltose (Bahmani et al. 2009b).

Plant Growth Regulators

In vitro shoot multiplication in apple is largely controlled by cytokinins, and 6-benzylaminopurine (BA) was found to be beneficial in most of the studies (Welander 1989). The requirement of auxins (Ward and Leyser 2004) or gibberellins was also reported to be useful for better shoot multiplication (Yepes and Aldwinckle 1994). The use of BA has been reported for the shoot multiplication in number of apple cultivars and also rootstocks (Welander 1989; Bartish and Korkhovoi 1997; Camargo et al. 1998; De Klerk et al. 2001; Chakrabarty et al. 2003; Amiri and Elahinia 2011). The low BA concentrations were reported to reduce the multiplication rate in various apple rootstocks ('M9', 'M26' and 'M27') and cultivars 'Macspur' and 'Akero' (Lane and McDougald 1982; Welander 1985). Further, it has been reported that higher concentration (above 10 μM) of BA resulted in thickening and swelling of shoots (Marin et al. 1993). In addition to BA, the effect of other cytokinins such as benzyladenine-9-riboside (BAR), N6-meta-hydroxy-benzyladenin (TOP) and thidiazuron (TDZ) has also been studied for shoot multiplication in apple (Van Nieuwkerk et al. 1986; Magyar-Tabori et al. 2001, 2002). In one of the studies, comparison was made between BA and TDZ on shoot proliferation in cv. 'Gala' (Van Nieuwkerk et al. 1986), and it was observed that size of leaves and shoots was smaller on medium supplemented with TDZ in comparison to BA (Van Nieuwkerk et al. 1986). In another experiment, where comparison was made between three cytokinins, it was observed that for rootstock 'MM.106' and cultivars 'Jonagold' and 'JTE-H', TOP and BAR are the best cytokinins in comparison with BA as these lead to 2.5-fold increase in number of shoots per explant and also result in minimum reduction in shoot length ((Dobranszki et al. 2000; Magyar-Tabori et al. 2001, 2002).

Apart from using cytokinin for shoot multiplication, other PGRs such as kinetin, gibberellic acid (GA_3) and indole-3-butyric acid (IBA) have also been tested in apple (Kaushal et al. 2005). The use of combination of BA (4.4 μM) and kinetin (7.5 μM) as compared to BA (4.4 μM) resulted in doubling of the rate of shoot multiplication (Modgil et al. 1999). This response was also found to be genotype dependent. For instance, the combination of BA and kinetin showed maximum benefit in cultivars 'Prima' and 'Galaxy', whereas no such benefit was observed in 'Jonagold' on the same medium (Dobranszki et al. 2000). The use of BA along with α-naphthaleneacetic acid (NAA) resulted in better shoot multiplication in three rootstocks, namely, M7, MM106 and MM111 (Fig. 1b). The MS medium supplemented with 2.5 μM BA and 0.5 μM NAA resulted in best shoot multiplication in actively proliferating cultures of these rootstocks.

Gelling Agent

Agar-agar is most commonly used gelling agent in the medium for apple (Pasqualetto et al. 1988). It was reported that agar affects shoot multiplication and can also regulate hyperhydricity depending upon the presence of high molecular weight fractions (Pereira-Netto et al. 2007; Kumar et al. 2003). Other gelling agents such as gelrite and phytagel have also been tested in apple (Zimmerman et al. 1995; Bommineni et al. 2001). Results revealed that phytagel is most suitable for shoot multiplication and proliferation of apple cultivars as compared to agar (Bommineni et al. 2001); however sometimes it may cause hyperhydricity.

In addition to these gelling agents, mixture of natural polymers in combination with agar has also been used (Babbar et al. 2005; Lucyszyn et al. 2005). It was observed that mixture of agar with guar gum, a commercial galactomannan, showed better shoot proliferation in apple as compared to agar (Lucyszyn et al. 2005). The occurrence of hyperhydric shoots was also largely reduced on medium solidified with mixture of agar and galactomannans (Lucyszyn et al. 2005).

2.3 Physical Factors

Light is the most important factor affecting plant morphogenesis (Kumar et al. 2003). Although reports are available on use of continuous illumination (38–105 μmol m^{-2} s^{-1}) (Noiton et al. 1992; Schaefer et al. 2002), a 16-h photoperiod was found to favour in vitro shoot multiplication of apple (Dalal et al. 2006; Soni et al. 2011; Magyar-Tabori et al. 2011; Bahmani et al. 2012; Mehta et al. 2014). However, some authors also reported better shoot multiplication when incubated in light for 8 h as compared to 16 h in apple rootstocks (Marin et al. 1993). Apart from photoperiod, intensity as well as quality of light is also known to play an important role in shoot multiplication and branching in apple (Li et al. 2001). It was also reported that reduction in the duration of light exposure but increase in the light intensity result in more shoot multiplication in apple (Zanandrea et al. 2006).

Emission spectrum of light is another important parameter known to influence axillary branching in apple rootstocks (Muleo and Morini 2006). Blue light is reported to reduce branching and promote apical dominance, whereas red light increases branching and inhibited apical dominance in apple rootstock 'MM.106' (Muleo and Morini 2006). The reason behind such a variation was demonstrated using rootstock 'M9' as interaction between blue light receptors and phytochrome (Muleo and Morini 2008).

Other physical factors affecting in vitro shoot growth includes temperature, relative humidity, ethylene accumulation in culture vessels, etc. (Bommineni et al. 2001; George and Davies 2008; Liu et al. 2009). Although, these are issues in culture of plant tissue, but such studies are limited.

2.4 Rooting of Microshoots

Rooting is a very important step in micropropagation and is a limiting factor in many woody plant species (Custodio et al. 2004; Kumar 1996). Rooting is regulated by a number of physiochemical and genetic factors (Pawlicki and Welander 1995). Indolebutyric acid (IBA) was beneficial for root induction in microshoots of apple. Sharma et al. (2007) studied the effect of different concentrations of IBA on root induction in microshoots of apple rootstocks 'M7' and 'MM.106'. The response observed from both cultivars varied significantly. It was further reported that IBA at higher concentrations induced profuse callusing at the base of microshoots, which was detrimental (Yepes and Aldwinckle 1994). Incorporation of 2.5 μM IBA in MS medium was found to induce rooting in more than 85% of microshoots of all the tested rootstocks and cultivars (Fig. 1C & D). The replacement of MS medium with LS medium supplemented with low IBA concentration successfully solve the problem of callusing and also improved rooting of apple microshoots (Welander 2006), with also higher acclimatization efficiency.

The position of microshoot is also seen as an important factor for determining the extent of rooting. A study conducted on rootstock 'G.65' showed that 100 percent rooting can be achieved through upright positioning of microshoots (Isutsa et al. 1998), and this position-based rooting effect was also found to be genotype dependent (Isutsa et al. 1998).

Various other auxins such as indoleacetic acid (IAA) and α-naphthalene acetic acid (NAA) have also been tested for their efficacy in apple rooting, and NAA was found to be more effective for root induction than IAA (Zimmerman and Fordham 1985). Interactive effect of BA and IBA on the root induction in apple has also been studied, but no indicative effect of BA was observed (De Klerk et al. 2001). There are reports about the role of amino acids and organic compounds such as arginine, phloroglucinol and sucrose on rooting of microshoots of apple (Teresa 1992; Xu et al. 2008; Sharma et al. 2000; Bahmani et al. 2009a). It was observed that arginine along with low concentration of IBA resulted in increased number of roots per microshoot (Xu et al. 2008). Phloroglucinol was found to have no effect on rooting. It was also reported that sucrose is the best carbon source for root induction in apple

rootstocks in comparison with fructose and maltose, and also its higher concentrations are beneficial (Bahmani et al. 2009a). The beneficial effect of high sucrose concentration on rooting has been observed in other plants also (Kumar et al. 1999, 2010).

2.5 Acclimatization

Micropropagated plants begin autotrophic life following transfer to field, and the success of acclimatization depends upon the shift from heterotrophic mode of nutrition to autotrophic mode of nutrition. Development in acclimatization studies started in apple with the first report published by Webster and Jones (1989) on 'M9' apple rootstock. A major challenge prevailing during hardening is chances of desiccation as well as contamination (Kumar and Rao 2012). In apple, the survival rate after in vitro rooting was reportedly low in comparison to direct rooting in peat pots (Webster and Jones 1989). The novel method of hardening was introduced by Bolar et al. (1998), where Jiffy-7 pellets, soaked in nutrient and plant protectant mixture, were used for transfer of microshoots to field. This method resulted in enhanced survival rates of important apple rootstocks 'M26' and cultivars 'Golden Delicious', 'Gala' and 'Liberty'. It was further reported that survival rate of apple cultivars can be increased by in vivo inducing roots in fog chambers at temperature of 24 °C (Isutsa et al. 1998). In year 2009, a major breakthrough in acclimatization procedure further intensifies research in apple rootstocks when 90% survival was observed in rootstocks 'M.7' and 'MM.106'on transfer of microshoots into coco peat mixture (Modgil et al. 2009). It was also observed that the use of sand and coco peat, vermicompost (50%) and sawdust (20%) mixtures resulted in low survival rates of apple rootstocks.

Another very important factor that influences the acclimatization of apple rootstocks was the stage of rooting. Microshoots at early stage of rooting (when roots just started to emerge) show better survival as compared to the microshoots with long root system (Fig. 1c). The elongation of roots in soil after transfer was found to be beneficial for acclimatization.

The season of transfer and the environmental conditions also significantly influenced the acclimatization of micropropagated plants. Plants transferred during late rainy season show better survival as compared to those transferred during early rainy season and winter. Establishment of plants before the onset of winter dormancy was beneficial for the survival and post survival performance.

3 Shoot Organogenesis in Apple

Shoot organogenesis and somatic embryogenesis are indispensable tools to undertake any plant improvement programme through biotechnological interventions (Ou et al. 2008). Initiatives on apple regeneration studies were taken as early as 1958 (Letham 1958) when callogenesis was observed from apple fruit tissue on white's medium in the presence of auxin. It was followed by work of Nitsch (1959) where

Gauthret's nutrient medium was used to induce callogenesis. However, in above-mentioned reports, shoot organogenesis was not observed from callus. Shoot organogenesis was first reported in apple using axillary bud as an explant (Pieniazek and Jankiewiez 1966). Since then, numerous explants have been used to induce organogenesis in rootstocks and other cultivars of apple (Lane 1978; Yepes and Aldwinckle 1994; Rustaee et al. 2007; Rehman et al. 2009). Some of the important studies are detailed below:

3.1 Leaf Segment Culture

Leaf disc and segment cultures were initially reported in cultivar 'Golden Delicious' by Mehra and Saroj (1979) where callus was induced on MS medium supplemented with NAA and IAA. Subsequently, it was also reported that organogenesis in apple can be promoted by use of lower concentrations of cytokinins (Fasolo et al. 1989). However, Dobranski et al. (2002) reported that higher BA concentrations (>6.2 mg/l) increased organogenic potential of leaf explants taken from apple cultivar 'Royal Gala' and rootstock 'M.26'. In addition to plant growth regulators, various factors studied, namely, leaf maturity, duration of photoperiod and explants, were also found to influence organogenesis in apple rootstock M.26 and cvs. 'Akero' and 'Mclntosh' (Welander 1988; James et al. 1988; Ferradini et al. 1996; Hyae et al. 1996). Further, the use of cefotaxime along with PGRs was reported to enhance shoot organogenesis from leaf explants (Ongjanov 1988). Moreover, the position of leaf explants on the microshoot has also been shown to effect organogenesis in rootstock 'MM.106' (Modgil et al. 2005). It was observed that younger leaf explants near shoot apex have high regeneration potential in comparison to other leaves.

Presently, shoot organogenesis in rootstocks was induced on using a two-stage culture of leaf explants of MM111 and M7. Initially callus was induced on MS medium variously supplemented with BA and 2,4-D (Fig. 2a, b). When about 1-month old calli were transferred to medium supplemented with higher concentrations of BA (>5 μM), shoot organogenesis was observed ((Fig. 2c, d). Initially, following transfer to BA supplemented medium nodular structures developed (Fig. 2c), which later on differentiate in actively growing shoots (Gupta 2011). However, the frequency of shoot induction was not very high. Further work to enhance the frequency of organogenesis and to establish the mode of organogenesis is required to be taken up.

3.2 Internodal Segments

The work on the use of internodal segments for shoot organogenesis was initiated in apple cultivar 'Cortland Melntosh' by Saad (1965), which was followed by other workers where callus was obtained at cut ends of stem; however regeneration could

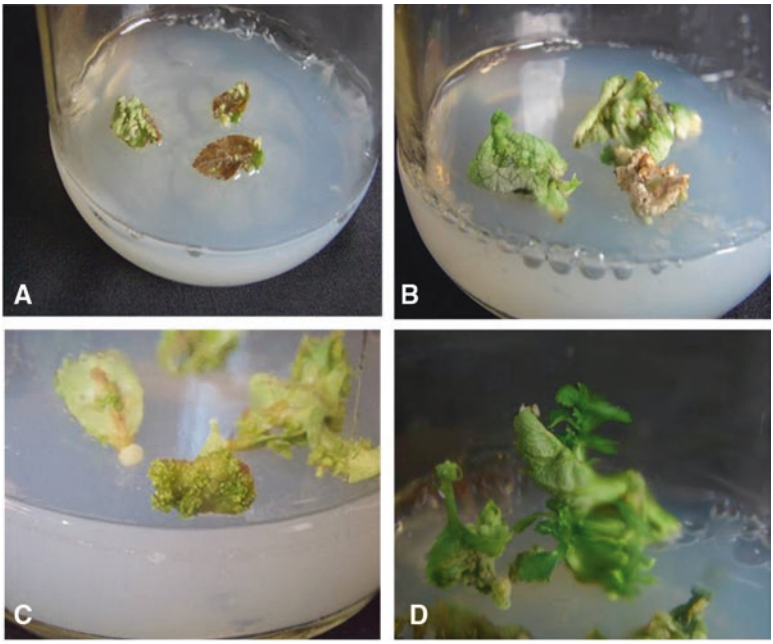

Fig. 2 Shoot organogenesis from leaf explants of apple rootstock 'MM111' (**a**–**b**) Callus induction from cut ends of leaf explants on MS medium supplemented with BA and 2,4-D (**c**) Calli developed nodular structures on BA rich MS medium (**d**) Actively growing differentiated microshoots

not be induced (Fujii and Nito 1972; Chong and Taper 1972; Mehra and Saroj 1979). First report on shoot organogenesis from callus induced from stem segment was published on apple cultivar 'Akero' by Evaldson (1985) and was achieved on MS medium supplemented with IBA and BA. It was reported that BA provided stimulus for growth and differentiation of shoot buds from callus. The role of various other cytokinins such as TDZ, zeatin and kinetin in shoot induction from internodal explants has also been reported (Korban et al. 1992). Moreover, pretreatment of explants with selective cytokinins prior to start inoculation is reported to enhance organogenic ability (Korban et al. 1992). In addition to above-mentioned factors, physical factors such as temperature, quality of light, photoperiod and light intensity were also studied for their effect on regeneration behaviour of apple (Liu et al. 1983; James et al. 1988; Magyar-Tabori et al. 2011; Hyae et al. 1996). Incubation of internodal segments of *Malus domestica* in dark for 14 days followed by transfer to 16/8 h light/dark photoperiod was observed to increase shoot organogenesis.

In the attempts to enhance organogenic potential, temporary immersion system has also been developed by Damiano et al. (2003). It was reported that daily short immersion period (2–3 hrs) in liquid medium followed by transfer on solidified medium enhance shoot organogenesis in apple cultivar 'Jork.9'. However, in most of the above-mentioned studies, the regeneration potential was found to be dependent

on choice of explant, genotype and the composition of the medium (Rout and Lucas 1996; Cheng et al. 2003; Eudes et al. 2003; Druart 1990; Fasolo et al. 1989; Famiani et al. 1994; Modgil et al. 2005).

Shoot organogenesis through callus phase is likely to increase the chances of somaclonal variations; thus, direct somatic embryogenesis and shoot organogenesis are often preferred regeneration pathways (Khilwani et al. 2016). Direct shoot organogenesis and somatic embryogenesis have been reported from various explants of different apple cultivars using cytokinins such as TDZ, BA, zeatin and kinetin (James et al. 1984; Welander 1988; Welander and Maheswaran 1992; Yepes and Aldwinckle 1994; Modgil et al. 1999; Magyar Tabori et al. 2010; Varsha 2008). The secondary embryogenesis has also been reported in apple (Diagny et al. 1996). It was observed that replacement of sucrose with maltose in the medium resulted in induction of secondary embryos in apple.

4 Genetic Transformation in Apple

The influence of the rootstock on the quality and quantity of the produce pave the way for the development of new rootstocks with improved characteristics (Zhu et al. 2001). Although many standard rootstocks with important characters have been selected in apple, there is a need for further undertaking the trait-specific modifications. Thus transgenics are often seen as an important method of incorporating desired characteristics such as early fruiting, dwarf height, fire blight resistance, etc. to apple rootstocks (Norelli et al. 2003). Genetic transformation was first attempted in apple using leaf as explant (James et al. 1989; Maheswaran et al. 1992). Transformation of apple has been achieved by various methods, including the direct uptake of naked DNA by protoplasts (Lee et al. 1995), and using *Agrobacterium*-mediated transformation (De Bondt et al. 1996; James et al. 1989; Maheswaran et al. 1992; Norelli et al. 1994; Martin et al. 1990; Song et al. 2000; Zhang et al. 2006). In apple cultivars such as 'Jonagold', 'Elstar', 'Gala', 'Braeburn', 'Merlijn' and 'Fuji', *Agrobacterium*-mediated genetic transformation was attempted, and various factors such as cocultivation period, pre-culture duration, bacterial culture density, carbon source, age and genotype of explant, etc. were optimized (De Bondt et al. 1994). It has been reported that explants infected with *A. tumefaciens* strain (EHA101) for 1 min and cocultivated for 4 days yielded maximum number of transformants and noticed that increasing the cocultivation period above 6 days leads to drastic decrease in transformation efficiency (De Bondt et al. 1994). Few of the studies on cloning and apple improvement are discussed in this chapter.

rol genes have been widely reported as modulators of plant growth and differentiation (Bulgakov 2008). Till date various attempts have been made to transform apple rootstocks with *rol* genes. During initial studies on apple transformation, rootstock 'M.26' was transformed with *rolA* gene to increase rooting and reduce stem growth (Holefors et al. 1998). Similar effect on rooting was observed when rootstock 'M.9/29' and 'M.26' were transformed with *rolB* gene (Zhu et al. 2001).

In addition to shoot growth and rooting, characters such as drought and salinity tolerance and reduced apical dominance were affected by *rolC* gene in apple (Zhang et al. 2006). There is a general approach of using selectable marker genes such as antibiotic resistance, herbicide resistance, etc. for successful identification of transformants. But, these selectable marker genes possess biosafety concerns (Miki and McHugh 2004). Many reports on development of marker-free technologies have also been published in apple (Zhu et al. 2004; Endo et al. 2001; Zuo et al. 2002; Degenhardt et al. 2006). Although these studies included use of compounds involved in shoot growth such as phosphomannose isomerase as selection markers instead of resistance genes, but the transformational efficiency was limited. More recently, a novel technique has been used to transform apple without the use of selectable markers (Malnoy et al. 2010). In this study, binary vectors pPin2ATT.35SGUSint + nptII or pPinMpNPR1.GUS-nptII with eliminated nptII expression cassette was used to carry out genetic transformation of leaf segments taken from apple rootstock M.26. Success of the experiment was completely dependent on efficiency of genetic transformation protocol developed for the rootstock (Malnoy et al. 2010).

5 Conclusion

Apple is an economically important fruit tree, propagated through grafting or layering using scion and selected rootstocks bearing desirable characteristics. Due to disadvantages associated with conventional propagation, protocols have been optimized for in vitro propagation of apple rootstocks. Various steps such as culture establishment, shoot organogenesis and proliferation, somatic embryogenesis, rooting and acclimatization have been optimized for some cultivars. As the response is genotype dependent, further studies are required in this direction. This will help in meeting the demand for healthy rootstocks for propagation and will also be instrumental for further trait-specific modifications. Work also needs to be intensified for trait-specific improvement of selected rootstock.

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Advances in Bamboo Biotechnology: Present Status and Future Perspective

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Abstract Bamboo, the most important natural and renewable resource of the world, has always been an integral part of the social and economic life of many Asian countries. Therefore, the annual demands for bamboos have already outcrossed the annual yields across the world. Increasing population pressure; indiscriminate exploitation by paper, pulp and fuel industry; and insufficient attempts to replenish and cultivate bamboos are further widening the gap between demand and supply. This has forced scientists to pay greater attention towards employment of advanced biotechnological tools for understanding, generating and improving bamboos. As a result, new insights into bamboos were gained through genomics, proteomics, nanotechnology and transgenic technology. The findings show the way for better utilization of improved bamboos in meeting the future needs of the world. The achievements highlighted in the present review pave the way for the betterment of bamboos for the next millennium.

Keywords Bamboo · Biotechnology · Genomics · Proteomics · Micropropagation

1 Introduction

Biotechnology involves multidisciplinary approaches in biological systems and can resolve various economical and industrial issues. Importantly, the field encompasses gene and genome analysis for potential industrial applications, transgenic technologies for crop improvement and functional and comparative genomics for phylogenetic analysis and gene function. Today, modern biotechnology has been revolutionized by diverse technological tools involved in bioprocess engineering, cellular engineering, bioinformatics, nanobiotechnology, etc. These have opened up various avenues for ecological and socio-economic improvements and have also

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facilitated diversified uses of plants. As a result, different underutilized plants with fast growth and abundant biomass have emerged as alternate resources of wood, textile, fuel and food. This is important in view of the ever-increasing population and consequent overall demand for natural resources.

In this regard, the woody grass, bamboo with fastest growth and long juvenility is particularly, important. Bamboos are considered as the 'green gold of the forest' as these have improved many facets of rural livelihood while strengthening urban sector. Bamboos have been traditionally used as construction material for rural housing, food and, last but not the least, handicraft products. In today's times, however, its uses have become more diverse. Thus, besides providing ecological solutions to global climate change through its carbon dioxide sequestration ability, about 2.5 billion people worldwide depend on bamboos (Lobovikov et al. 2007; Peng et al. 2013). There are many documented applications of bamboos that have exploited their fast growth rate and high renewable capacity (Salam 2008). Presently, bamboos have emerged as one of the most important renewable resources for food,

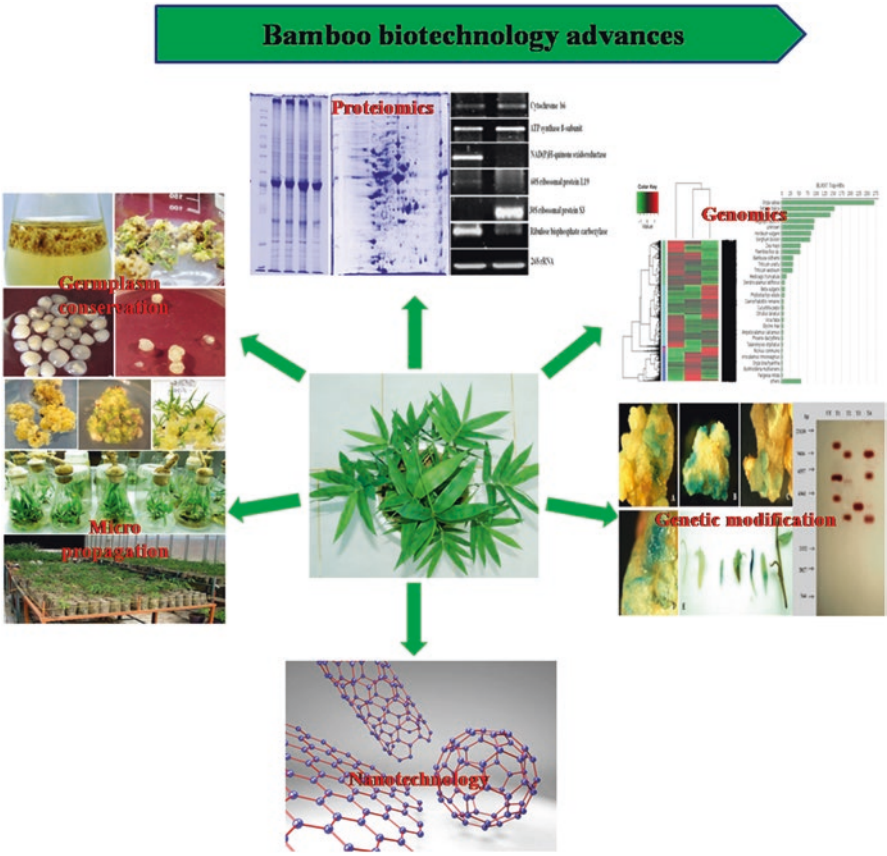

Fig. 1 Bamboo biotechnology advances

fuel, paper, pulp, textile, plywood, etc. According to an estimate, 20 million tons of bamboos are required per annum for commercial ventures (Scurlock et al. 2000). Thus, it is not surprising that several multidisciplinary approaches are being employed in bamboos. This includes genomics, proteomics and several other emerging fields such as nanobiology, etc. (Fig. 1).

2 Genomics

Plant genomics is a recent advancement in the field of plant biotechnology. Until recently, plant biologists were focused towards the development of transgenic plants and finding phenotypic and genotypic variations. Recently however, work has shifted towards the whole genome level. With the advent of recent new NGS technologies, the plant genomic studies have become more cost-effective and detailed (Wang et al. 2010; Bhattacharyya et al. 2013). It has also revealed the dynamic structure of genome organization, expression and interaction at the single gene level. Today, genomics has permeated every aspect of plant biology and is substantially expanding the understanding of plant genomes at the structural, functional, epigenomic and comparative levels. Many model plants have been successfully sequenced, and their wide range of biological data sets are now publicly available (Wang et al. 2010). With these continuous improvements, many workers are now focusing on non-model plants having high economic importance. Until now, about 34 flowering plant species have been sequenced (Kaufmann and Busch 2013). Recently, the first genome of a gymnosperm was made available (Kaufmann and Busch 2013). Among the flowering plants, bamboos are the most unique monocarpic non-timber plants from the grass family. The genomic resources for almost all major lineages of grasses have accumulated rapidly, but bamboos remain the only large subfamily of Poaceae with little genomic information available in databases (Zang et al. 2012). This seriously hampers one's ability to take the full advantage of the wealth of grass genomic data. In the last 5 years, however, many genomic studies have been carried out on different bamboo species, and attempts have been made to understand the complexity of the gene regulation and networking in bamboos. For effective comparative studies, the first insight into the gene and genome structures of bamboos were obtained through cloning and sequencing of 10,608 putative full-length cDNAs (FL-cDNAs), primarily from the moso bamboo, *Phyllostachys heterocycla* cv. *pubescens* (Peng et al. 2010). To date, this represented the third largest FL-cDNA collection of all plant species. Analysis of FL-cDNA sequences showed that bamboo diverged from its close relatives, i.e. rice, wheat and barley through an adaptive radiation. Gui et al. (2010) also showed high genomic synteny between bamboo and rice/sorghum suggesting that rice and sorghum are probable models for decoding Bambusoideae genomes. Construction of bamboo genetic population and genetic map is difficult because bamboo flowering is unpredictable, and the comparative genomic approach is more important for studies on the bamboo genome.

Bamboos have two unique properties associated with its rapid growth (5–20 m in 2–4 months) and unpredictable flowering behaviour (40–120 yrs). Thus, all earlier studies were focused on the growth, anatomical structure, hormone distributions, chemical and physical characteristics of bamboo culms, in vitro flowering, etc. (Lee and Chin 1960; Nadgauda et al. 1990; Chambers et al. 1991; Zheng et al. 1998; He et al. 2002; Li et al. 2007; Yu et al. 2008; Yu et al. 2011; Wen et al. 2011; Kaur et al. 2014). Although these studies have provided many clues related to high growth rate and flowering in bamboos, a comprehensive description of molecular mechanism at the gene level is urgently required. Therefore, many workers have employed EST's and transcriptomics in order to understand the mechanism of rapid growth and flowering in bamboos. In this regard, Zhou et al. (2001) reported 13 differentially expressed EST's based on homology search. They also reported an absolute requirement of meristem initiation and proliferation followed by a high level of protein synthesis, cellular respiration, cell wall synthesis, regulation of activated methyl cycle, gibberellin and brassinosteroid biosynthesis and their signal transduction pathways. Further reports of different bamboo species on shoot growth by Peng et al. (2013) and Bhandawat et al. (2017) confirmed and gave more detailed information about these key regulatory pathways (transcription factors, plant hormones, cell cycle regulation, cell wall biogenesis and cell morphogenesis) using RNA-seq data. These provided a better insight into the rapid growth of bamboos and also offered candidate gene resources for bamboo improvement.

Another area which drew the attention of different researchers was an understanding of the molecular mechanism of bamboo flowering. Zhang et al. (2012) for the first time studied the de novo transcriptome data of flower development in *Dendrocalamus latiflorus* and identified several putative transcription factor families such as the GRF, SBP-box, BHLH, ARF, bZIP, zf-HD, NAC, WRKY, MYB and MADS box. Several genes related to flowering pathways such as the photoperiod, the vernalisation, the gibberellin and the autonomous were identified along with the meristem identity-related genes. Further, to increase the knowledge regarding floral development in bamboos, transcriptomic studies were carried out in species such as *Phyllostachys edulis*, *Guadua inermis*, *Otatea acuminata*, *Phyllostachys aurea* and *Lithachne pauciflora* (Gao et al. 2014; Wysocki 2016).

3 Proteomics

In order to complement the whole genomics studies, proteomic studies were also carried out because the same has the potential to contribute significantly towards unravelling the problems of plant biology systems. Since the protein set of a cell is the final response regulators and represents the functional part of a genome, proteomics approach is often employed for an elaborate and finer picture of a cell's response. The proteome of a system can thus provide the dynamics, functions, comparative analysis and interactions of proteins. Hence, proteome analysis is often used to validate the huge amount of data obtained through transcriptomics. Recently, Kaur et al. (2015) first attempted to study the poorly understood and enigmatic

process of flowering in bamboo. The MS/MS and subsequent homology search revealed the various proteins involved in metabolism, regulatory pathways, signalling and transportation, stress and, most importantly, flowering. Further reports by Louis et al. (2015) elucidated the involvement of stress, mobile elements and signal transduction as the major markers during floral transition in *Bambusa vulgaris* and *Dendrocalamus manipureanus.*

4 Nanotechnology

Nanotechnology is a new interdisciplinary field of science which deals with the nanometric (> 100 nm) dimensions and paves the way to understand function and properties of matter at the nanoscale. It provides unique and novel insights in many disciplines of sciences, i.e. medicine, physics, chemistry, biology, engineering, mathematics, material sciences, etc. (Roco et al. 1999; Scott and Chan 2002; Kulzer and Orrit 2004). It has a wide range of applications in research, agriculture and industries. Nowadays, it is emerging as a dynamic new industry accompanying many sectors like energy, materials, electronics, manufacturing and biomedical to strengthen the global economy. Products derived from nanotechnology are known as nanomaterials, and more than 800 nanomaterial products were available in the market (Al-Halafi 2014; Safiuddin 2014; Zhou 2014). In these days, new application of nanotechnology is the formation of nontoxic, environment-friendly, cost-effective, less energy-intensive nanomaterials using plant system (Ahmad et al. 2015). In this regard until now few higher angiosperm plants are being exploited in which bamboos having high ecological and commercial value show a wide application. The first application report of nanotechnology using bamboos was the formation of bamboo-like carbon nanostructures by Erkoc (2006). He had shown the structural and electrical strength of them and found high stability and endothermic nature of carbon nanostructure of bamboos. Thereafter many workers reported silver nanoparticles and carbon nanospheres formation from bamboo leaf extract and bamboo charcoal, respectively, based on bamboos' high antimicrobial and antifungal properties (Yasin et al. 2013; Das and Saha 2012). Silver nanoparticles and carbon nanospheres have enhanced chemical and physical properties as compared to its macro particles. They have many applications in the field of electronics (Lu et al. 2007; Mitin et al. 2008), biosensing (Mirkin et al. 1996), photonics, optoelectronics, pharmaceuticals (Baruwati et al. 2009; Ahmad et al. 2011), textiles, water treatment (Murphy et al. 2008), cosmetics, DNA sequencing (Cao et al. 2001), wound healing and prevention of HIV (Singla et al. 2017; Murphy et al. 2008). Recently few workers have explored two new applicabilities of bamboo in very different aspects, i.e. in construction and in nanobiocomposites dressings (Ahmad et al. 2015; Singla et al. 2017). They have shown that for the construction purpose, bamboos' carbonized micro-sized inert particles can be used as an additive in cement composites for the enhancement of flexural strength, ductility and fracture energy properties. The second applicability is the use of natural resource of cellulose in dressing materials for wound healing, i.e. from plants. The workers have provided

first time the use of plant cellulose nanocrystals as wound dressing material made from bamboo leaves *Dendrocalamus hamiltonii* and *Bambusa bambos*. Bamboo was selected because of its fastest growing and of high source of cellulose properties (David 1984). However the hydrophilic nature of CNCs lacks antimicrobial activity (Peng et al. 2016). Therefore, they have applied in situ approach for the development of nanobiocomposites (NCs) in which impregnation of silver nanoparticles (AgNPs) from *Syzygium cumini* leaf extract a biological reducing agent into CNCs matrix was carried out. Furthermore in this regard, NCs in ointment and film forms were tested for its in vitro antimicrobial and in vivo topical wound healing traits and showed that hydrophilic nature would keep wounded tissue moist by controlling wound exudates, ultimately enhancing tissue repair, along with antibacterial and anti-inflammatory properties of AgNPs accelerating tissue repair.

5 Transgenics: Genetic Engineering

Tailor-made transgenic plants have been shown to play crucial roles in the improved utilization of various crop plants. This has been possible through extensive optimization of the genetic transformation process. Besides its many applications in plant biology and crop modification, transgenics have provided new solutions to specific problems. Hence, genetic transformation of bamboos was attempted by different workers, and the difficulties encountered in transforming this highly complex monocot genome were elucidated (Sood et al. 2011). Low competence due to rapid differentiation of monocot cells, lignification, reduced cell division and elimination of transgenes during integration into host genome are considered to be the major limitations of *Agrobacterium*-mediated genetic transformation of monocots (Kahl 1982; Graves et al. 1988; Frame et al. 2002). This accounts for the low success rates in bamboo transformation and the availability of very few scientific reports on transgenic production in bamboos. Both the direct delivery and the *Agrobacterium* methods were attempted by different workers for the genetic transformation of different bamboo species. Till now, there are only very few reports on genetic transformation of bamboo. Douglas et al. (1985) showed attachment of *A. tumefaciens* to bamboo cell walls and, further, revealed that the kinetics of attachment to monocotyledon and dicot cell walls were very similar (20% of added bacteria were attached after approximately 2 h). This thereby, signified that attachment is not a limiting step. Later, Wu and Feng (1999) reported transient GUS or GFP expression in intact cells of callus induced from root cells of *Bambusa beecheyana* Munro var. *beecheyana* transformed by electroporation. Further, in 2011 Ogita et al. developed a particle bombardment-mediated transformation protocol in *Phyllostachys nigra* suspension cells. The report described stable expression of fluorescent proteins in the transformed suspension cells. However, the first successful report on *Agrobacterium*-mediated genetic transformation of bamboo was by Sood (2013) only. The researchers used an efficient somatic embryogenesis system to transform the solid culmed bamboo, *Dendrocalamus*

hamiltonii and developed the first ever transgenic bamboo plant expressing the *gus* reporter gene. The group then used the method to develop transgenic *D. hamiltonii* plants expressing a gene encoding thaumatin-like protein from *Camellia sinensis* (Cs-*tlp*). The protein encoded by the *tlp* gene belongs to group-5 of PR-proteins and are induced in plants in response to abiotic and biotic stresses and also elicitors and developmental signals (Malehorn et al. 1994; Hu and Reddy 1997; Thatcher et al. 2005). Hence, the transgenic *D. hamiltonii* plants were found to be cold stress tolerant. The plants also exhibited a series of morphological, physiological, biochemical and molecular changes under cold stress. Cold stress is a major environmental factor that adversely affects growth and productivity in bamboo species such as *D. hamiltonii.* The species is particularly sensitive to cold stress and experience mild to severe cold injury. Hence, cold stress-tolerant transgenic *D. hamiltonii* was a major achievement. This was followed by a recent report by Qiao et al. (2014) who developed the transgenic Ma bamboo plants expressing cold tolerant bacterial CodA gene encoding choline oxidase. However, a lot of work is yet to be done for actual utilization of an improved transgenic bamboo.

6 Micropropagation of Bamboos

Micropropagation is a commercially established technology that demands the production of a large number of genetically identical plants. It relies on the phenomenon of cell totipotency wherein, a single cell or a tissue regenerates into a whole organism. The technology has been used for the clonal production of several plant species for commercial ventures, conservation and germplasm exchange. Considerable achievements have also been made in the micropropagation of bamboos since the first report on in vitro seed germination in *Dendrocalamus strictus* (Alexander and Rao 1968). Thereafter, different routes such as adventitious shoot formation, organogenesis and somatic embryogenesis were successfully implemented for bamboo propagation using both juvenile and mature explants (Tables 1 and 2). The micropropagated bamboo plants have been extensively used in commercial ventures including the paper and pulp industry. Recently, slow growth method was reported in *D. hamiltonii* using the liquid paraffin overlay method for medium-term preservations (Kaur et al. 2012). In addition to this, many workers have reported the use of in vitro grown shoots for in vitro flowering in different bamboo species. These include bamboos such as *B. bambos*, *B. arundinacea*, *B. vulgaris*, *B. ventricosa*, *D. brandisii*, *D. giganteus* and *D. strictus and B. edulis* (Nadgauda et al. 1990, 1997; Chambers et al. 1991; Rout and Das 1994; Ansari et al. 1996; John and Nadgauda 1999; Ramanayake et al. 2001; Lin et al. 2003, 2004, 2007). Recently, Kaur et al. (2014) used an in vitro flowering system in *D. hamiltonii* for understanding different factors related to flower development and floral transition. The group also used to study the proteome changes related to floral transition in the plant in an attempt to identify markers and/or specifiers of flowering in *D. hamiltonii* (Kaur et al. 2015).

Table 1 Micro propagation response using seeds, embryos and young inflorescence as explants

S. No.	Plant	Explant used	Medium + sucrose 3.0% (unless and otherwise specified)	PGRs used (μM)	Response	References
1.	*Dendrocalamus strictus*	Seeds	MS	TDZ 2.22	Shoot formation and in vitro flowering	Singh et al. (2000)
2.	*Thamnocalamus spathiflorus*	Zygotic embryos/nodal segments	MS + 2%	BAP 5.0 + IBA 1.0 IBA 150	Shoot multiplication Rooting	Bag et al. (2000)
3.	*D. asper*	Seeds/nodal cuttings	MS	BAP 0.44–66.6 BAP 13.2 NAA 16.2 or IBA 49.0	Shoot initiation Shoot multiplication Rooting	Arya et al. (2001)
4.	*D. strictus*	Seeds	½ MS	BAP 8.8 IBA 9.8	Multiple shoots Rooting	Reddy (2006)
5.	*Phyllostachys meyeri*	Seeds	Sterilized water, PPM (0.1%) Modified ½ MS (liquid)	– –	Multiple shoots Rooting and acclimatization	Ogita et al. (2008)
6.	*D. asper*	Immature inflorescence	MS	30.8 BA 13.2 BAP 49.0 IBA	Shoot initiation Shoot multiplication Rooting	Arya et al. (2008a)
7.	*D. asper*	Roots, leaves and nodal segments	MS	2,4-D 30.0 BAP 20.0 IAA and NAA 5.0–25.0	Embryogenic callus Germination of somatic embryos Rooting	Ojha et al. (2009)
8.	*D. hamiltonii*	Seeds	MS	2,4-D 4.5–13.5 BAP 8.88 + Kn 4.65 + NAA 5.37 IBA 24.5	Embryogenic calli Somatic embryos proliferation, shoot differentiation and subsequent growth Rooting	Zhang et al. (2010)

9.	*D. farinosus*	Seeds	MS	2,4,5-T 7.82 + Kn 0.93 + IBA 1.96 Kn 11.4 + IBA 1.96 IBA 1.96 + IAA1.42	Callusing Plant regeneration Rooting and green house acclimatization	Hu et al. (2011)
10.	*Bambusa oldhamii*	Seeds/nodal explants	MS (liquid) AdS 40 mg/l ½ MS	BAP 4.4 IBA 9.84 + NAA2.69	Multiple shoots Rooting and acclimatization	Thiruvengadam et al. (2011)
11.	*Ochlandra wightii*	Seeds	½ MS ½ MS + 4% MS	BAP 2.22 BAP 2.22 + TDZ 2.22 Kn 4.65 BAP 8.8+ Kn 2.35	Germination of embryos Shoot multiplication Rhizome induction Shoot multiplication from nodal segments	Bejoy et al. (2012)
12.	*D. giganteus*	Seeds	MS ½MS	BAP 8.9–13.3 13.3 BAP + 1.0 IBA 15.0 IBA for three subculture and transfer to ½ MS with 25.0 IBA and 0.05 BAP	Bud break Shoot multiplication Rooting	Devi et al. (2012)
13.	*D. hamiltonii*	Seeds	MS	BAP 35.0 BAP 10.0 IBA 100.0	Shoot initiation Shoot multiplication Rooting	Arya et al. (2012)
14.	*D. membranaceus*	Seeds	MS	BAP 8.8+ Kn 2.3	Multiple shoot formation and rooting	Brar et al. (2013)
15.	*P. pubescens*	Zygotic embryos	MS	2,4-D 18.12 + Zeatin 0.46 Zeatin 22.0–31.92 NAA 11.74	Callus initiation Regeneration Rooting	Yuan et al. (2013)

Table 2 Micropropagation response using shoot buds, nodal and intermodal segments, roots, leaf sheaths, etc

S. no.	Plant	Explant used	Medium used +sucrose concentration 3.0% unless or otherwise specified	PGRs and other additives used (μM)	Response	References
1.	*D. strictus*	Shoots	MS liquid	TDZ 0.454–4.54 TDZ 2.27	Shoot proliferation In vitro flowering	Singh et al. (2000)
2.	*D. giganteus*	Nodal segments	MS + 2%	BAP 26.6	Shoot proliferation and in vitro flowering	Ramanayake et al. (2001)
3.	*D. hamiltonii*	-Do-	½ MS MS	BAP 11.1 NAA 5.37/IBA 4.9 + 2,4-D (2.26) + phloroglucinol (0.1 mM) + AC 0.3% + choline chloride (18.9, 56.7) + IBA 2.45, IAA 0.57 or NAA 0.54/coumarin 61.2 alone or in combination with IBA (2.45), IAA 0.57 or NAA 0.54	Shoot multiplication Slightly brownish, short, thick roots/ less hairy white roots with secondary and tertiary roots/ unbranched, profuse roots	Sood et al. (2002)
4.	*D. asper*	Nodal segments and seeds	MS	BA 0.44 + NAA13.2 NAA16.2 or IBA 49.0	Shoot initiation and multiplicationRooting	Arya et al. (2002)
5.	*D. hamiltonii*	Nodal segments	MS Sucrose 8%	BAP 4.44 + 2,4-D 4.53 BAP 11.1	Somatic embryogenesis Maturation Germination	Godbole et al. (2002)
6.	*G. angustifolia*	-Do-	MS + 10.0 mg/l inositol	BAP 11.0	Regeneration of axillary buds	Marulanda et al. (2005)
7.	*D. giganteus*	Shoots, spikelets and roots	MS + 4%	2,4-D 33.9, NAA 16.1 2,4-D 33.9 alone or with NAA 40.3	Callus induction Cell suspension	Ramanayake and Wanniarachchi (2003)

8.	*B. nutans*	In-vitro shoots	MS	2,4-D 5.0 + BAP 2.5+ ABA 1.0 2,4-D 5.0 + BAP 2.5 BAP 5.0 + NAA 1.25	Somatic embryogenesis lmaturation Germination	Kalia et al. (2004)
9.	*B. edulis*	Nodal and internodal segments of in vitro plantlets	MS + 6% + CM (0.1%v/v) Sucrose (3%)	Kn 9.2, 2,4-D 13.6, TDZ 0.046, 2,4-D 13.6 TDZ 0.445	Somatic embryogenesis Maturation Germination	Lin et al. (2004)
10.	*Bambusa balcooa*, *B. nutans*, *B. salarkhanii*, *B. vulgaris*, *B. vulgaris var. striata* and *Thyrsostachys oliveri*	Nodal segments	MS ½ MS	4.4–22.0 BAP 5.4–16.2 NAA + 4.9–24.5 IBA	Multiple shoots Rooting	Islam and Rahman (2005)
11.	*P. nigra*	Shoots	½ MS	2,4-D 3.0	Callus	Ogita, (2005)
12.	*Pseudoxytenanthera stocksii*	Nodal segments	MS MS ½ MS	NAA 2.68 + BAP 4.4 Ascorbic acid 283.93, citric acid 118.10, cysteine 104.4 glutamine342.24 + NAA 2.68 + BAP 2.21 IBA 4.90 + BA 0.44	Multiple shoots Shoot proliferation Rooting	Sanjaya et al. (2005)
13.	*B. wamin*	-Do-	MS	BAP 22.0	Multiple shoots induced	Arshad et al. (2005)
14.	*B. balcooa*	Axillary buds	MS (liquid) ½ MS	BAP 11.25, Kn 4.5 IBA 1.0	Shoot multiplication Rotting	Das and Pal (2005)
15.	*Guadua angustifolia*	Nodal segment	MS	PPM 2.0 ml/l BAP 13.32 BAP 22.2	Bud sprout Multiple shoot induction Lateral shoot production and rooting	Jimenez et al. (2006)
16.	*B. vulgaris*	-Do-	MS (modified)	BAP 8.88 IBA 98.0	Shoot formation Rooting	Ndiaye et al. (2006)

(continued)

Table 2 (continued)

S. no.	Plant	Explant used	Medium used +sucrose concentration 3.0% unless or otherwise specified	PGRs and other additives used (μM)	Response	References
17.	*B. vulgaris*	-Do-	MS	BAP 17.76 Pretreatment of TDZ 2.22 for two to three subcultures then IBA 14.7	Shoot formation Rooting	Ramanayake et al. (2006)
18.	*B. bambos*	Embryonic axes of caryopses	MS + 2.0% MS + 5%	BAP 5.0 BAP (2.5, 5.0), +GA_3 0.1 + NAA 50.0	Multiple shoot initiated and rooting Rhizome induction	Kapoor and Rao (2006)
19.	*B. glaucescens*	Nodal segments	MS	BAP 5.0, Kn 15.0 IBA 25.0	Initiation, shoot multiplication Rooting	Shirin and Rana (2007)
20	*B. balcooa*	Pseudo spikelets	MS	2,4-D 4.5 BAP 22.2	Callusing Shoot regeneration	Gillis et al. (2007)
21	*B. oldhamii*	Meristems	MS	TDZ 0.45 NAA 10.74–26.85	Initiation, shoot multiplication Rooting	Lin et al. (2007)
22	*B. tulda*	Nodal segments	MS (liquid)	BAP 12.0, IAA 0.1, glutamine 100 Coumarin 40.0	Initiation, shoot multiplication Rooting	Mishra et al. (2008)
23	*D. asper*	Nodal tissues/ basal part of leaves	MS + 2% MS + 6%	2,4-D 30.0 2,4-D 9.0, IAA 2.85, BAP 0.88 ABA 5.0 BAP 4.4, GA_3 2.8	Embryogenic calli Somatic embryo induction Maturation Germination	Arya et al. (2008b)
24	*Oxytenanthera abyssinica*	Nodal segment	MS	BA 22.20, NAA 1.08 IBA 39.2	Multiple shoots Rooting and acclimatization	Diab and Mohamed (2008)

25	*B. nutans*	-Do-	MS + Glucose (88 mM) for 3 days followed by MS 88 mM sucrose	IBA 49.0	Axillary bud proliferation and high frequency rooting	Yasodha et al. (2008)
26	*D. hamiltonii*	Nodal segments Seeds	MS	BAP 4.44 IBA 98.0 BAP30.8 IBA 98.0 pulse	Shoot proliferation rooting Shoot proliferation rooting	Arya et al. (2009)
27	*D. hamiltonii*	Nodal segments	MS	BAP 8.0, NAA 1.0 IBA 100.0 for 10 days further shifted MSO	Bud sprouting Shoot multiplication Rooting	Agnihotri and Nandi (2009)
28	*D. asper*	-Do-	MS	2,4-D 4.5 + BAP 4.4 BAP 30.8 2,4-D 4.5 + IAA 5.7 + NAA 5.4	Callus Multiple shoots rooting	Ali et al. (2009)
29	*Melocanna baccifera*	-Do-	MS (liquid) ½ MS	BAP 15.0 + Kn 3.0 IBA 25.0	Multiple shoots Rooting	Kant et al. (2009)
30	*B. balcooa, B. bambos, D. asper, D strictus, D. stocksii, G. angustifolia.*	-Do-	MS (liquid) ¼ MS	(Ascorbic acid 50.0 mg/l + citric acid 25.0 mg/l + cysteine 25.0 mg/l) + NAA (0.54–1.35) + BAP (4.4–11.0) IBA 4.9–9.9/NAA (5.4–10.8)	Multiple shoot Formation Rooting	Rathore et al. (2009)
31	*Arundinaria callosa*	Nodal segments	MS ½ MS	BAP 13.3 + IBA 1.0 IBA 25.0+ BAP 0.05	Multiple shoots Rooting	Devi and Sharma (2009)
32	*B. balcooa*	-Do-	MS	BAP 4.4 BAP 4.4 + NAA 13.2	Multiple shoots Rooting	Mudoi and Borathakur (2009)
33	*A. gigantea*	-Do-	MS	IBA 0.1 + TDZ 0.01	Multiple shoots	Baldwin et al. 2009

(continued)

Table 2 (continued)

S. no.	Plant	Explant used	Medium used +sucrose concentration 3.0% unless or otherwise specified	PGRs and other additives used (μM)	Response	References
34	*B. nutans*	-Do-	MS MS MS +2%	2,4-D 4.52 + BAP 4.44 TDZ 0.499 + NAA 10.74 NAA 16.11	Somatic embryogenesis Germination Rooting	Mehta et al. (2011)
35	*B. nutans*	-Do-	MS ½ MS liquid MS	BAP 4.4, Kn 2.32 BAP 13.2, Kn 2.32, IBA 0.98 IBA 9.8, IAA 2.85, NAA 2.68	Bud break Shoot multiplication Rooting	Negi and Saxena (2011)
36	*B. bambos*	Young bamboo shoots and nodal explants	MS	BAP 2 0.0 + Kn 5.0 IAA 1.5/ IBA 1.0	Multiple shoots Rooting	Nayak et al. (2010)
37	*P. bambusoides*	Nodal explants/ leaf sheaths	MS MS+ 2% glucose	Picloram 32.8	Callus and embryogenesis Rhizogenesis	Komatsu et al. (2011)
38	*Gigantochloa atroviolaceae*	Nodal segment	MS (liquid) MS MS	BAP 25.0 BAP 20.0 + NAA 3.0 IBA 35.0	Bud break Multiple shoots Rooting	Bisht et al. (2010)
39	*D. giganteus*	-Do-	MS+ 9% CM MS ½ MS MS	BAP 26.6 + Kn 0.46 BAP 4.4 + 2,4-D 4.52 + NAA 5.37 BA 11.10 + Kn 2.32 IBA 98.41	Multiple shoots Callus induction Caulogenesis Rhizogenesis	Yasodha et al. (2010)
40	*B. balcooa*	-Do-	MS	BA 4.4 and 6.6 NAA 18.9 and 21.6	Multiple shoots Rooting	Sharma and Sharma (2011)
41	*B. tulda*	-Do-	MS	BA 4.4 NAA 27.0	Axillary shoot formation Rooting of shoots	Sharma and Sarma (2013)

42	*D. asper*	-Do-	MS (liquid) + AdS 40 mg/l MS	BAP 22.0 IBA 4.9	Shoot multiplication Rooting and acclimatization	Banerjee et al. (2011)
43	*D. asper*	-Do-	MS MS ½ MS	BAP 15.0 10 BAP + 75 AdS IBA5.0 + NAA 5.0	Shoot initiation Shoot multiplication: Rooting, acclimatization	Singh et al. (2012a)
44	*D. hamiltonii*	-Do-	MS ½ MS	TDZ 1.5, ascorbic acid 56.0 IBA 25.0 + Choline chloride 36.0	Shoot multiplication: Rooting, acclimatization	Singh et al. (2012b)
45	*D. hamiltonii*	-Do-	MS ½ MS	BAP 5.0 + 2,4-D 5.0 BAP 5.0 + 2,4-D 7.5 IBA 5.0 IBA 20.0	Embryogenic callus Somatic embryogenesis and germination Rooting Rhizome formation	Bag et al. (2012)
46	*D. asper*	Internodal segments		BA 2.2 BA 8.8 Kn 2.35 + NAA 16.2	Multiple shoot initiation Shoot multiplication Rooting	Shroti et al (2012)
47	*D. asper*	Nodal segment	MS	BAP 8.86, AdS 13.5 IBA 14.76+ NAA 3.67	Shoot multiplication Rooting	Nadha (2013)
48	*D. strictus*	-Do-	MS	BAP 17.6 + 15 mg/l AdS IAA 5.7 + IBA 14.7 + NAA 27.0	Multiple shoots Rooting	Pandey and Singh (2012)
49	*B. bambos*	-Do-		BAP 4.4 + Kn1.16 IBA 9.80	Multiple shoots Rooting	Anand et al. (2013)
1.50	*D. membranaceus*	Seeds		BAP 8.8 + Kn 2.3	Multiple shoot formation and rooting	Brar et al. (2013)
		Nodal explants	MS +2.0% ½ MS + 2.0%	BAP 4.4, Kn 1.16 BAP 4.4, NAA 5.37	Shoot multiplication Rooting	
51	*B. tulda*, *M. baccifera*	Nodal segments	MS	BAP 13.2 Kn 9.4 + BAP 12.12 IBA 14.7 + coumarin 68.4	Bud breaking Shoot multiplication Rooting and rhizome production	Waikhom and Louis 2014

(continued)

Table 2 (continued)

S. no.	Plant	Explant used	Medium used +sucrose concentration 3.0% unless or otherwise specified	PGRs and other additives used (μM)	Response	References
52	*B. balcooa*	-Do-	MS	BAP 8.8 + Kn 4.7	Axillary shoot bud proliferation	Khan et al. 2014
53	*B. arundinacea*	-Do-	MS + CM 4% + 4% MS+ $AgNO_3$ 2.0 mg/l	BAP 13.2 + IBA 2.45 IBA 14.7	Shoot bud initiation Rooting	Venkatachalam et al. 2015
54	*Drepanostachyum falcatum*	-Do-	MS	BAP 15.4 IBA 31.85	Shoot proliferation Rooting	Saini et al. 2016
55	*D. hamiltonii*	Shoot tips	MS MS ½ MS	2,4-D 13.5, BAP 4.4, glutamine 500 mg/l, casein hydrolysate 500 mg/l BAP 4.4, Kn 1.41, NAA 1.62 IBA 14.7	Callus induction Differentiation of callus Rooting and acclimatization	Zang (2016)
56	*B. bamboos*	Nodal segments	MS MS + 4% sucrose ½ MS	BAP 8.8, TDZ 4.5 BAP 8.8 + TDZ 4.5 + CM 10%IBA 12.25 + NAA 13.5	Shoot initiation Shoot multiplication Rooting of shoots	Raju and Roy (2016)

AC activated charcoal, *AdS* adenine sulfate, *MS* Murashige and Skoog medium (1962), *MSO MS* basal, *CM* coconut milk

7 Conclusion

With increasing demand and awareness about the vast potential of this fast-growing renewable resource of the future, bamboos have been subjected to different biotechnological interventions either for basic understanding of their growth and behaviour or commercial utilization of this valuable resource. The past decade, in particular, has seen an upsurge in bamboo biotechnology for better insight into the mechanisms that govern the fast process of growth or the enigmatic process of reproduction and flowering. All the past and present approaches in bamboos have culminated into better understanding, conservation and improvement of bamboos for future.

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Fundamental Facets of Somatic Embryogenesis and Its Applications for Advancement of Peanut Biotechnology

Suprabuddha Kundu and Saikat Gantait

Abstract Peanut (*Arachis hypogaea* Linn.) is one of the most vital crops providing predominant supply of protein, vitamins and fats, along with other necessary nutrients. Various factors, responsible for induction, maintenance, multiplication of the embryogenic cultures, as well as maturation and conversion of somatic embryos (SEs) into complete plants have been discussed in this review. In order to find the present trends and thriving methodologies for the development of somatic embryogenesis, a lot of emphasis has been given to the economically important species. It has been reported that from young meristematic tissues like immature embryos and leaves of legumes, SE can be induced comparatively in a easier way. However, there are multiple constraints that limit the usage of somatic embryogenesis-based biotechnological applications on legumes, such as low rate of embryo formation, reduced germination, inadequate conversion into plantlets and somaclonal variation. These hindrances, nonetheless, may significantly be diminished in future, since the effective plant growth regulators with specific morphogenic targets are becoming available for experimental purposes. Existing reports reveal that somatic embryogenic systems, having superior germination and regeneration ability shall have direct usage in large-scale propagation and several other crop improvement features. With increasing knowledge of different morphogenic processes, involving differentiation of zygotic embryos, it is possible that improvement of this technology having practical efficacy may be applicable for the important peanut genotypes.

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Abbreviations

2,4-D	2,4-dichlorophenoxy acetic acid
IAA	indole-3-acetic acid
B5	Gamborg's medium
BA	N_6-benzyladenine
BAP	N^6-benzylaminopurine
BLB	Bulbil-like body
CPPU	forchlorfenuron
GA_3	Gibberellin A_3
IBA	indole-3-butyric acid
Kinetin	6-furfurylaminopurine
MS	Murashige and Skoog
MZEL	Mature zygotic embryo-derived leaflet
NAA	α-naphthalene acetic acid
PEDCs	Pre-embryogenic determined cells
PGR	Plant growth regulator
SE	Somatic embryo
TDZ	*N*-phenyl-*N*′-(1,2,3-thiadiazol-5-yl) urea or thidiazuron
Zeatin	4-hydroxy-3-methyl-terms-2-butenyl aminopurine

1 Introduction

Peanut (*Arachis hypogaea* Linn.) is an important edible oil seed crop, rich in protein, growing over 20 million hectors around 108 tropical and subtropical countries, with a yearly production of 28 million ton seeds (Fao 2007). On account of slight genetic variability found in the peanut germplasm, traditional methods of plant advancement technique have resulted in partial achievement regarding production of disease-tolerant cultivars. Such failure often arises during interspecific crosses like post-fertilization barriers resulting in embryo or seed abortion due to which progress of cultivated crop species is hampered. Tissue culture has its own restrictions wherein it does not have valuable methods for plant recovery from in vitro cells and tissues. However, regeneration of plant using somatic embryogenesis and organogenesis is more constructive than plant transformation. Embryo resembling structures, i.e. somatic embryos (SEs), are obtained from somatic cells by-passing the normal fertilization process that are genetically similar to the parent tissue and are hence considered as clones (Gantait and Vahedi 2015). Somatic embryogenesis may follow direct or indirect pathway. When preceding embryo production callus is

formed upon dedifferentiation of organized tissues, it is called indirect SE. However, when SE is directly formed from organized tissue superseding callus formation, the process is direct somatic embryogenesis (Slater et al. 2003). In spite of the dissimilar way of development, the physiological and anatomical properties of SE are similar to zygotic embryos (Zimmerman 1993). Bandyopadhyay and Hamill (2000) further supported the above reported fact with their work on *Eucalyptus nitens*, wherein, it was stated that both zygotic and SEs have strong resemblance with respect to size, morphology and cellular organization. Furthermore, the effectiveness of transformation procedures and in vitro organogenesis are responsible for successful regeneration of transgenic crops (Sharma and Vanamala 2000). Being a genotype-specific crop, in vitro propagation of peanut is tiresome and needs precise methods for each type. Somatic embryogenesis is preferred over direct organogenesis due to its high transformation rate, and also the formation of chimeric plants can be reduced appreciably (Stefaniak 1994). The standardization of plant regeneration protocols through callus induction or direct SE induction would surely help in the large-scale high quality propagation of peanut cultivars. The present chapter emphasizes on the progress made in the somatic embryogenesis of different peanut genotypes (Table 1) that will help biotechnologists, conservation biologists and plant physiologists who intend to investigate other non-listed peanut types/genotypes or to increase the scope of research for the presently investigated species.

2 In Vitro Regeneration

A comprehensive method of somatic embryogenesis includes induction of callus or direct SE, SE maturation and their conversion into complete plantlets. The common method for inducing SE is to culture an appropriate explant in a nutrient medium enclosing an auxin intended for callusing and subsequent somatic embryogenesis. Maturation of SE requires the transfer of callus to a cytokinin- or auxin-free medium. Nonetheless, the prerequisite for stimulation of embryogenesis may significantly vary with the species or cultivars. A specific selection and manipulation of the explants, basal nutrient medium, plant growth regulators (PGRs), as well as other physical factors are responsible for the successful somatic embryogenesis, which has been elaborately discussed below and schematically presented in Fig. 1.

2.1 Influence of Explant Source

Accomplishment of high rate of somatic embryogenesis in peanut species became possible because of the switch in emphasis from manipulation of medium to selection of suitable explant. The explant selection is a vital factor on which the success of most tissue culture research depends. In peanut, as also found in other groups of plants, immature, meristematic tissues evidenced to be the most appropriate explant for inducing SE. For instance, immature embryonal axes (Little

Table 1 Impact of explants, media formulations and culture conditions on in vitro somatic embryogenesis of peanut (*Arachis hypogaea* L.)

Cultivar	Explant	Basal media	Carbon source	PGR	Response	Success/remarks, experimental outcome	Reference
A. hypogaea L.	IC	B5	2% sucrose	0.5–1.0 mgl^{-1} picloram	SE	50–60% SEs were produced	Ozias-Akins (1989)
A. hypogaea L. cv. Florigiant	IZE	MS	2.5% sucrose	3 mgl^{-1} 4-amino-3,5,6-trichloropicolinic acid	SE	62% of the explants produced SEs	Mckently (1991)
	SE	½MS		1 mgl^{-1} GA_3	Reg	40% of the SEs of normal shape germinated from the explant cultures within 21 days of transfer	
A. hypogaea cv. AT127	IL	MS	3% sucrose	40 mgl^{-1} 2,4-D + 0.2 mgl^{-1} kinetin	Primary SE	Primary SEs were fused along the axes with no distinct cotyledons	Baker and Wetzstein (1992)
	Primary SE			5 mgl^{-1} 2,4-D + 0.2 mgl^{-1} kinetin	Secondary SE	Secondary SEs had single axes with two cotyledons. Maximum embryogenesis of 14.6% was obtained after a 15 d incubation on induction medium	
A. hypogaea L. cv. AT127	IC	MS	3% sucrose	40 mgl^{-1} 2,4-D	SE	An average of two SEs per cotyledon was produced	Durham and Parrott (1992)
	SE	MS		w/o PGR	Reg	15.3% SEs converted into plantlets. 10-day desiccation following SE development enhanced germination	

A. Hypogaea L. cv. McRan, OAC Garroy, OAC ruby	Intact seed	MS	–	TDZ	SE	SE development and proliferation	Gill and Saxena (1992)
A. hypogaea cv. 7 genotypes	EA	MS	3% sucrose	0.5 mgl^{-1} picloram	SE	The rate of cultures producing SEs and its number per responding explant was higher for all genotypes with EA	Ozias-Akins et al. (1992)
	SE			25 mgl^{-1} BAP	Reg	The percentage of cultures forming shoots ranged from 5% for Georgia red to 71% for Tifrun	
A. hypogaea L. cvs. McRan, ruby And tango	Seedling	MS	3% sucrose	10 µM TDZ	SE	SEs developed after 4 to 6 weeks of seedling culture. Higher concentrations (50–100 µM) of TDZ were found to be inhibit the induction of SEs	Saxena et al. (1992)
	SE	MS		w/o PGR	Reg	30–50% of the SEs developed into plantlets that had well-formed shoots and roots	

(continued)

Table 1

Cultivar	Explant	Basal media	Carbon source	PGR	Response	Success/remarks, experimental outcome	Reference
A. hypogaea L. *fastigata* type cv. JLM-1	IC; EA	MS	6% sucrose	90.4 µM 2,4-D	SE	48.9% cultures responded and 9.2 ± 1.0 SEs per responding culture. Total 203 SEs were produced	Eapen and George (1993)
	SE			22.6 µM dicamba or NAA	Reg	Highest plant conversion frequency, i.e. 25%	
A. hypogaea L. cv. JLM-I	Immature EA, IC	L-6 salts (Kumar et al. 1988)	6% saccharose	50 mgl^{-1} NAA	SE	The highest number of responding cultures was produced on medium containing NAA (50 mgl^{-1}), while the highest average number of SEs (9.3) per culture was produced on medium with 2,4-D (10 or 20 mgl^{-1})	Eapen et al. (1993)
				10–20 mgl^{-1} 2,4-D			
A. Hypogaea L. cv. Toalson and Florunner	IZE	MS	3% sucrose	0.5 mgl^{-1} picloram	SE	Initiation of SE	Ozias-Akinset al. (1993)
A. Hypogaea L. cv. AT127	IC	MS	3% sucrose	5, 10, 20 or 40 mgl^{-1} 2,4-D	SE	Highest induction of SE	Wetzstein and Baker (1993)

A. hypogaea L. cv. AT127	IC	MS	3% sucrose	5 mgl^{-1} 2,4-D	SE	94% embryogenesis was obtained. however, the mean number of SEs per embryogenic explant was greater at 40 mgl^{-1}. As auxin level increased in induction medium, percent embryogenesis decreased	Baker and Wetzstein (1994)
A. hypogaea L. cv. AT127	Immature zygotic Cotyledon	MS	3% sucrose	7.5 mgl^{-1} 2,4-D	SE	2 to 4 times more SEs were obtained with 2,4-D than NAA. SEs produced under a 16 h photoperiod were tough, woody and difficult to separate. Those produced under a 0-h photoperiod were succulent	Baker et al. (1994)
A. hypogaea. J.L.24	IL	MS	6% sucrose	20 mgl^{-1} 2,4-D	EM	EM induction was highest (78.9%)	Chengalrayan et al. (1994)
	EM	MS	6% sucrose	3 mgl^{-1} 2,4-D	SE	SEs developed within 20 days from 90% of the embryogenic masses transferred to medium	
	SE	½MS	2% sucrose	w/o PGR; 0.25% AC	Reg	50% SEs converted into plantlets	

(continued)

Table 1

Cultivar	Explant	Basal media	Carbon source	PGR	Response	Success/remarks, experimental outcome	Reference
A. hypogaea L. cv. JL-24	IL	MS	6% sucrose	90 µM 2,4-D	EM	There was a steady increase in fresh weight and triglyceride content of the tissue up to week 16. In contrast, transfer of SEs to fresh medium after 8 weeks of culture led to embryo germination and exhibited development of roots and shoots, as well as a sharp decline in TG levels with increases in weight	Mhaske and Hazra (1994)
	EM			13.6 µM 2,4-D	SE		
A. hypogaea L. cv. Tango, Garroy and ruby	IC	MS	3% sucrose	4.0 µM forchlorfenuron	SE	100% explants produced SEs. Highest number of SEs (18.3) was produced. frequency and number of SEs were higher for younger seedlings (up to 9 days), and seedlings older than 21 days failed to produce SEs	Murthy and Saxena (1994)
	SE			w/o PGR	Reg	Converted into whole plantlet	

A. hypogea L. cv. AT127	IC	MS	3% sucrose	20 mgl^{-1} 2,4-D	SE	Over 90% primary embryogenesis and 41–46% repetitive embryogenesis were obtained 12 weeks after initiation by maintaining embryogenic cultures	Baker and Wetzstein (1995)
A. hypogaea L. cv. GK-7	EA	MS	3% sucrose	20 mgl^{-1} 2,4-D	SE	94% embryogenesis was obtained with a mean number of 7.6 embryos per explant. Embryos obtained from cultures grown in the dark were easier to remove from the explant than those under a 16-h photoperiod	Baker et al. (1995)
A. hypogaea L. (14 genotypes)	EA	MS	2.5% sucrose	12.42 μM 4-amino-3,5,6-trichloropicolinic acid	SE	Response ranged from 10% to 58%. A highly variable number of SEs formed from the individual responding explants within each genotype	McKently (1995)

(continued)

Table 1

Cultivar	Explant	Basal media	Carbon source	PGR	Response	Success/remarks, experimental outcome	Reference
A. hypogaea L. cv. JL 24	IL	MS	6% sucrose	90.5 µM 2,4-D	EM	Embryogenic mass induction was the highest	Chengalrayan et al. (1997)
	EM			13.6 µM 2,4-D	SE	SEs developed within 20 days from the embryogenic masses transferred to medium	
	SE		2% sucrose	22.7 µM TDZ	Reg	Substitution of BA and kinetin with 22.7 mM TDZ increased plant recovery from 86% to 92%	
A. hypogaea L. cv. Tango	Intact seed	MS	3% sucrose	10 or 20 µmol l^{-1} TDZ	SE	After 2 weeks of exposure to TDZ, SEs developed at the hypocotyledonary notch region of the cultured seedlings	Murch and Saxena (1997)
A. hypogaea L. cv. GK7	Leaflet	MS	3% sucrose	136 µM 2,4-D + 0.93 µM kinetin	SE	Leaflets that were 5–7 mm long had a greater embryogenic response (67%) than smaller or larger leaflets	Baker and Wetzstein (1998)

A. Hypogaea L. (cv. 16 genotypes)	MZEL	MS	6% sucrose	90.5 µM2,4-D	EM	22–90% variation occurred in case of SE development	Chengalrayan et al. (1998)
	EM			13.6 µM2,4-D	SE		
	SE			w/o PGR	Reg	In 10 of the 16 genotypes, shoots and roots differentiated simultaneously in PGR-free medium to produce plantlets	
A. hypogaea L. cv. JL 24	IL	MS	0.175 M sucrose	20 mgl^{-1} 2,4-D	EM	EM induction	Mhaske et al. (1998)
		EM		3 mgl^{-1} 2,4-D	SE	SE developed after the embryogenic masses transferred to the fresh medium	
A. Hypogaea L. cv. VRI-2, TMV-7	ILDC	MSL	3% sucrose	5 mgl^{-1} 2,4-D + 1 mgl^{-1} BAP	SE	The highest frequency of SE was 85.3% in VRI-2 and 72.3% in TMV-7 cultivar	Venkatachalam et al. (1998)
	SE	MS		0.5 mgl^{-1} NAA + 0.5 mgl^{-1} BAP	Reg	Frequency of plantlet regeneration is low, i.e 15.3% in VRI-2 and 9.5% in TMV-7 cultivar	

(continued)

Table 1

Cultivar	Explant	Basal media	Carbon source	PGR	Response	Success/remarks, experimental outcome	Reference
A. Hypogaea L. cv. Georgia green	IC; EA	MS	3% sucrose	10 µM TDZ	SE	100% morphogenic callus was produced. concentration greater than 10 µM TDZ caused primary morphogenic calli to turn brown and inhibited growth	Gill and Ozias-Akins (1999)
	SE			5 µM TDZ	Reg	Gradual reduction in TDZ concentration and exposure to light was necessary for shoot organogenesis	
				1.44 µM GA_3 + 2.32 µM kinetin		100% regeneration and largest number of elongated shoots was obtained with 3.71 cm in length	
A. hypogaea L. cv. Gajah And NC-7	EA	MS	3% sucrose	5 µg ml^{-1} picloram	SE	6.3 and 14.3 SE per explant for Gajah and NC-7, respectively	Livingstone and Birch (1999)
	SE		2% sucrose	10 µg ml^{-1} BAP	Reg	Regeneration rates increased to 22% for Gajah and 30% for NC-7	

A. hypogaea L. cv. Tango	Seedling	MS	3% sucrose	10 µmol l^{-1} TDZ	SE	TDZ had characteristic thickened, stunted root systems and enlarged green cotyledons. SEs developed in the hypocotyledonary notch region	Murch et al. (1999)
A. hypogaea L. cv. TMV-2	Cotyledon	MS	3% sucrose	22.19 mM BAP + 2.68 mM NAA	SE	Maximum frequency of somatic embryogenesis reached to 80.2%	Venkatachalam et al. (1999a)
	SE	½MS		w/o PGR	Reg	Highest regeneration	
A. hypogaea L.; VRI-2 and TMV-7	IL	MS	6% sucrose	20 mgl^{-1} 2,4-D + 0.5 mgl^{-1} BA	SE	The highest frequency of embryogenesis was 69.2% and 53.6%, in VRI-2 and TMV-7 cultivars, respectively	Venkatachalam et al. (1999b)
	SE		2% sucrose	2.0 mgl^{-1} BA +0.5 mgl^{-1} NAA + 0.3% AC	Reg	SE germination frequency was 84.3% with VRI-2 and 76.9% with TMV-7	
A. hypogea L. cv. Tango	Seedling	MS	3% sucrose	10 µmol l^{-1} TDZ	SE	SE was induced at the hypocotyledonary notch region Inclusion of purine analogs 2,6-diaminopurine, azaadenine or azaguanine to the thidiazuron amended medium inhibited the embryogenic response	Victor et al. (1999b)

(continued)

Table 1

Cultivar	Explant	Basal media	Carbon source	PGR	Response	Success/remarks, experimental outcome	Reference
A. hypogaea L. cv. Tango	Intact seed	MS	3% sucrose	10 µmol l^{-1} TDZ	SE	SEs produced on the hypocotyledonary notch region of peanut seedlings wherein a maximum of 47 SEs/seedling were produced within 5 weeks of culture	Victor et al. (1999a)
	SE			50 µmol l^{-1} BAP	Reg	Differentiation into multiple shoots	
A. hypogaea L. cv. AT120, GK7, 59–4144 and VC-1	EA	MS	3% sucrose	83.0 µM picloram +124.4 µM centrophenoxine	SE	Centrophenoxine and picloram at higher concentrations were the most effective treatments for both percentage of responding explants and total SE per explant. AT120 and VC1 yielded more clusters of SEs than GK7 and 59–4144	Little et al. (2000)
A. hypogaea L. cv. TMV-2	Cotyledon	MS	3% sucrose	5 mgl^{-1} BAP + 0.5 mgl^{-1} NAA	SE	Maximum frequency of SE formation	Venkatachalam et al. (2000)
				0.5 mgl^{-1} BAP	Reg	Green healthy shoots emerged from the SEs	
A. hypogaea L. cv. J 11	IEA	MS	3% sucrose	40 mgl^{-1} 2,4-D + 1 mgl^{-1} NAA	SE	100% somatic embryogenesis with 9.5 SE per explant	Radhakrishnan et al. (2001)

A. hypogaea L. cv.J.L.24	IL	MS	6% sucrose	90.5 µM 2,4-D	EM	EM induction was highest	Joshi et al. (2003)
	EM	MS	6% sucrose	13.6 µM 2,4-D	SE	SEs developed from the embryogenic masses	
	SE	MS	2% sucrose	22.7 µM TDZ	Reg	79.21% SEs converted into plantlets	
A. hypogaea L. cv. VRI 4	EA	MS	3% sucrose	27.5 mgl^{-1} Picloram	Direct SE	97% SE induction with 23.6 ± 0.28 embryoids per explant	Bhanumathi et al. (2005)
	Leaf explant			2 mgl^{-1} 2,4-D + 1 mgl^{-1} kinetin	Indirect SE	84% SE induction through suspension culture	
	SE			0.2 mgl^{-1} BAP + 0.2 mgl^{-1} IBA	Reg	75.8% of plant regeneration	
A. Hypogaea L. cv. DRG-12	IZE	MS	3% sucrose	18.09 µM 2,4-D	SE	100% induction of SE with 18.3 SE per explant	Roja Rani and Padmaja (2005)
	SE			26.62 µM BA +0.54 µM NAA	Reg	80% shoot induction	
A. hypogaea L. cv. DRG-12	Zygotic EA	MS	3% sucrose	18.1 µM 2,4-D	SE	100% frequency with 18.3 SEs per explant was observed	Roja Rani et al. (2005)
A. hypogaea cultivar JL24	IZE	MS	3% sucrose	40 mgl^{-1} 2,4-D	SE	Proliferative SE was produced	Athmaram et al. (2006)
A. hypogaea L. cv. Co-5 And co-7	Hypocotyl explant	MS	40 g dm^{-3} sucrose	20 mg dm^{-3} 2,4-D	SE	Maximum embryogenesis with 9 SE per explant	Muthusamy et al. (2007)
	SE			2.0 mg dm^{-3} BAP + 0.25 mg dm^{-3} NAA	Reg	Maximum 78.6% germination	

(continued)

Table 1

Cultivar	Explant	Basal media	Carbon source	PGR	Response	Success/remarks, experimental outcome	Reference
A. Archeri	EA	MS	3% sucrose	8.8 µM BAP	SE	214 SE per explant	Pacheco et al. (2007)
A. Porphyrocalix				17.6 µM BAP		41.8 SE per explant	
A. Appressipila				8.8 µM BAP		19.2 SE per explant	
A. hypogaea L.	Leaflet	MS	0.18 M Sucrose	13.6 µM 2,4-D + TDZ (2.27–45.41 µM)	SE	2,4-D (13.6 µM) induced SE In lower dose (2.27 µM) of TDZ, 73% of cultures responded with the protrusions appearing in a single row around the equatorial region. With higher dosage, the structures were not restricted to the equatorial region but were more scattered	Joshi et al. (2008)
	SE			13.6 µM 2,4-D + TDZ (2.27–45.41 µM)	Reg	Lower concentrations of TDZ (2.27–9.08 µM) resulted new buds and further shoot formation in PGR-free medium. But higher TDZ (13.62–45.41 µM) turned the buds and globular structures necrotic	

A. Hypogaea	IZE	MS	3% sucrose	18.1 µM l^{-1} 2,4-D	SE	Maximum SE derived from 60-day-old cultures of immature zygotic embryo	Roja Rani et al. (2009)
A. hypogaea L. cv. SB-11	Axillary meristem	MS	6% sucrose	90.5 µM 2,4-D	EM	89.3% turned into EM	Singh and Hazra (2009)
	EM			13.6 µM 2,4-D	SE	89% of the EM converted into SE after 2 weeks	
	SE			w/o PGR	Reg	62% SE converted into complete plantlets	
A. Hypogaea L. cv. DRG-12	EA	MS	3% sucrose	4 mgl^{-1} 2,4-D	SE	100% SE with 18.3 SEs per explant that were big, healthy, succulent and green in colour	Venkatesh et al. (2009)
A. Hypogaea L. cv. BARD-479	IC	MS	3% sucrose	19 mgl^{-1} picloram	SE	91% somatic embryogenesis with 27 SE per explant	Iqbal et al. (2011)
	SE			0.15 mgl^{-1} BAP + 0.2 mgl^{-1} IAA	Reg	81% of plant regeneration was observed	
A. hypogaea L. (Guihua 26, 771, 1026, 83,836)	Leaflet	½MS	4% sucrose	10 mgl^{-1} 2,4-D	SE	Guihua 26 represented the highest SE induction rate of 62.8%	Jiang et al. (2013)
	SE			3 mgl^{-1} BAP + 0.8 mgl^{-1} NAA + 5 mgl^{-1} GA_3	Reg	Highest rate of plantlet regeneration	

(continued)

Table 1

Cultivar	Explant	Basal media	Carbon source	PGR	Response	Success/remarks, experimental outcome	Reference
A. hypogaea L. (Serenut 4 T, Serenut 1R and Acholi-white)	Mature embryo axes	MS	3% sucrose	5–30 mgl^{-1} 2,4-D	EC	Acholi white and Serenut 4 T gave the best response at 5 mgl^{-1} whereas Serenut 1R showed best response at a concentration of 30 mgl^{-1}	Okello et al. (2015)
	EC			2.5 mgl^{-1} BAP + 0.5 mgl^{-1} NAA	Reg	Over 50% shoot regeneration among all the cultivars	
A. hypogaea L. cv. Heiyuzhen	Cotyledon	MS	3% sucrose	10 mgl^{-1} 2,4-D	BLB-like SE	Formed BLBs with a high frequency of average 26 BLBs	Xu et al. (2016)
	BLB				Reg	BLBs spontaneously developed into multiple shoots (3–5 shoots per BLB) without changing induction and incubation conditions	

2,4-D 2,4-dichlorophenoxy acetic acid, *AC* activated charcoal, *B5* Gamborg's medium, *BA* N^6-benzyladenine, *BAP* N^6-benzylaminopurine, *BLB* bulbil-like body, *Ca* callus, *EA* embryonal axes, *EC* embryogenic callus, *EM* embryogenic mass, GA_3 gibberellin, A_3 *IAA* indole-3-acetic acid, *IBA* indole-3-butyric acid, *IC* immature cotyledons, *IEA* immature embryonal axes, *IL* immature leaflet, *ILDC* immature leaflet-derived callus, *IZE* immature zygotic embryo, *LI* light intensity, *MS* Murashige Skoog basal medium, *MSL* Murashige Skoog liquid medium, *MZEL* mature zygotic embryo-derived leaflet, *NAA* α-naphthalene acetic acid, *PGR* plant growth regulator, *PP* photoperiod, *Reg* regeneration, *SE* somatic embryo, *TDZ N*-phenyl-*N′*-(1,2,3-thiadiazol-5-yl)urea or thidiazuron

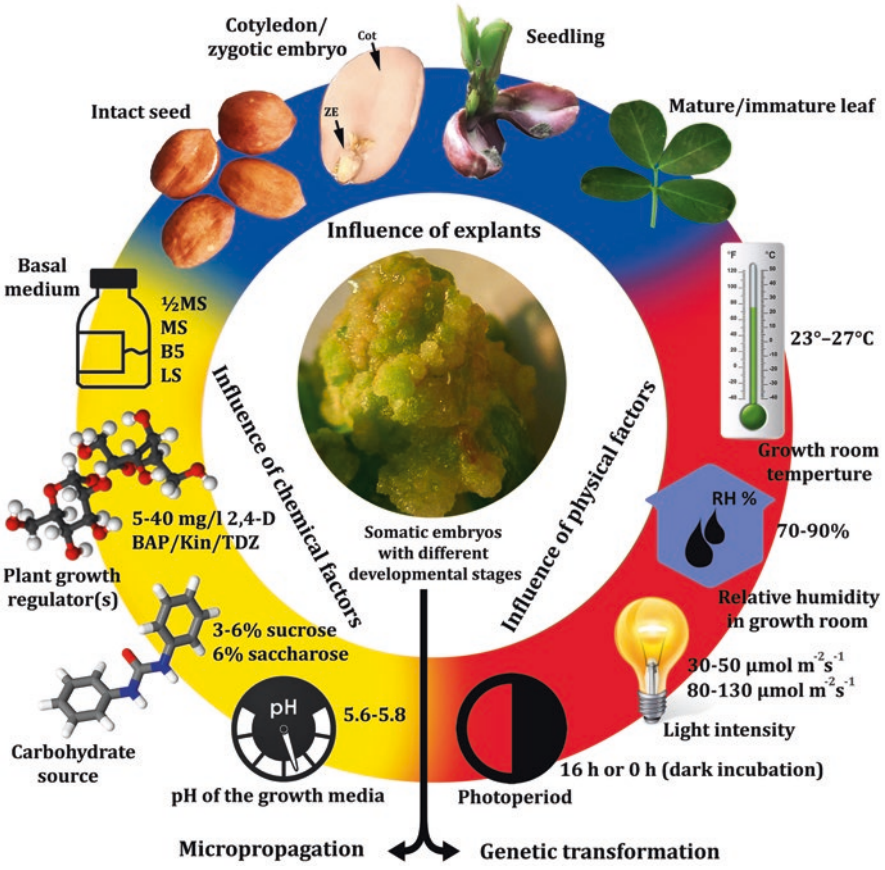

Fig. 1 Schematic presentation, illustrating the influence of explants, physical as well as chemical factors on somatic embryogenesis and its application in peanut (*Arachis hypogaea* L.)

et al. 2000; Radhakrishnan et al. 2001; Venkatesh et al. 2009), zygotic embryos (Mckently 1991; Roja Rani and Padmaja 2005; Athmaram et al. 2006; Roja Rani et al. 2009) and cotyledons (Wetzstein and Baker 1993; Venkatachalam et al. 2000; Iqbal et al. 2011) were the most responsive explants for the induction of SE in the majority of examined reports (Table 1). The cells of zygotic embryo that hold embryogenic competence are known as pre-embryogenic determined cells (PEDCs). SEs were produced from an array of explants such as seeds (Gill and Saxena 1992; Murch and Saxena 1997), intact seedlings (Murch et al. 1999; Saxena et al. 1992), leaflets (Baker and Wetzstein 1998; Bhanumathi et al. 2005; Joshi et al. 2008), hypocotyls (Muthusamy et al. 2007) and mature zygotic embryo-derived leaflet (MZEL) (Chengalrayan et al. 1998) in different peanut species. Singh and Hazra (2009) designed an experiment for somatic embryogenesis to investigate the mature zygotic embryo axis derived plumule containing three meristems. They found that embryogenic masses and SEs were induced from the caulogenic meristems in the

axils. Even if less critical, the genetic make-up of explant tissue plays a regulatory function in the induction of somatic embryogenesis. Genotypic variations could be as a result of different endogenous levels of growth hormones. Ozias-Akins et al. (1992) reported somatic embryogenesis and embryogenic callus formation of seven peanut genotypes including one Valencia, three Spanish and three Virginia botanical types, from immature cotyledon as well as embryo axis explants. There were noteworthy differences among the genotypes regarding SE initiation, subculture capacity and complete plant regeneration by means of a single media sequence. Moreover, SE development from cotyledons showed superior plant regeneration than the embryo axis. Additionally, using 16 genotypes of peanut, Chengalrayan et al. (1998) also showed a genotype-dependent variation in responses at each stage of SE development, including the phase of embryo conversion to whole plantlets.

2.2 Influence of Basal Media

The composition of basal media containing both organic and inorganic constituents strongly influences the rate of somatic embryogenesis and plant development. Majority of the researchers recommended semi-solid full strength Murashige and Skoog (1962) (MS) medium for SE induction in peanut (Little et al. 2000; Chengalrayan et al. 2001; Roja Rani et al. 2005; Muthusamy et al. 2007; Joshi et al. 2008; Roja Rani et al. 2009; Singh and Hazra 2009; Iqbal et al. 2011) (Table 1). Modification in the MS medium, for example, reduction of MS salts to one half, one third or three fourth, was not tested for SE in any of the genotypes. Even employment of liquid MS medium was reported by Venkatachalam et al. (1998), since the cost of production in commercial scale is much less in liquid medium. On a contrary note, other media types were seldom reported. Ozias-Akins (1989) employed B5 medium (Gamborg et al. 1976) for SE and regeneration, and Eapen et al. (1993) utilized L-6 basal medium (Kumar et al.1988) for the induction of SEs. Moreover, there are no reports available on the comparison between diverse basal media and its effect on SE.

2.3 Influence of Carbohydrate Sources

Carbohydrates are one of the most essential components as an energy source necessitate for growth and development (Gamborg et al. 1976) and provide carbon skeletons for various biosynthetic pathways as well. Further, presence of sucrose in the culture medium is fundamental for different metabolic activities of the cell. The nutritional requirements and the capability of plant tissues to absorb sucrose differ from species to species. Murashige and Skoog (1962) accounted that the usage of 3% (*w/v*) sucrose is superior over 2% or 4% during in vitro culture. Most of the reports validate the use of 3% sucrose in MS medium for SE induction (Murch et al.

1999; Gill and Ozias-Akins 1999; Little et al. 2000; Roja Rani et al. 2005; Athmaram et al. 2006; Venkatesh et al. 2009; Iqbal et al. 2011; Xu et al. 2016) (Table 1). Interestingly, there are many reports where higher concentration of 6% sucrose yielded much better results (Chengalrayan et al. 1994; Chengalrayan et al. 1998; Chengalrayan et al. 2001; Joshi et al. 2003; Singh and Hazra 2009). However, there are reports where 2% sucrose showed promising results in SE (Ozias-Akins 1989) and enhanced the regeneration frequency of somatic embryos (Chengalrayan et al. 1994; Chengalrayan et al. 1997; Livingstone and Birch 1999; Chengalrayan et al. 2001; Joshi et al. 2003). Carbohydrate sources other than sucrose, for instance, glucose and maltose, have not been tested in case of peanut, but 6% saccharose was reported in the experimentation of Eapen et al. (1993). From these reports it is obvious that plants can readily make use of carbohydrate in the form of sucrose. But, species specificity and formulation of maintenance medium might have supplementary effect on the performance of carbohydrate. Although several data are available concerning the uptake and consumption of exogenous carbohydrates by explants, yet, data on the associations between the trialling of source of carbon in the culture medium and the modification of sugar composition in in vitro cultured tissues are lacking.

2.4 Influence of Physical Factors

2.4.1 Light

It plays a chief role in morphogenesis of the in vitro plantlet all the way in association with photoperiod, light intensity and spectral wavelength. Generally, light released from cool- or warm-white fluorescent lamps is provided on in vitro cultures of peanut during somatic embryogenesis. Analysis of literature (Table 1) indicates that light intensity of 30–50 μmol m^{-2} s^{-1} is to be sufficient for the induction of callus and SE (Chengalrayan et al. 1997; Murch et al. 1999; Chengalrayan et al. 2001; Roja Rani et al. 2005; Muthusamy et al. 2007; Joshi et al. 2008). Some of the researchers found higher light intensity of 80–130 μmol m^{-2} s^{-1} to be optimum for SE (McKently 1995; Livingstone and Birch 1999; Roja Rani and Padmaja 2005). But, Eapen et al. (1993) and Eapen and George (1993) described that 12 μE m^{-2} s^{-1} was sufficient for indirect organogenesis. Contrastingly, dark incubation of the explants was also found effective (Little et al. 2000; Radhakrishnan et al. 2001; Athmaram et al. 2006). Lately, Xu et al. (2016) investigated the effect of light on somatic embryogenesis. It was evident that no bulbil-like body (BLB)-shaped SEs were induced from explants cultured on MS medium with all the considered concentration series of 2,4-D or NAA, at 25 °C under 16 h photoperiods with diverse light intensities (40, 80, 120, 160 or 180 μmol m^{-2} s^{-1}), signifying that light may regulate SE induction in peanut. Interestingly, the BLB-like SE induction from cotyledon was attained in the dark, signifying the importance of dark incubation.

2.4.2 Temperature

Not much consideration has been taken into the influence of temperature during SE induction and regeneration. No direct investigations were conducted on the effect of temperature, but the applied temperature regimes have depended generally on the amenities of different laboratories. The temperature under which different peanut varieties were cultured is summarized in Table 1. In most of the cases, a temperature range of 24–26 °C has been maintained (Venkatachalam et al. 2000; Radhakrishnan et al. 2001; Bhanumathi et al. 2005; Iqbal et al. 2011).

2.4.3 Others

Physical factors like carbon dioxide, ethylene and relative humidity in the culture vessels influence the in vitro culture as well (Gantait et al. 2014, 2015, 2016). The rate of carbon dioxide and ethylene accumulation differs based on the volume of culture vessel, explant type and culture environment, for instance, temperature and light. The relative humidity in addition to temperature has an interactive effect. The culture environment with lower humidity optimizes the rate of transpiration in plantlets and persuades positive morphogenesis. Even though a range of 70–90% relative humidity has been maintained in case of peanut somatic embryogenesis, the methodical experimental data concerning the role of relative humidity is not available. Since, the relative humidity in a culture vessel is regulated by the interior water content of the medium, light intensity and temperature, it is essential to compute these significant factors by a numerical model as established by Chen (2003).

2.5 Influence of PGRs on Somatic Embryogenesis

PGRs are the most significant factor for manipulating growth and morphogenesis in a plant tissue (Gantait et al. 2014, 2015, 2016). Among the PGRs, auxins are extensively used for inducing SEs and form a central part of the basal nutrient media. Auxins uphold the growth of calli, cell suspensions and other morphogenetic changes, mainly in association with cytokinins. In view of the fact that they are able to initiate cell division, they are concerned in the development of meristems that results in either unorganized mass of tissue or defined organs. Necessity for any other PGR for the induction of SE is largely dependent upon the developmental state of the explant tissue. The most frequently employed technique for somatic embryogenesis includes the induction of callus in an auxin-supplemented medium and subsequent formation of SEs upon shift of callus to a medium of low level of PGR. Particularly, auxin promotes callus proliferation and suppresses differentiation, whereas the deduction or decline in auxin level allows SE formation to progress. Table 1 presents a collection of research works that has been conducted

to analyse the effectiveness of different PGRs on the induction of SE in peanut. Somatic embryogenesis was induced in several peanut genotypes by an array of auxins, with 2,4-dichlorophenoxy acetic acid (2,4-D) being the most effective (Eapen and George 1993; Mhaske et al. 1998; Roja Rani et al. 2005; Muthusamy et al. 2007; Xu et al. 2016). Singh and Hazra (2009) described a method to obtain SEs from the determined organogenic buds of the axillary meristem. They found that an exposure of 2 weeks in primary medium containing 90.5 μM 2,4-D suppressed the shoot tip differentiation for the time being which then recovered the capability to form the shoot on withdrawal of 2,4-D. But, an exposure of 4 weeks in primary medium supplemented with 90.5 μM 2,4-D holds back the differentiation of shoot tip irreversibly. No shoot formation was observed from the tips in any of the cultures that were in secondary medium enclosing 13.6 μM 2,4-D. The efficacy of other auxins, such as indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA), has not been reported. However, α-naphthalene acetic acid (NAA) (Eapen et al. 1993) and picloram (Ozias-Akins et al. 1992; Little et al. 2000; Bhanumathi et al. 2005; Iqbal et al. 2011) had been used by some of the researchers and found effective results. Radhakrishnan et al. (2001) reported that double dose of auxins, i.e. 40 mgl^{-1} 2,4-D + 1 mgl^{-1} NAA, induced 100% somatic embryogenesis with 9.5 SE per explant. Nevertheless, combinations of auxin and cytokinin were found superior. Cytokinins have been shown to have promontory effects on the induction of SEs. Combination of N^6-benzylaminopurine (BAP) (Venkatachalam et al. 1998; Venkatachalam et al. 1999a) or 6-furfurylaminopurine (kinetin) (Baker and Wetzstein 1992; Baker and Wetzstein 1998) or thidiazuron (TDZ) (Joshi et al. 2008) with 2,4-D was found to induce more number of SEs than 2,4-D when used alone. Interestingly, there are also many instances where TDZ (a cytokinin-like substance) alone happened to induce somatic embryogenesis more competently (Murch and Saxena 1997; Murch et al. 1999; Gill and Ozias-Akins 1999). Victor et al. (1999a) employed several other cytokinins such as 6-(4-hydroxy-3-methyl-but-2enylamino) purine (zeatin), kinetin, diphenylurea or 2-isopentenyladenine and compared for morphogenic potential, but only seedlings exposed to TDZ and BAP produced regenerants. Interestingly, some rarely employed PGRs marked its role for successful somatic embryogenesis. Mckently (1991) evaluated NAA, 4-amino-3,5,6-trichloropicolinic acid, 2,4-D and 2,4,5-trichlorophenoxyacetic acid and observed highest production was occurred on medium containing 3 mgl^{-1} 4-amino-3,5,6-trichloropicolinic acid. Likewise, Murthy and Saxena (1994) analysed the competence of forchlorfenuron (CPPU), a substituted phenylurea compound, for inducing SEs in peanut. They found that exposure of seedlings for 2 days to CPPU induced SEs, but the most effectual treatment was the media fortified with 4.0 μM CPPU. Rate of embryogenesis and the number of SEs were higher for younger seedlings, in spite of the CPPU concentrations. Little et al. (2000), moreover, reported that picloram as well as centrophenoxine induced comparable numbers of globular stage and total SEs from each genotype, while dicamba was not as much effective.

2.6 *Influence of PGRs on Regeneration*

Frequent published reports propose a vital role of PGRs in SE maturation and its regeneration in peanut. Similar to zygotic embryos, the accessibility of food reserves and existence of germination-associated proteins along with various phytohormones that control germination and conversion of SEs to seedlings. Addition of low doses of auxins in conjunction with cytokinin is evident to boost shoot numbers in several varieties. Conversion or regeneration of SEs is dependent on media enclosing cytokinins as the foremost PGR, auxins in lower concentration and also Gibberellin A_3 (GA_3) in some instances. A variety of cytokinins such as N^6-benzyladenine (BA), kinetin or zeatin have been used for SE regeneration. In most work on shoot regeneration, BA was used as the superior cytokinin source either alone or mainly in association with an auxin source (Ozias-Akins et al. 1992; Chengalrayan et al. 2001; Bhanumathi et al. 2005; Roja Rani and Padmaja 2005; Muthusamy et al. 2007; Iqbal et al. 2011) (Table 1). The PGR used during the induction phase can play an important role in the embryo to-plant conversion step. According to some reports, SEs induced on media supplemented with cytokinins alone show suppression of root development, despite the normal development of cotyledonary leaves (Kaparakis and Alderson 2002). Hence, an amalgamation of cytokinin and auxin is compulsory for the adequate formation of both shoots and roots from SEs. However, other cytokinins like TDZ (Chengalrayan et al. 1997; Joshi et al. 2003) or GA_3 (Mckently 1991; Gill and Ozias-Akins 1999) were hardly employed. Jiang et al. (2013) also confirmed that adding GA_3 in plant induction medium is beneficial to plant regeneration. Chengalrayan et al. (1997) compared the effect of BAP, kinetin and TDZ and found that MS medium supplemented with 8.9 mM BA and 14 mM kinetin resulted in 86% of the SEs developed shoots. But, substitution of BA and kinetin with 22.7 mM TDZ increased plant recovery from 86% to 92% and exhibited multiple shoots. There are various instances where basal medium devoid of PGR promoted organogenesis much efficiently (Durham and Parrott 1992; Saxena et al. 1992; Chengalrayan et al. 1994; Murthy and Saxena 1994; Chengalrayan et al. 1998; Singh and Hazra 2009). Joshi et al. (2008) also accounted that, on transferring cultures to the medium lacking PGRs, the explants from 2,4-D with lower concentrations (2.27–9.08 μM) of TDZ responded quicker as well as the protrusions differentiated and elongated to form shoots.

3 Acclimatization of In Vitro Regenerated Plantlets

The definitive success of any in vitro culture program relies on a consistent acclimatization protocol that makes sure of low cost as well as high survival frequencies. Quick dehydration of in vitro regenerated plantlets and susceptibility to fungal and bacterial infections limit the acclimatization procedure. For the appropriate hardening of in vitro regenerated plantlets, it generally takes about 4–6 weeks to become

accustomed according to regular environmental circumstances. Most of the reports supported the acclimatization of plantlets by moving them to a combination of sand plus compost mixture at a fraction of 1:1 or 1:2 (*v/v)* (Chengalrayan et al. 1997; Little et al. 2000; Joshi et al. 2008; Singh and Hazra 2009). Yet, vermiculite added to the combination of soil and sand improved the survival rate (Radhakrishnan et al. 2001). Moreover, Baker and Wetzstein (1992) and Baker et al. (1995) acclimatized the plantlets in a vermiculite and perlite mixture (1:1). Incorporation of organic manures to the potting substrates proved superior in regard to vigour and survival. Muthusamy et al. (2007) moved the regenerated plantlets into the sand, red soil and organic manure in the proportion of 1:1:1 and noticed high percentage of survivals. But, none of the reports point towards the requirement of any particular treatment, like spraying of an insecticide or fungicide.

4 Genetic Transformation

Genetic transformation is an attractive tool in the present era for introducing novel agronomic traits into peanut species and cultivars. Experiments conducted on peanut in respect to genetic transformation are still limited and mainly based on *Agrobacterium*-mediated and particle bombardment methods. Venkatachalam et al. (2000) developed a competent transformation procedure for peanut. Cotyledons were cocultured for 2 days with *Agrobacterium tumefaciens* LBA 4404 strain harbouring the pBI121 binary vector enclosing *uidA* (GUS) and *nptII* genes and incubated on an embryo induction medium supplemented with 5.0 mgl^{-1} BAP, 0.5 mgl^{-1} NAA, 300 mg ml^{-1} cefotaxime and 75 mg ml^{-1} kanamycin. The presumed transformed SEs were transferred to the medium with decreased kanamycin (50 mg ml^{-1}) for additional growth. Multiple shoots emerged from the SEs with a transformation efficiency of 47% on MS medium having 0.5 mgl^{-1} BAP and 50 mg ml^{-1} kanamycin. Transformation was validated by GUS activity and polymerase chain reaction (PCR) analyses. Incorporation of T-DNA into the plant's nuclear genome was further authenticated by southern hybridization by means of *nptII* gene probe. On the other hand, Ozias-Akins et al. (1993) developed the transgenic crop through microprojectile bombardment of embryogenic tissue. They incorporated a *hph* gene under the control of the CaMV 35S promoter, giving resistance to hygromycin. Approximately, 12% of the bombardments lead to the successful recovery of a transgenic cell line. The integration of foreign DNA in hygromycin-resistant callus as well as regenerated plants has been validated by PCR and southern hybridization analysis. Resistance for hygromycin was expressed in transformed plants in its leaflets from which continued to be green when cultured on hygromycin rich basal medium. However, leaflets from non-transformed plants, i.e. control, became brown within 3 weeks on the medium containing hygromycin. Athmaram et al. (2006) also executed the transformation by means of particle bombardment with a plasmid enclosing a Bluetongue VP2 gene (*BTVP2*) along with neutralizing epitopes. Assortment for kanamycin-resistant SEs was started

after 12 days of bombardment on the medium supplemented with 25 mgl^{-1} kanamycin. Plantlets with 12.38% kanamycin resistant were obtained from bombarded SEs. In this case the production of transgenic peanut was mostly focused on analysing an enhanced gene transfer as well as somatic embryogenesis regeneration technique and to generate the Bluetongue outercoat protein that encompasses the neutralizing epitopes.

5 Problems Encountered During Somatic Embryogenesis

5.1 Low-Frequency Callus Induction

The major difficulties during callus formation of peanut involve the low rate of callus induction and reduced regeneration as observed by various groups of researchers. There had been a great discrepancy in the protocols reported so far, which need to be standardized and should be universal. MS medium was considered as the basal nutrient medium; some researchers employed 2,4-D alone to induce callus from cotyledonary nodes (Iqbal et al. 2011), while other group of researchers found that callus derived from hypocotyls on 2,4-D alone could not proliferate further (Muthusamy et al. 2007). Seven-day-old leaf explants produced embryogenic callus on the medium containing MS salts, B5 vitamins, 2,4-D (2 mgl^{-1}) and BAP (1 mgl^{-1}) combination. At this concentration as high as 84% embryogenic callus induction was observed (Bhanumathi et al. 2005). However, the calli often died after subculture that is one of the major limitations. On the other hand, the combination of PGRs differed significantly in different trials, and the efficiency as well as validity of callus induction was uncertain. In fact, there are loads of factors influencing callus induction, like the genotype from which explants are obtained, age of explant and level of endogenous hormones in addition to culture conditions. Genotype is a vital factor that to a great extent affects callus induction in peanut. Pacheco et al. (2007) induced calli from seed explants of *Arachis archeri*, *A. appressipila* and *A. porphyrocalix* in response to BAP. Embryo axes resulted in friable embryogenic calli from the hypocotyl region, after around 15 days of incubation. The highest rate of callus initiation was noted in the explants of *A. porphyrocalix*. All the three species also developed friable calli from embryonic leaflets in response to all BAP concentrations but at dissimilar frequencies. Calli of *A. porphyrocalix* and *A. archeri* were green, friable surface but compact at inside. In contrast, explants of *A. appressipila* formed pale yellow and highly friable calli. Moreover, calli of *A. appressipila* and *A. archeri* underwent differentiation and formed SEs, while calli of *A. porphyrocalix* did not show embryogenic competence. Interestingly, in the study of Xu et al. (2016), the leaf, stem, root and hypocotyl explants failed to induce SEs under the implemented culture conditions, but only cotyledons (with or lacking plumule and epicotyl) successfully induced SE in peanut. Hence, the selection of appropriate explants could be critical for plant SE induction as well.

5.2 Asynchronized Somatic Embryogenesis

Somatic embryogenesis in peanut is frequently observed to be asynchronous. Most of these protocols are incompetent, probably because of morphological anomaly in the apical meristem of the embryos as observed in the cultivar JL-24 (Chengalrayan et al. 1997, 2001). This is to a certain extent as a consequence of dissimilar association between the cultures and nutrient media (mostly PGRs) that result in the induction of SEs at different time intervals. It is also as a consequence of two types of secondary embryogenesis, first, in which secondary SEs grow straight on the primary SEs and second, where SEs form callus and then forms new SEs. Further, Baker and Wetzstein (1994) reported the irregularity in SE induction wherein minimum embryo development took place when the explants were cultured on induction nutrient media, but enhanced embryogenesis were observed only after relocating the cultures to the basal secondary medium. They further accounted that repetitive embryogenesis decreased with time after induction, due to proliferation of non-embryogenic callus and precocious germination of SEs. Little et al. (2000) observed a noteworthy effect of genotype and auxin on the number of shifted clusters of 6-week-old globular SEs that formed repetitive secondary SEs following 4 weeks transfer to the unchanged medium. Similarly, Chengalrayan et al. (1997) noted that SEs, induced from immature leaves of peanut exhibited a high rate of morphological defect. After relocating these SEs to PGR-free medium, roots were emerged. However, abnormalities were obvious in SEs induced from cotyledons, embryo axes or from the radicle end. Singh and Hazra (2009) also noted that at the end of 2 weeks of incubation period, bipolar embryos were observable, but the SEs were ceased to exist. SE formation was asynchronous, and different developmental stages could be visible through the histological documentation. Roja Rani and Padmaja (2005) also confirmed abnormal as well as asynchronous SE at a frequency of 43.3% on MS medium containing 72.4 μM 2,4-D. Joshi et al. (2008) reported the asynchronous growth and differentiation of SE originating in the medium containing 2.27 μM TDZ along with 2,4-D. Moreover, explants transferred from the higher doses of TDZ, where there were predominant globular structures, were found less responsive in respect to differentiation into mature SE. More than a few of these structures dedifferentiated and became necrotic whereas some turned green with a few differentiating into shoot primordia.

5.3 Limited Regeneration of Somatic Embryos

Complicatedness in the regeneration of SE is also a key setback that is the ultimate stage in organogenesis of peanut. Low frequency of conversion is a significant drawback to the exercise of somatic embryogenesis for in vitro regeneration of many plant species. Baker and Wetzstein (1992) found the conversion percentage as low as 2% only. It is the fact that morphology of SEs influenced the regeneration

pattern of plants. SEs having broad, fan-shaped fused axes produced plantlets with fused stems and numerous branches, while SEs comprising single axis produced plants with single stem and a more stable morphology. Culture conditions like PGR dosage and duration of incubation may have an effect on SE morphology, event of secondary embryogenesis as well as conversion into complete plantlets. Joshi et al. (2003) in his report stated that the TDZ-induced SE showed stunted shoot growth and was recorded for almost 79% of the SEs. Pacheco et al. (2007) studied the comprehensive germination of SEs with the development of both shoots and roots but obtained the same at a very low rate. Further, SEs, at distinctive phase of development, exhibited a partial conversion process, producing only shoots regardless of the presence of root apical meristem. Mature SEs of *A. porphyrocalix* and *A. archeri* demonstrated the maximum shoot developmental frequencies. But in *A. appressipila*, the highest shoot growth rates were observed in immature embryos. Furthermore, shoots of *A. appressipila* and *A. porphyrocalix* moved to MS basal medium containing various concentrations of IAA did not induce roots irrespective of the concentrations. Alternatively, shoots of *A. archeri* significantly produced roots with the influence of the PGR concentration and the developmental stage of the SE. Venkatachalam et al. (1999a) noticed that though the SEs showed both shoot and root meristems, synchronized initiation of shoot and root was occasionally observed only. More often, shoots are produced from the SEs, which consequently induced roots on the unchanged medium when cultured for 4 to 6 weeks. Also, adventitious roots were produced in some of the cultures. Interestingly, Xu et al. (2016) accounted that individual BLB-like SEs can instinctively produce multiple shoots and turn into green without altering induction or incubation conditions. As BLB is believed to be a new structure for SE, and the regeneration competence of SE is also high (about 103 shoots per cotyledon), it is practical to expect that transformation of peanut based on the BLB-mediated SE regeneration could be of superior technique.

6 Conclusion

Significant progression has occurred in the recent past in regard to the regulation of somatic embryogenesis in peanut. This technique accounts for the better understanding of physiological, biochemical as well as molecular events taking place during SE formation and subsequently the conversion of a somatic cell into an embryogenic cell. Nevertheless, utilizing this morphogenic mode for large-scale propagation along with genetic transformation is limited only to a few species. Much research, chiefly in the fields of embryogenesis in cell and protoplast cultures and repetitive embryogenesis, is needed to get better the efficiency of the established somatic embryogenic methods. Likewise, more thrust ought to be applied to optimize the protocols in favour of somatic embryogenesis of new commercially imperative species and cultivars. Lastly, reduction of somaclonal variation must be kept in focus, since the genetic uniformity between the regenerated plants will

eventually conclude the efficacy of somatic embryogenesis for crop improvement and propagation.

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Somaclonal Variation for Sugarcane Improvement

Pooja Manchanda, Ajinder Kaur, and Satbir Singh Gosal

Abstract Cell and tissue culture approaches serve as an important and an easily accessible source for the creation and utilization of variability in sugarcane improvement programmes. This variation arising from cell and tissue cultures is called somaclonal variation that can be genetic/epigenetic in nature. Somaclonal variation is considered as a complex phenomenon resulting from various genetic and cellular mechanisms under in vitro conditions. The induction of variation under in vitro conditions either through shock treatment or in stepwise manner generates useful variability without sexual recombination. Due to limited genetic system and/or narrow genetic base, somaclonal variation is more rewarding in sugarcane. During plant propagation process under laboratory conditions, the genomic shock is induced which can result in the activation of various transposons, retrotransposons, chromosomal changes, methylation and demethylation of DNA leading to somaclonal variation. Desirable variants (disease resistant, herbicide resistant, drought tolerant, salt tolerant, antibiotic resistant, etc.) have been isolated in sugarcane through in vitro selection. The frequency of variation can be further enhanced using physical and chemical mutagens. 'Ono', 'Phule Savitri' and 'VSI 434' are the sugarcane varieties released through the process of somaclonal variation.

Keywords Callus · Epigenetic · Genetic · Regeneration · Somaclonal variation · Sugarcane

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1 Introduction

The tissue culture approaches such as micropropagation and somatic embryogenesis can be employed to obtain a large number of plants in a limited space (Krishna et al. 2008; Eftekhari et al. 2012). The major benefit of plant production through these methods is the uniformity and genetic fidelity of plants that can be employed for commercial use (Krishna and Singh 2013). According to Scowcroft (1985), 'Clonal uniformity is now recognized as the exception rather than the rule'. This is because any process involving in vitro tissue culture may result in variation of any type; this is a serious concern for the propagators whose goal is to produce plantlets exactly alike the mother parent (clones), e.g. in micropropagation. The variations in tissue culture-derived plants are now considered as a ubiquitous phenomenon in commercial nurseries. These variant plants were termed as 'phenovariants' by Sibi (1976). The importance of tissue culture-induced variation was first given by Skirvin and Janick (1976) in horticultural crops. The scope of improvement in asexually propagated crops like sugarcane through conventional method is limited due to its highly polyploid nature, narrow genetic base and long breeding cycle. Since the existence of genetic variability in a population serves as the starting point for crop improvement, there is a need to widen the crop genetic base through conventional and modern methods of plant breeding, which require the use of wild relatives for crossing, tissue culture for induction of variation and genetic transformation for incorporation of desired genes. In fact, generation of variations through tissue culture approaches has served as a potential adjunct for crop improvement as such variability represents a pool upon which selection pressure can be imposed to select desired forms of a clone. The non-uniformity in clones is one of the predominant responses when plant regeneration occurs through adventitious pathways (Shepard et al. 1980). Plants derived from callus cultures and undifferentiated cells have been reported to exhibit a high frequency of variations (Bairu et al. 2011; Currais et al. 2013). The tissue culture-induced variations are best referred to as somaclonal variations and are defined as the genetic/epigenetic variations observed among the plants raised through tissue culture procedures under in vitro conditions. The concept of somaclonal variation was given by Larkin and Scowcroft (1981). Nowadays, it has become one of the key tools for plant science research. Protoclonal, gametoclonal and mericlonal variations are the terms which are often used to describe somaclonal variations in plants obtained from protoplast, anther and meristem cultures, respectively (Karp 1994; Chen et al. 1998). Therefore, the term somaclonal variation comprises different forms of variations that are derived from tissue culture procedures (Bajaj 1990), and a general term 'somaclones' is used for the plants regenerated from any form of cell culture (Larkin and Scowcroft 1981). These variations are manifested as somatically or meiotically stable events. In asexually propagated crops, the potential of tissue culture for inducing somaclonal variation was first recognized in sugarcane and later in potatoes. Somaclonal variation has been observed in many crop species such as apple, potato, tomato, sugarcane, rice, wheat, maize, etc. With the cultivation of sugarcane crop continuously for 20–25 years,

significant losses have occurred in crop yield and sugar recovery. Increased susceptibility to various diseases in old elite varieties is another cause of concern. The induction of somaclonal variation in sugarcane can be used to increase tolerance to various biotic and abiotic stresses, cane yield and sugar production. The first somaclone in sugarcane was produced by Heinz (1973) for resistance to Fiji disease from the callus cultures of clones H 37–1933 and H 50–7209. Somaclonal variations have also proved useful for inducing variability for economically important characters such as percent sucrose and tillering, which is beneficial for obtaining better genotypes (Sreenivasan and Sreenivasan 1984a, b). Table 1 reviews the various studies conducted for the creation of variation in sugarcane.

Table 1 Generation of variants through somaclonal variation and in vitro mutagenesis in sugarcane

Plant material	Remarks	Reference
Isd 37, Isd 39, Isd 40	The mutant plants regenerated from ethyl methanesulfonate (EMS, 0.01 M)-treated calli showed variation in internodes, millable canes, stalk colour, etc. from the source material	Mahmud et al. (2016)
VMC 7616, PS 862	Mutation was induced in calli using EMS (0.1%, 0.3%, 0.5%), and plantlets were obtained through in vitro selection. Aluminium tolerance of PS 862 was found to be higher as compared to VMC 7616	Purnamaningsih and Hutami (2016)
Isd 37, Isd 38, Isd 39, Isd 40	Assessed red rot-resistant somaclones for variation through RAPD and SSR markers	Mahmud et al. (2015a)
Isd 37, Isd 38, Isd 40	Used RAPD markers for identification of somaclonal variants	Mahmud et al. (2015b)
Co740	Obtained NaCl-tolerant plants through gamma ray-induced mutagenesis and in vitro selection of embryogenic calli	Nikam et al. (2015)
BL4	The somaclones K-250, K-265, K-251, K-109, K-106, K-300 and K-315 exhibited improved sugar yield per plant in comparison to BL4	Raza et al. (2014)
NIA98, BL4, AEC82–1026	Studied somaclonal variation in sugarcane clones obtained from callus cultures using RAPD markers	Seema et al. (2014)
HSF-242, S2003-US-623, S2003-US-633	Somaclones exhibited variability for reaction to sugarcane mosaic virus (SCMV) disease	Abdullah et al. (2013)
BF-162	Assessed red rot-resistant somaclones for variability through RAPD and SSR markers	Shahid et al. (2012)
CoJ 64	The tissue culture process resulted in enhanced variability among the somaclones for morphological traits and sucrose content	Sobhakumari (2012)
CoC 671	Somaclones derived from CoC 671 calli had useful traits, e.g. smut resistance, superior yield traits and early maturity	Dalvi et al. (2012)

(continued)

Table 1 (continued)

Plant material	Remarks	Reference
CoS 88230	Four out of 50 somaclones, SC-15, 20, 22 and 30, were better in red rot resistance than the source variety	Kumar et al. (2012)
NIA-98, NIA-0819, BL4	Induced genetic variability through in vitro mutagenesis of callus cultures using gamma rays	Yasmin et al. (2011)
S97US297	Assessed red rot-resistant somaclones and parent material for genetic variation using RAPD and SSR markers	Shahid et al. (2011)
B4362	Selected brown rust-resistant mutants through mutation induction in callus cultures and buds	Oloriz et al. (2011)
CP48–103	Obtained salt-tolerant variants from embryogenic calli evaluated on selective culture medium containing different levels of NaCl	Shomeili et al. (2011)
NIA-98, NIA-2004, BL4	2,4-D resulted in more genetic variability in callus cultures as compared to other auxins	Khan et al. (2009)
S. officinarum x *S. spontaneum* hybrids	Obtained subclones having commercial potential from callus cultures	Rajeswari et al. (2009)
CoJ 88, CoJ 64	Calli were screened under in vitro conditions against red rot pathogen. Somaclones regenerated from resistant/tolerant calli exhibited better disease resistance than the parents in the field	Sengar et al. (2009)
Co 86032	The irradiated calli selected on NaCl medium accumulated more proline than non-irradiated calli exposed to salt treatment	Patade and Suprasanna (2009)
CoJ 64, CoJ 83, CoJ 86	60 Gy dose of gamma radiation was optimum for in vitro mutagenesis of callus and callus-derived shoots	Kaur and Gosal (2009)
Co 94012, VSI 434 somaclones	The amplicon pattern of VSI 434 and Co 94012 with two RAPD primers were different from parent variety CoC 671	Tawar et al. (2008)
CoJ 88	Three somaclones exhibited moderate resistance to two *C. falcatum* pathotypes, whereas source variety CoJ 88 was susceptible	Singh et al. (2008)
CoC 671	Plantlet regeneration was highest from non-irradiated calli followed by 10 and 20 Gy irradiated calli on NaCl selection medium. In total, 147 plantlets were selected from different salt concentrations	Patade et al. (2008)
CoC 671	Partial desiccation improved plant regeneration in irradiated embryogenic callus cultures	Suprasanna et al. (2008)
CoC 671	Somaclones derived from callus cultures were better in cane yield, sugar yield, etc. as compared to parent variety	Doule et al. (2008)
GT54–9, Phil8013, G98–28, G98–24	Callus cultures generated new variability that was confirmed using RAPD markers	El-Geddawy et al. (2008)

(continued)

Table 1 (continued)

Plant material	Remarks	Reference
CP 77400	Obtained red rot-resistant lines from calli irradiated with gamma rays	Ali et al. (2007)
CoC 671	Observed genetic polymorphism between salt- and drought-tolerant lines from the parent plant (control) using RAPD markers	Yadav et al. (2006)
CP65–357	Developed stable NaCl (68 mM)-tolerant callus cultures from salt-sensitive cultivar CP65–357 through in vitro selection	Gandonou et al. (2006)
CoC 671	Employed in vitro mutagenesis of embryogenic callus cultures for selection of salt- and drought-tolerant lines	Patade et al. (2006)
CoC 671	Evaluated somaclones for agronomic and quality traits	Doule (2006)
Q77N1232, Co6519, Cadmus	Somaclonal variants had improved tolerance to drought with no inferior agronomic traits	Wagih et al. (2004)
CP-43/33	Salt-tolerant somaclones had a number of desirable morphological traits	Khan et al. (2004)
CP 70–321, LCP85–384, HoCP 85–845	Stalk diameter and weight were lower; stalk population was higher for callus-derived plants as compared to bud-raised plants in CP 70–321	Hoy et al. (2003)
PR62258, V64–10, V71–51	Obtained glyphosate-tolerant cell suspension line by in vitro selection	Zambrano et al. (2003a)
B6749	Obtained SCMV-resistant clones through in vitro mutagenesis of calli of susceptible cultivar B6749	Zambrano et al. (2003b)
CoJ 64, CoJ 83, CoJ 86	Induced somaclonal variation for agronomic traits in sugarcane	Kaur et al. (2001)
Isd-16	Obtained three clones moderately resistant to red rot and three clones tolerant to water logging	Samad et al. (2001)
CoS 91279	Identified moderately resistant and resistant somaclones to red rot regenerated from toxin-selected calli	Singh et al. (2000)
CP-43/33 (COL-54 hybrid)	Plants derived from calli irradiated with gamma rays (0.5 Kr) showed positive effect on agronomic traits linked with yield and sugar content	Khan et al. (2000)
AEC81–8415, BL4	Tested sugarcane clones for callus induction and in vitro mutagenesis	Khan et al. (1998)
C87–51	Obtained two somaclones resistant/tolerant to eyespot regenerated from callus of C87–51 (highly susceptible to eyespot disease)	Ramos Leal et al. (1996)
Sugarcane cultivars	Embryogenic callus-derived plants showed very few polymorphisms using RAPD markers; epigenetic effects result in transient morphological changes in regenerated plants	Taylor et al. (1995)

(continued)

Table 1 (continued)

Plant material	Remarks	Reference
PR62258	Identified SCMV-resistant somaclones from the susceptible source variety and susceptible somaclones using RAPD markers	Oropeza et al. (1995)
CP 74–383	Tissue culture regenerated plantlets had high frequency of variants as compared to plants raised through conventional method	Burner and Grisham (1994)
CoC 671	Clones of callus-derived plants exhibited higher cane yield and sucrose content as compared to source variety	Dhumale et al. (1994)
B43–62	Calli-derived plants showed higher rust severity, thinner stalks and smaller leaves as compared to control	Peros et al. (1994)
Intergeneric hybrid clone (H83–9998)	Nine somaclones had higher yield than the hybrid	Nagai et al. (1991)
Co 413, 54 C9	Produced clones resistant to sugarcane mosaic potyvirus from callus cultures	Fahmy (1990)
Rust-susceptible sugarcane variety	Obtained somaclones resistant to rust through callus culture	Sreenivasan et al. (1987a)
Sugarcane	Obtained somaclonal variation for resistance to smut disease	Sreenivasan et al. (1987b)
H69–9092	Plants regenerated from colchicine-treated callus cells were aneuploids	Nagai et al. (1986)
S. officinarum interspecific hybrids (CP 65–357, CP 72–356)	Majority of the variation arising from tissue culture was not under genetic control	Laurens and Martin (1986)
S. officinarum x *Sclerostachya fusca* hybrid	The clones exhibited morphological variations and structural aberrations	Sreenivasan and Sreenivasan (1984a)
Sugarcane	Isolation of somaclones with improvement in yield	Liu et al. (1984)
Q101	Obtained a high frequency of toxin-tolerant variants regenerated especially from cultures exposed to eyespot toxin	Larkin and Scowcroft (1983)
B4362	Preliminary inoculation trial depicted variation in the degree of rust resistance in somaclones derived from callus cultures	Liu et al. (1983)
F 177	Nine somaclones were tested for smut resistance, and only one (76–5530) had 43.8% infection rate as compared to 88.2% in donor	Liu and Chen (1981)
Saccharum-Zea hybrid	Callus cells and plants derived from callus exhibited variation in chromosome number	Sreenivasan and Jalaja (1981)
F156, F164	Callus-derived lines had improved yield and genetic changes as shown by the isoenzyme patterns	Liu and Chen (1978)
Sugarcane	Identified somaclones having disease resistance	Heinz et al. (1977)

Table 1 (continued)

Plant material	Remarks	Reference
58 sugarcane varieties	Callus-derived plants of eight varieties differed from their source varieties in the frequency of morphological changes and variation in chromosome number	Liu and Chen (1976)
Pindar	Isolated Fiji disease-resistant clones from callus culture	Krishnamurthi and Tlaskal (1974)
H 37–1933, H 50–7209	More variation was observed in plants raised from callus cultures of H 50–7209 than in H 37–1933	Heinz and Mee (1971)
H 57–1627	Colchicine-treated cells resulted in polyploid, mixoploid and diploid plants	Heinz and Mee (1970)
Five *Saccharum* spp. hybrids	Cell suspension cultures showed variation in chromosome number	Heinz et al. (1969)

2 Induction of Somaclonal Variations

The studies on tissue culture in sugarcane started during the year 1961 in Hawaii. Callus cultures were first established in sugarcane by Nickell (1964). Somaclonal variations in sugarcane usually occur in callus cultures and cell suspension cultures, which can be established by transferring the friable callus into liquid medium which is constantly agitated and maintained through periodic cycles of subculturing. Generally, regeneration of plantlets is carried out from old (long-term maintained) callus cultures, which are then transferred to soil and screened for variations in the greenhouse or field. Somaclonal variants for traits such as high yield can be screened at an adult plant stage. The scheme for induction of variation in somatic tissues and selection of somaclonal variants in sugarcane is shown in Fig. 1. Induction of somaclonal variation is usually carried out in elite cultivars or breeding lines. In vitro selection can also be carried out at cellular level for some traits by growing cells from cell suspensions and calli on a medium supplemented with various antibiotics and chemicals for providing different biotic and abiotic stresses. For example, a medium supplemented with polyethylene glycol or mannitol can be used for screening of drought-tolerant trait, sodium chloride for salt tolerance and phytotoxin or specific fungal culture filtrate for disease resistance. Only a few cells are capable of growing on a medium supplemented with these agents and regenerated. Thus, a large number of cells can be screened in a small space (Petri dish). This process provides another advantage of reduction in time for generation of potential plants or somaclonal variants. There are basically two methods of selection using in vitro process of culturing (Rai et al. 2011):

- *Long-term treatment in stepwise manner*, in which cultures are exposed to different concentrations of a selective agent, i.e. the stress level of cultures is gradually increased

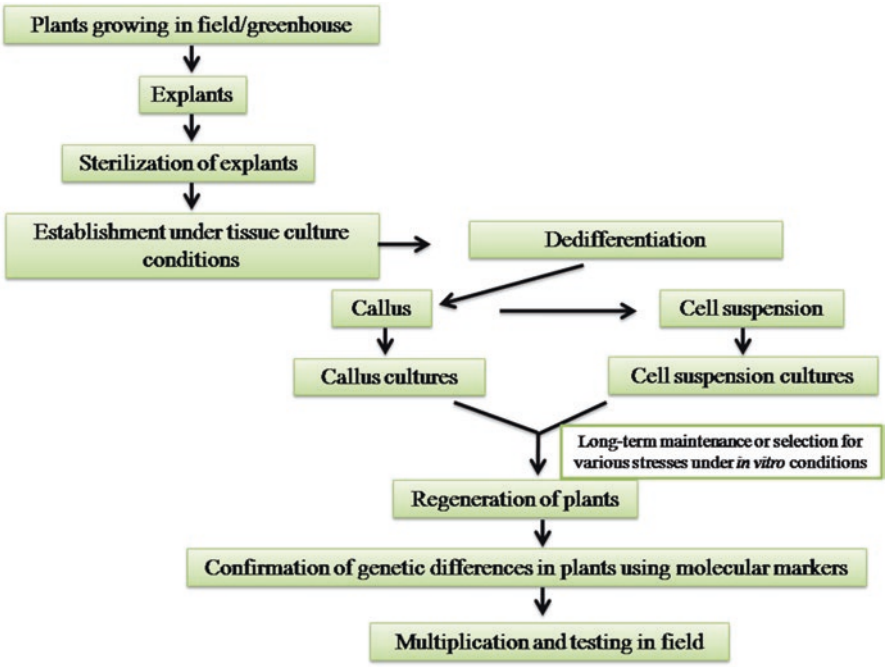

Fig. 1 Scheme for induction and selection of somaclonal variation in sugarcane

- *Shock treatment*, in which cultures are grown on a medium supplemented with high concentration of a selective agent and only those cultures which would tolerate that level will survive and be selected (Purohit et al. 1998)

These methods result in the induction of genetically stable variations among cells, tissues and organs (Mohamed et al. 2000). Sometimes, under high selection pressure, the explants may lose the ability to regenerate into plantlets (Tal 1994). This problem can be circumvented by the use of highly morphogenic explants which have an excellent ability to regenerate into plantlets completely. Genetic stability of the variations induced under in vitro conditions is confirmed by field testing. Remarkable progress has been made in recent years for the selection of plants for salinity tolerance and disease resistance using in vitro screening process.

3 Importance of Somaclonal Variations in Sugarcane

The main aim of sugarcane breeders is to develop improved varieties with good ratooning ability, low fibre level and high sucrose content and resistance to insect-pests and diseases (Jackson 2005). One of the prerequisites for any crop improvement programme is the generation of genetic variation and selection of plants with

desirable traits (Brown and Thorpe 1995). Conventional breeding by artificial mating of selected parents is one of the key approaches for the creation of genetic variability. In sugarcane, it is difficult to carry out the intended crosses for several reasons, viz. (i) different genotypes differ in fertility and produce small seeds (Poehlman and Sleper 1995) that are fertile only when produced under favourable environmental conditions (James 2004); (ii) the flowering time does not coincide in different genotypes (Selman-Housein et al. 2000); and in North Indian conditions, sugarcane usually does not flower, and (iii) cross-pollination occurs through anemophily and pollen viability is also very less. Further, it takes as long as 13–15 years for the release of a variety in sugarcane due to its complex polyploid nature which may result in masking of alleles of desirable genes at the same locus in other chromosome sets (Butterfield et al. 2001). In this regard, tissue culture methods hold a significant importance for the creation, conservation and utilization of genetic variability for the improvement of sugarcane. Generation of somaclonal variations through in vitro culture techniques has the advantage of selecting plants with desirable traits in a short period of time (Duncan and Widholm 1990). The exposure of cultured cells to physical and chemical mutagens may result in enhancing the frequency of somaclonal variations by induction of mutations (Maluszynski et al. 1995), Liu and Chen (1981), Ahloowalia and Maretzki (1983), Rodriguez et al. (1984) and Srinivasan and Vasil (1985) observed that sugarcane plantlets regenerated from cell cultures showed variation for various traits like cane yield, sugar content, stalk number, plant height and leaf shape.

4 Causes of Somaclonal Variations

Somaclonal variations can be epigenetic or genetic in nature. Epigenetic changes are the physiological changes that are temporary, nonheritable and reversible (Kaeppler et al. 2000). Epigenetic variations are also called developmental variations as these are induced during various stages of tissue culture process (Evans et al. 1984) and usually occur due to carryover effect of growth regulators in tissue culture medium, e.g. cellular adaptation involving loss of auxin, cytokinin or vitamin requirements by the callus (Jackson and Lyndon 1990; Skirvin 1978). Occurrence of thorns in juvenile *Citrus* (Hartmann and Kester 1983) is an example of epigenetic variation which involves expression of certain genes present in the genome of the plant. Male fertility, transient dwarfism and partial fertility are some other examples of epigenetic variations (Moore et al. 1991). A somaclone of sugarcane (*Saccharum officinarum* L.) resistant to eyespot was isolated by Maretzki (1987). The resistance to eyespot remained stable for 10 years through asexual propagation and then disappeared (Smith and Drew 1990) due to epigenetic nature of the trait. Genetic variations are the ones that are useful to plant breeders because these are permanent, stable during the sexual cycle and heritable and may occur in somatic tissues due to pre-existing genetic variation. The pre-existing variations can be due to non-uniformity in multicellular explants (harbouring different types of

cells as parenchyma, cortex, phloem and xylem parenchyma). For commercial use, the somaclonal variations obtained through tissue culture process must involve useful characters and be inherited stably through successive generations, i.e. the variations should be heritable. Genetic variations occur via an undetermined genetic mechanism (Larkin and Scowcroft 1981) and may involve a combination of factors. Some of the causes of genetic variations are chromosomal breakage, chromosomal changes, mitotic crossing over, apparent 'point' mutations, gene amplifications and deamplifications, transposable element activation, virus elimination and altered expression of multigene families that are briefly given below.

4.1 Chromosomal Breakage

Under in vitro conditions, the control of normal cell cycle (which helps in preventing division of cell before the completion of DNA replication) gets disrupted resulting in the breakage of chromosomes (Phillips et al. 1994). As a consequence, various deletions, duplications, transitions and transversions occur (Duncan 1997). Chromosomal breakage may result in the activation of various transposable elements in maize (Freeling 1984). Therefore, chromosomal breakage due to excision and insertion of transposable elements becomes one of the causes for the occurrence of somaclonal variations. Secor and Shepard (1981) obtained 65 clones in potato derived from leaf protoplasts that differed from the parent variety with respect to different traits. They concluded that this variation might be due to the occurrence of various transposition events.

4.2 Chromosomal Changes

Chromosomal changes in tissue culture-derived plants have been observed with respect to chromosome number and structure. Occurrence of aneuploids and polyploids has been observed in triticale, ryegrass, oats, potato, etc. In embryogenic cultures of maize, delayed separation of sister chromatids led to the formation of chromosome bridges and chromosome fragments (Fluminhan and Kameya 1996). Chromosomal aberrations were observed during in vitro propagation in bread wheat (*Triticum aestivum* L.), and the morphogenic potential is also lost due to structural rearrangements and extensive chromosomal loss (Karp et al. 1987). The most commonly observed aberrations are the structural chromosomal changes in the tissue-cultured plants which gives an overview of the karyotypic changes (Lee and Phillips 1988). The two theories which explain the origin of rearrangements in chromosome structure during tissue culture are the nucleotide pool imbalance and heterochromatin replication (Bryant 1976).

4.3 In Vitro Process of Propagation

Disorganized growth phase in tissue culture is one of the factors that result in somaclonal variation (Rani and Raina 2000). Generally, the greater is the departure from the organized growth, the greater are the chances of mutations (Araujo et al. 2001; Cooper et al. 2006). During in vitro growth, explants are under extreme stress which increases the frequency of mutations (Kaeppler and Phillips 1993; Shepherd and Dos Santos 1996). Further, during explant establishment, highly mutagenic processes occur which can result in developmental and heritable variations (Lorz et al. 1988). Direct somatic embryogenesis is the preferred pathway for obtaining genetically uniform plants in sugarcane (Manchanda and Gosal 2012) than indirect somatic embryogenesis and organogenic differentiation, because lower level of methylation exists in DNA in the initial stages of development as compared to later stages (Sahijram et al. 2003). Indirect somatic embryogenesis process involves an intermediate callus phase which is formed through uncontrolled cell divisions (Vazquez 2001); hence, more chromosomal variability is observed in callus phase (Saravanan et al. 2011). In direct somatic embryogenesis, dedifferentiation (callus) step is absent, and cultures give rise to normal plants identical to the mother plant (Peschke and Phillips 1992).

4.4 Source of Explant

The explant source used for in vitro propagation has a major effect on the extent of somaclonal variation. The occurrence of somaclonal variation also depends upon the origin of explant, method of regeneration and the source of regenerants (Ahuja 1998). The explants from differentiated source tissues like leaves, stems, roots, nodes, etc. are more prone to variations than the explant sources having pre-existing meristems, such as axillary buds and shoot tips (Duncan 1997). Karp (1994) reported that somatic mutations present in the donor plant, from which explant is taken, also serve as a source of somaclonal variation. Generally, the greater is the time of maintenance of cultures in vitro, the larger are the chances of occurrence of somaclonal variations. Krikorian et al. (1993) regenerated a stable variant in plantain named as ‘Superplantano’ from a chimeric variant of cultivar ‘Maricongo’. The process of initiating in vitro process of clonal propagation from fresh explants helps in reducing the extent of somaclonal variation.

4.5 Concentration of Different Plant Growth Regulators in the Medium

Suitable concentration and combination of auxins and cytokinins are essential for the efficient outcome of the micropropagation process (Letham and Gollnow 1985). High concentration of cytokinin benzylaminopurine (BAP) at 15 mg/l

resulted in an increase of chromosome number in a somaclonal variant 'CIEN BTA-03' derived from *Musa* spp. cultivar 'Williams' (Gimenez et al. 2001). 2,4-D (2,4-dichlorophenoxyacetic acid), the most commonly used auxin used for the induction of callus and cell cultures, is often associated with abnormalities such as polyploidy (Mohanty et al. 2008) as it may result in endoreduplication due to stimulation of DNA synthesis (Nehra et al. 1992; Ahmed et al. 2004). Polyploidy may also be induced in cell cultures due to imbalance in the concentration of auxins and cytokinins. There occurs a positive correlation between increase in DNA methylation rate and addition of auxins into the culture medium of cell suspension cultures (LoSchiavo et al. 1989). The 2,4-D and kinetin are the two most commonly used plant growth hormones, which result in differences in chromosome numbers and cause chromosomal aberrations (Daub 1986). The plant growth hormones result in an increased rate of cell division in the callus cultures. Cryptic chromosomal changes, polyteny, endopolyploidy and amplification of DNA sequences are some of the major changes which occur in the genome of plants during somatic differentiation (D'Amato 1977; Larkin and Scowcroft 1981).

4.6 Cytoplasmic Genetic Changes

Cytoplasmic genetic changes involving mitochondrial and chloroplast DNA are responsible for genetic variations in many crops. In maize, the cytoplasmic traits were evaluated by studying genetic changes in mitochondrial DNA. For example, most of the maize genotypes carrying Texas male sterile cytoplasm (controlled by mtDNA) are susceptible to *Drechslera maydis* race T, the causal agent of southern corn leaf blight. To recover resistant cytoplasmic male sterile lines, selection for resistance to *Drechslera* toxin was carried out under in vitro conditions. Among the regenerated plants, resistance was associated with reversion to male fertility. Significant differences were observed in the plants after analysis of restriction endonuclease pattern of mtDNA (Gengenbach et al. 1977).

4.7 Altered Expression of Multigene Families

The multigene families encode a large number of plant genes which occur in clusters at one or more positions in the genome. It has been evidenced in gliadin storage proteins of wheat (Larkin et al. 1984) and hordeins of barley (Breiman et al. 1987) that in vitro cultural conditions may regulate the expression of multigene family in such a way that one of the members of the family that earlier expressed some of the agronomically important trait, e.g. zeins, gliadins, etc., gets activated.

4.8 Instability Caused due to Methylation

DNA methylation is one of the mechanisms which result in the high frequency of tissue culture-induced variation. Methylation of DNA is a type of epigenetic variation that arises due to enzymatic modification resulting from the addition of a methyl group to cytosine. Variations due to DNA methylation conditions result in chromatin changes which cause quantitative variation (Phillips et al. 1994). It occurs by modulating the effects of multiple loci and causing chromosomal breakage due to alteration in timing of replication (Selker and Stevens 1985). During in vitro culture process, variations occur in total methylation levels as well as due to methylation at specific sites during culture process. During regeneration of plants from cultured tissues, various rearrangements occur in DNA (Muller et al. 1990), resulting in DNA methylation that acts as a major source of somaclonal variation, which is mitotically stable and meiotically heritable. Brown et al. (1991) reported differences in callus regenerated plants of maize due to methylation patterns. Unique methylation profiles were obtained in maize regenerants derived from the same cultured explant (Kaeppler and Phillips 1993). Extensive DNA hypomethylation has been reported in proliferating cultures of carrot using roots as explants (Arnhold-Schmitt 1993).

4.9 Departure from Organized Growth

While using organized explants, such as meristems, the organized growth from the meristematic tissue is maintained. In contrast, calli-derived plantlets regenerate from the cells, which are devoid of any organized growth. Therefore, the chances of occurrence of variation are higher in such plantlets. Most of the variations expressed under in vitro conditions are due to the damage caused by oxidative stress (Cassells and Curry 2001; Tanurdzic et al. 2008). As a result, elevated levels of pro-oxidants or reactive oxygen species (ROS) such as hydroxyl, hydrogen peroxide, superoxide and peroxy and alkoxy radicals are produced. The ROS are involved in altered hypermethylation and hypomethylation of DNA (Wacksman 1997), deletions and substitutions in DNA (Czene and Harms-Ringdahl 1995) and changes in chromosome number such as aneuploidy, polyploidy, etc. The production of callus from an explant and further its regeneration into a complete plantlet are asexual processes and involve only mitotic divisions; therefore, theoretically, no variation is expected from the clonal multiplication through micropropagation or somatic embryogenesis. But an unexpected phenomenon of occurrence of random spontaneous variation has found its usefulness in the creation of novel variants (Bouharmont 1994; Predieri 2001). DNA amplification (Karp 1995; Tiwari et al. 2013), organellar DNA changes (Cassells and Curry 2001) and segregation of chimeral tissue pre-existing in the explant (Ravindra et al. 2012; Nwauzoma and Jaja 2013) are some of the other causes of somaclonal variation. Figures 1 and 2 show the generation of somaclonal

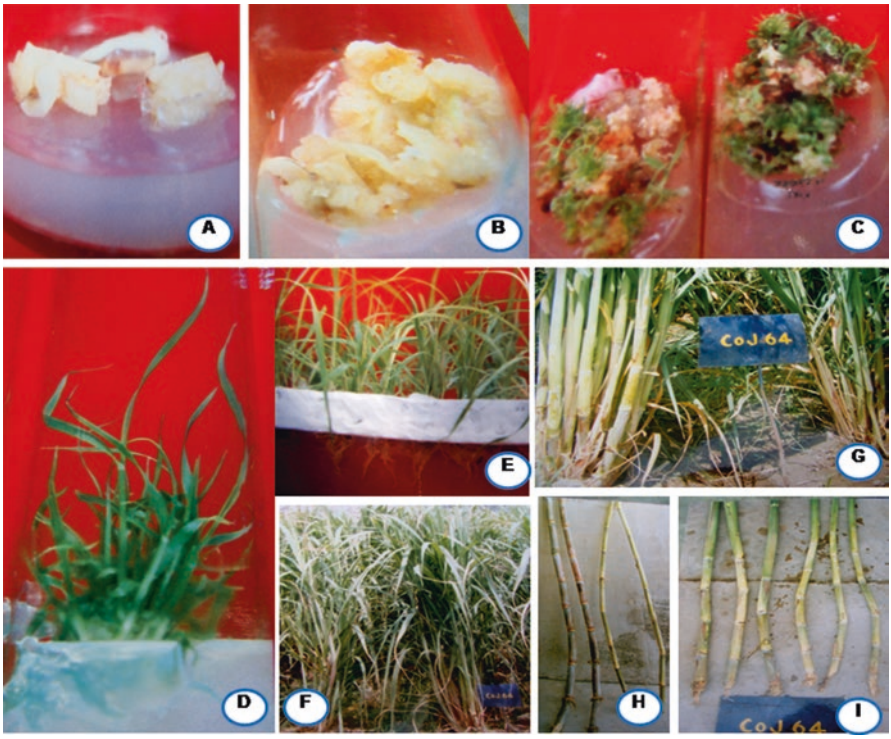

Fig. 2 Induction of somaclonal variation in sugarcane variety CoJ 64 (**a**) Cultured young leaf segments showing callus initiation from cut ends (**b**) Callus proliferation (**c**) Shoot regeneration from long-term maintained callus cultures (**d**) Shoot elongation (**e**) Root induction in shoots (**f**) Callus-derived plants growing in the field (**g**) Variation for tiller number in the field-grown plants (**h**) Variation for cane girth (**i**) Variation for internode length

variation for various agronomic traits in plants of sugarcane variety CoJ 64 regenerated from callus cultures induced from young leaf segments.

5 Molecular Bases of Somaclonal Variation

Plant regeneration through indirect somatic embryogenesis involves dedifferentiation of cells (callus formation) and redifferentiation of cells (regeneration) during which somaclonal variations occur (Arencibia et al. 1999) comprising both genetic and epigenetic changes. Callus formation from the explants depicts disorganized growth of cultured tissue which allows for various genetic rearrangements and variations in the regenerated plants (Reisch et al. 1981). Larkin and Scowcroft (1981) proposed that any disturbance in the cell cycle is responsible for the occurrence of variations in the plants as the cell is the basic structural and functional unit of an organism and plays a key role in its growth and morphogenesis (Bairu et al. 2011). Karp (1992) also suggested that anything that disturbs the routine process of cell cycle results in somaclonal variations.

For example, plant growth hormones (auxins, cytokinins, etc.), light, temperature and agitation rate of cell suspension cultures affect the normal cell cycle through their influence on cell organization and mitosis (Jones et al. 1989). The variation may result due to pre-existing variation in the somatic cells that is depicted when regeneration of plants takes place from the dedifferentiated tissues. The variation observed in somaclones can be due to the expression of some genic regions in the genome, which got suppressed at some stage of development. Somaclonal variations may arise from single gene mutations which result due to transitions (purine to purine or pyrimidine to pyrimidine) or transversions (purine to pyrimidine or pyrimidine to purine). Mitotic crossing over is also one of the causes of molecular changes that result in somaclonal variations including both symmetric and asymmetric variations. New genetic variations may occur from single gene mutations and mitotic crossing over, followed by segregation resulting in the phenotypic expression of recessive genes.

Altered methylation of DNA is one of the major causes of epigenetic changes (Adams 1990; Miguel and Marum 2011). Changes due to methylation were first reported in rice by Brown et al. (1990). Stress induced under in vitro culture conditions may activate various transposable elements (repetitive sequences dispersed throughout the genome); insertion of transposable elements has been reported to cause changes in maize (Chourey and Kemble 1982), tobacco (Lorz and Scowcroft 1983), alfalfa (Groose and Bingham 1984) and wheat (Ahloowalia and Sherington 1985). Tanurdzic et al. (2008) reported that various quiescent transposons and retrotransposons occur in the genomes of many plant species, and these become activated during tissue culture processes resulting in different chromosomal rearrangements. According to McClintock (1984), the cultured cells of rice are under traumatic and stressful conditions, and the genomic shock induced during in vitro regeneration process enhances transcription of retrotransposons (Hirochika et al. 1996). Brettell and Dennis (1991) observed that an inactivated transposable element became activated at a high frequency in culture and the activation frequency was higher as compared to seed-derived plants.

Heritable ‘gene inactivation’ is another factor that results in somaclonal variation (Karp 1993), which arises due to ‘putative mutation’; however when this plant with an inactivated gene is hybridized with a normal plant carrying active gene, the inactivity is reversed. Occurrence of homozygous mutations is another type of somaclonal variations. Examples of putative homozygous mutants obtained through in vitro culturing include a jointless-pedicel mutant in tomato (Evans and Sharp 1983), a yellow-seeded mutant in *Brassica juncea* (George and Rao 1983), two dwarf plants in rice (Sun et al. 1983), a white-grained mutant in wheat (Larkin 1985), etc.

6 Detection of Somaclonal Variations

Detection of somaclonal variation is important to select the somaclones which have all the desired characters of the parent cultivar with the addition of the trait that it lacks. Phenotypic, cytological, biochemical and molecular analyses provide an

overview to investigate the extent and type of somaclonal variations. These help in detecting useful variants with efficient agronomic traits, such as resistance to various biotic and abiotic stresses. Earlier, phenotypic/morphological detection was carried out to identify somaclonal variants by observing physical differences, such as altered leaf morphology, plant height and abnormal pigmentation (Israeli et al. 1991). 'Dwarf off-types' were obtained in banana that were detected by observing leaf index (leaf length/width) and stature after 4 months of transferring them in the field (Rodriguez et al. 1998). In sugarcane cultivar CoJ 64, morphological variation was observed in the somaclones consecutively for 3 years for stalk diameter, length, sucrose content, leaf length, etc. (Sobhakumari 2012). However, the morphological detection of somaclonal variations is a time-consuming process and environment sensitive (Bairu et al. 2011). Cytological analysis is used for determining the numerical and structural variation in the chromosomes (Abreu et al. 2014). It provides direct proof of change in the nuclear composition of an organism. Somaclonal variations among the in vitro regenerants are detected using complex microscopic procedures and flow cytometry; out of the two, flow cytometry is more widely employed for observing chromosomes (Dolezel et al. 2004). However, it is a time-consuming process, and the compounds used for preparing cytosolic suspensions interfere with the quantitative DNA content (Krishna et al. 2016). The absence of a set of DNA reference international standards also limits the use of flow cytometry for studying somaclonal variation (Dolezel and Bartos 2005). The phenotypic differences in an organism are as a result of biochemical variation which depicts the differences between proteins and isoenzymes. Isoenzymes are the different forms of the same enzyme whose amino acid sequence is different but catalyses the same biochemical reaction. The differences in the profiles of various proteins and isoenzymes are depicted as polymorphism in the genetic constitution of the organism. In sugarcane, the variation among somaclones was studied with respect to malate dehydrogenase, superoxide dismutase and peroxidase enzymes (Weising et al. 2005). The protein and isoenzyme analysis has been used to a limited extent to detect changes in the in vitro regenerated plants because the isoenzymes are limited in number, tissue-specific and influenced by the environment.

To determine the extent of deviation from the mother plant, molecular marker technology is a reliable and valuable tool because molecular markers are environment-independent, highly specific, large in number and reproducible. These are able to detect insertions, deletions and single nucleotide polymorphisms. Evans et al. (1984) reported that the frequency of occurrence of morphological variations is much lower as compared to variations at the DNA level. Restriction fragment length polymorphism (RFLP) was the first molecular markers to be reported and used for detecting DNA polymorphisms. These involve the use of restriction endonucleases followed by gel electrophoresis to separate out the resulting fragments. Random amplified polymorphic DNA (RAPD) involves the use of arbitrary short primers to amplify the target regions of the genome. Tawar et al. (2008) detected variations among the sugarcane somaclones, VSI 434 and Co 94012, and the parent variety CoC 671 using RAPD markers. Other PCR-based markers are the microsatellites or SSRs (simple sequence repeats) that are being routinely used for the detec-

tion of genetic variation in sugarcane. These are tandemly repeated short DNA motifs present in eukaryotic and most of the prokaryotic genomes. Using these markers, the somaclonal variants will show polymorphism between individuals due to differences in the number of repeat units at a particular microsatellite locus (Coggins and O'Prey 1989). Pandey et al. (2012) carried out molecular characterization of the micropropagated sugarcane plantlets generated through direct organogenesis using RAPD and SSR markers to assess their genetic fidelity; the plantlets showed the same profile as that of the mother plant, thus showing no instance of somaclonal variations. Nowadays, high-throughput next-generation sequencing technologies such as Helicos and Applied Biosystems SOLiD have provided a new impetus for the detection of somaclonal variations.

7 Advantages of Somaclonal Variation

The occurrence of somaclonal variation offers the following advantages:

- The creation of genetic variability is an essential component for any plant breeding programme. Somaclonal variation is a useful plant tissue culture tool that provides for uncovering the genetic variability that has been widely used for the improvement of sugarcane; otherwise, it takes 10–15 years for improving a sugarcane variety through conventional breeding.
- Somaclonal variations help in the development of new plant varieties resistant to various biotic and abiotic stresses, such as disease, drought and high salinity (Yusnita et al. 2005). Thus, the induction of somaclonal variations may result in the generation of novel mutants.
- Variability induced in cell and tissue cultures can be used to select high-yielding cell cultures for the synthesis of secondary metabolites on a commercial scale.
- Somaclonal variation can arise from different regions of the genome, which are not accessible through mutation breeding and conventional plant breeding (Karp 1992).
- Somaclonal variation is a cheaper method as compared to somatic hybridization and genetic transformation and does not require any containment facility.
- Gametoclonal variations induced during the sexual cycle of F_1 hybrid through meiotic recombination uncover new and unique gene combinations resulting from transgressive segregation.
- The frequency of occurrence of somaclonal variation is higher as compared to spontaneous mutations and induced mutagenesis (Gavazzi et al. 1987), which accelerate the process of classical breeding approaches for the selection of new genotypes.
- The frequency of somaclonal variations can be increased through the mutagenic treatment of large population of cells, somatic embryos and apical buds.

8 Limitations of Somaclonal Variation

Various problems arise due to somaclonal variation, an account of which is given below:

- One of the serious disadvantages of somaclonal variation occurs in processes like somatic embryogenesis and micropropagation which require uniformity and genetic fidelity of plants. It is not suitable for complex agronomic traits like yield, quality, etc.
- Most of the somaclonal variations occur in an uncontrolled and unpredictable manner.
- Most of the traits obtained through somaclonal variation are random, unstable, epigenetic in nature and nonheritable.
- Occurrence of somaclonal variation is a genotype-specific process as it is dependent upon the regeneration potential of calli derived from the explants of different cultivars.
- Extensive field trials are required for the selection of suitable somaclonal variants.
- Variants with pleiotropic effects can develop.
- The instance of somaclonal variation is mostly successful in crops with narrow genetic basis (e.g. vegetative reproducers, apomicts, etc.).
- It is comparatively difficult to predict the outcome of somaclonal variation experiment.
- The evaluation of variants generated through tissue culture approaches should be carried out in multiple environments to ensure the stability of desirable traits over generations.

9 Varieties Released in Sugarcane Through Somaclonal Variation

In sugarcane, only a few varieties have been released by the use of somaclonal variation. Due to quantitative inheritance of agronomic traits, the frequency of obtaining favourable mutation through tissue culture in terms of increased sucrose content and yield in sugarcane is limited. Till date, only three cultivars have been released through somaclonal variation in sugarcane. ‘Ono’, a sugarcane cultivar resistant to Fiji disease, was developed from Pindar, a susceptible cultivar by carrying out in vitro selection (Krishnamurthi and Tlaskal 1974). ‘Phule Savitri’ (Co 94012), somaclonal variant of a popular cultivar CoC 671, is the first sugarcane variety that has been released in India by somaclonal variation for cultivation in Maharashtra state. It is early in maturity and has high sucrose content and moderate resistance to diseases, viz. red rot and smut (Jalaja et al. 2006). Another somaclonal variant of sugarcane obtained from variety CoC 671 is VSI 434 released by

Vasantdada Sugar Institute, Pune (Tawar et al. 2016). It has increased cane yield and commercial cane sugar content by 2-fold and 1.3-fold, respectively, as compared to the source variety. Besides, it has moderate resistance to red rot. The distinctness of VSI 434 from CoC 671 was evident from morphological and molecular analyses.

10 In Vitro Mutagenesis

An alternative route for sugarcane improvement is through induced mutagenesis of in vitro cultures. The well-adapted sugarcane varieties can be further upgraded by the induction of mutations. In fact, the application of physical and chemical mutagens on embryogenic callus cultures helps in capitalizing the in vitro-induced somaclonal variation. The conventional mutagenesis is carried out using seeds or buds, where the major limitation is screening of large mutagenized population. In vitro mutagenesis helps in overcoming this problem (Micke et al. 1990) as uniform treatment is provided to all the cells of callus/explants, and selection of desirable mutants is carried out by adding selective agent to the medium used for culturing mutagen-treated calli/explant (Constantin 1984), thus requiring lesser space for screening of mutants. It also increases the frequency of mutations (Evans and Sharp 1983) as a large number of plant material/cells can be subjected to in vitro mutagenesis, and largely in vitro-induced mutagenesis yield dominant mutations (Larkin and Scowcroft 1983). Further, in vitro techniques allow the exposure of a large number of explants and multiplication of selected mutants in a small space. Both physical (ionizing radiations like gamma rays, X-rays, etc.) and chemical (ethylmethanesulphonate, sodium azide, etc.) mutagens have been successfully used in sugarcane to induce mutations (Kenganal et al. 2008; Koch et al. 2010). However, gamma irradiation is the most commonly used mutagen for inducing variation using embryogenic calli as explants. Up to 90% of the mutant varieties of different crops have been released through gamma irradiation of in vitro cultures (Micke et al. 1990). Radiosensitivity tests are carried out for the detection of optimal dose of gamma rays and X-rays for inducing mutagenesis. Induction of variations through mutation is a random process, and the plants regenerated from embryogenic calli subjected to in vitro mutagenesis can also display reduced vigour and other undesirable traits, such as albinism, hyperhydration, etc. (Koch et al. 2010), which may result due to DNA methylation, gene inactivation or reactivation of silent genes, instability of chromosomes, transitions, transversions, etc. (Larkin and Scowcroft 1981). Two methods may be followed to carry out irradiation of in vitro cultures:

- *Chronic irradiation*, in which the explants are exposed to low dose of irradiation over extended periods of time
- *Acute irradiation*, in which irradiation of explants or calli is carried out at higher dose over short periods of time

Novak (1991) reported that in vitro culture techniques combined with mutation induction is one of the effective methods for plant improvement. Till now, 13

varieties of sugarcane, namely, CCe 10582, CCe 183, CCe 283, CCe 483, Co 6608 mutant, Co 8153, Co 85017, Co 85035, Co 997 mutant, Guifu 80–29, Guitang 22, Nanei and Yuetangfu 83–5, have been registered in mutant variety database of the International Atomic Energy Agency, Vienna. Molecular markers such as RAPD, RFLP and AFLP (amplified fragment length polymorphism) are used for the identification and analysis of mutant plants.

In conclusion, availability of genetic diversity in the germplasm of a crop serves as the starting point for its improvement. Various landraces and wild relatives are available in most of the crops and offer sufficient genetic variation for the development of an improved cultivar. Recombination through hybridization results in the improvement of most of the sexually propagated crops (Mascarenhas 1991). In sugarcane, development of elite varieties through recombination is difficult due to narrow genetic pool, complex genome, low fertility and long breeding cycle (Lakshmanan et al. 2005). Somaclonal variations mutually complement the conventional plant breeding techniques as these are a source of new variability that involves multiplicity of genetic, biochemical and cytological factors. Somaclonal variation is a phenomenon which is observed in all crops. Some tissue culture-based companies maintain a separate space for 'somaclones' in an effort to obtain useful variants. Thus, somaclonal variation introduced through the process of tissue culture becomes one of the key tools for the development of superelite varieties in sugarcane. In vitro mutagenesis can be used to further increase the frequency of variation in callus/shoot cultures of sugarcane.

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Developing Stress-Tolerant Plants Through In Vitro Tissue Culture: Family Brassicaceae

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Abstract Plant stress is an unfavourable environmental constraint affecting the plant growth and development leading to a worldwide loss in agricultural productivity. The environmental stress on plants can be biotic or abiotic. Biotic stress involves the damage caused by various living organisms including bacteria, viruses, fungi, parasites and insects affecting the crop yield. Abiotic stress involves various environment factors including drought, salinity, heavy metal, high temperature and low temperature stress that affect the plant growth and development, leading to reduced crop yield. Brassicaceae being grown in arid and semiarid regions is severely affected by both biotic stresses and abiotic stresses. Various agronomic practices, conventional breeding methods and biotechnological approaches were used for the management of various stresses and development of stress tolerance in Brassicaceae. However, all these methods were found to be undesirable and less successful. Recently, tissue culture approach has proved to be more convenient and cost-effective technique for the development of stress tolerance in plants. The technique operating under controlled environmental conditions with less time and space has very high potential for the development of various stress-tolerant crop plants and is used for the understanding of physiology and biochemistry of plants cultured under various environmental stress conditions. Using tissue culture technique, various stress-tolerant *Brassica* crops are developed. Various stress-tolerant lines of Brassica were obtained by in vitro selection. These stress-tolerant lines of Brassicaceae showed improved tolerance to both biotic and stresses along with better yield. Therefore, in vitro selection technique provides new opportunities for improving stress tolerance in Brassicaceae for environmental sustainability.

Keywords Tissue culture · Abiotic stress · Somatic hybridization · Brassicaceae · In vitro

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1 Introduction

Stress on plants is an unfavourable environmental constraint affecting the productivity in plants leading to a worldwide loss in agricultural productivity (Haag 2013; Rai et al. 2011). The stress on plants can be biotic/abiotic or both. Biotic stress is the damage caused by living organisms such as viruses, bacteria, fungi, insects, parasites and weeds reducing 25% of crop yield worldwide (Savary et al. 2012). Abiotic stress is the multiplex of factors in a specific environment affecting the plant (Kayum et al. 2016) and detrimental to the plant growth and development and reduces crop yield by more than 50% (Acquaah 2007). In many regions of the world, salinity and drought are becoming more devastating and may lead to more than 50% of extreme salinization of the arable land by the year 2050 (Wang et al. 2003).

Brassicaceae, commonly known as crucifers, is an important vegetable group comprising about 100 species, including mustard (*Brassica juncea* L.), rapeseed (*Brassica napus* L.), turnip rape (*Brassica rapa* L.) and cabbage (*Brassica oleracea* L.) that are grown mainly for oil, vegetables, fodder and condiments (Ashraf and McNeilly 2004). Brassica is a good source of antioxidants due to the presence of high phenolics and glucosinolate content (Jahangir et al. 2009). Brassicaceae is grown in both arid and semiarid regions and is severely affected by both biotic stresses, including bacteria, viruses and fungi, and abiotic stresses, including cold, heat, salinity and drought. Bacterial soft rot disease being the most destructive disease leads to serious damage and yield loss across all the members of Brassicaceae (Kayum et al. 2016). The fungal pathogen *Leptosphaeria maculans* leads to a most destructive disease (stem cankers) in Brassicaceae, particularly *B. rapa* and *B. napus* (Fitt et al. 2006; Howlett et al. 2001). The wilt disease caused by *Fusarium oxysporum* causes a huge loss in quality and quantity of oilseed Brassica (Gaetan 2005).

Various agronomic practices, including the altering of sowing time, soil amendment, irrigation by quality water, etc., can be used for the management of various abiotic stresses. However, these strategies are not economical and sustainable for vegetable production (Farooq et al. 2009; Hashmi et al., 2015). Therefore, there is a need to look for the permanent solution for the protection of vegetable and other crop plants against stress. The conventional breeding method can be used for the integration of genes of interest from inter-crossing species into the crop for the development of stress tolerance; however, these methods proved less successful with undesirable results (Purohit et al. 1998). Moreover, biotechnological approaches can be used for the development of stress-tolerant crop plants. The genetic transformation involves the transfer of gene (responsible for stress tolerance) from distant gene pools in many plant species for development of stress-tolerant crop plants. This genetic engineering for the development of stress-tolerant plants could be fast track for the improvement of crop varieties; however, the major limitation of this approach is the low transformation efficiency, silencing of transgene and reduced gene expression (Rai et al. 2011). Recently, tissue culture approach has proved to be a convenient and cost-effective tool for development of

stress-tolerant plants. This technique operates under controlled laboratory conditions with limited time and space having high potential for the development of stress-tolerant crop plants and leads to the better understanding of biochemistry and physiology of plants cultured under severe environmental conditions (Rai et al. 2011; Pérez-Clemente and Gómez-Cadenas 2012). Using in vitro tissue culture technique, various stress-tolerant *Brassica* crops are developed. In *Brassica*, stress-tolerant lines have been obtained by in vitro selection (Rai et al. 2011). Using partially purified culture filtrates, B. napus showing resistance to *Alternaria brassicicola* has been obtained (MacDonald and Ingram 1985), and *B. napus* showing resistance to *Phoma lingam* has been obtained through embryonic culture (Sacristan 1982). Salt-tolerant *B. juncea* and *B. napus* have been obtained by using in vitro culture (Kirti et al. 1991; Rahman et al. 1995). Through in vitro selection, drought-tolerant *B. juncea* (Gangopadhyay et al. 1997) and metal (Mn + Zn)-tolerant *Brassica* spp. (Rout et al. 1999) have been obtained. In this chapter, the devastating effect of environmental stress on Brassicaceae and the progress made towards development of stress-tolerant crop plant of Brassicaceae through in vitro tissue culture have been discussed.

2 Environmental Stress

Defined by Lichtenthaler (Lichtenthaler 1996), plant stress is 'any unpleasing condition or substance that have an impact or halts plant's metabolism, growth or development'; by Strasser as 'a state of altered equilibrium caused by factors'; and by Larcher as 'alterations in physiological state of plant that occur when species are exposed to unusual admonishing conditions that need not correspond a menace to life but will bring on an alarm outcome' (Gaspar et al. 2002). Equivalent to 'stress' and 'strain' in mechanics, 'stress factor' and 'stress' are used commonly by plant scientists. Regardless of terminology, stress factors (or stresses) approaching from outside demands separation from stresses (or strains) generating within an organism. Factors which stimulate stress can be biotic, which upshot from living beings, such as insects and fungi, or abiotic, resulting from inanimate factors, such as drought, intense temperatures, salinity and pollutants (heavy metals, etc.). The harmony between sensitivity and tolerance may ascertain whether a stress factor has a positive (eustress) or negative (distress) effect (Kranner et al. 2010). Besides, short-term and long-term (persisting) stresses need to be differentiated, along with the 'low stress events' that can be partly counterbalanced by acclimation, adaptation and repair and intense or chronic stress events that result inappreciable harm leading to cell and plant death (Gordon et al. 1992; Lichtenthaler 1996). Therefore, with increasing duration and severity of stress, plant responses to stress will change.

2.1 Abiotic Stress

2.1.1 Drought Stress

Inadequate accessibility of water, i.e. drought, is presumptively the most familiar stress that terrestrial plants undergo. It will affect critical metabolic functions and troubles turgor pressure at cellular level thereby affecting cell wall formation and cell expansion which are indispensably more sensitive to water limitation. The damage due to oxidative stress gets amplified by coinciding high radiation and heat together. Hence, a general feature of nearly all abiotic stresses comes up with the increased concentrations of reactive oxygen species (ROS), and acclimation to the stress usually corresponds to beefing up of the anti-oxidative system (Tippmann et al. 2006). For instance, studies in maize have shown that increased anti-oxidative enzyme activity explicitly correlates with enhanced plant drought tolerance (Malan et al. 1990). Plant growth regulator (PGR), abscisic acid (ABA), is one of the crucial messengers during drought stress. Upregulated during drought stress, a specific ABA responsive element (ABRE) has been identified in promoters of few genes (Wang et al., 2018). Dehydration-responsive element (DRE), included among the ABA-independent signal transduction pathways, has been brought up by probing into drought-induced genes. Moreover, the volatile PGR, ethylene (ET), piles up during drought stress and may be involved in initiation of specific gene expression, in contrast to the cytokinin, which has antagonistic effects. Drought-induced genes encode proteins which play a key role in osmotic, metabolic or structural adjustment as well as in checking the damage and repair functions (Ingram and Bartels 1996). Nevertheless, most of the drought-induced genes have not been characterized so far, and disclosure of their roles may give novel insights to shielding mechanisms of plant during drought conditions.

2.1.2 Temperature Stress

Depending on the limited thermo-sensitivity of the plant species, plants execute their vital functions at optimum characteristic temperature range. Temperatures above and below the optimum range severely affect critical cell structures and functions, such as membrane integrity, enzyme activity and cell division. As mild stress, pretreatment with temperatures outside optimum range can substantially increase the thermo-tolerance of plants. Therefore, cold and heat acclimation is accomplishable (Thomashow 1999). Temperature stresses can be categorized as heat stress and cold stress including freezing stress.

Ranging from moderate effects like oxidative stress and increased transpiration to deadly consequences for the plant species, like tissue collapse and plant death, higher temperatures affect plant's survival and fitness. Under these circumstances, various events occur: synthesis of small heat shock proteins (HSPs) in larger abundance which act as molecular chaperones (Vierling 1997), enhancement in Ca^{2+} influx and accumulation of ROS in various parts of the cell (Doke et al. 1996).

The suboptimal temperature, at which the plant suffers trimmed down enzyme activity and possibly shortage of water availability, also is termed as the chilling stress, although the temperature is higher than the freezing point of water. Metabolic processes, substrate diffusion rates, impairing enzyme reactions, membrane structures and associated transport properties are mainly touched by cold stress (Tippmann et al. 2006; John et al., 2016).

Plants go through freezing stress, when temperatures fall down the zero degrees. Protoplast membrane structures are damaged, mechanically injured and ultimately killed by the expanding ice crystals due to the freezing of water intracellularly; and due to extracellular freezing, the severe dehydration of the protoplasm occurs. Encoded by a category of cold-induced 'cold-regulated genes' (COR), hydrophilic polypeptides and associated proteins stabilize membranes against freeze-induced injury and thereby aid in freezing acclimation (Artus et al. 1996). Presumptively by ethylene induction, protective antifreeze proteins (AFP) pile up after cold and drought treatment (Yu et al. 2001; Yu and Griffith 2001; Griffith et al. 2005). Two signalling pathways have been reported to get activated during cold-regulated gene expression in plants, an ABA-independent pathway (Capel et al. 1997) and an ABA-dependent pathway (Lang et al. 1994).

2.1.3 Salt Stress

Impaired water and nutrient uptake and reduction in photosynthetic activity and growth are the usual consequences of ample concentrations of salt (i.e. ions, mostly Na^+) in the soil solution. Additionally, larger salinity can result in adverse Ca^{2+} or K^+ to Na^+ ratio, peroxidation of membrane lipids and fatal intracellular Na^+ concentrations (Levitt 1980). Secondary oxidative stress of salt stress tolerance is ostensibly very important for stress tolerance: hence, a number of salt-tolerant species not only enhance the activity of antioxidant enzymes but also accumulate them as well in response to salt stress, in contrast to salt-sensitive species. One salt stress-specific signalling pathway gains a peculiar worth for the ionic stress element at the cellular level. In *Arabidopsis,* the SOS (salt overly sensitive) pathway has been studied thoroughly which involves Ca^{2+}-dependent protein kinases (Zhu 2000). Additionally, other significant determinants of salt tolerance like the vacuolar Na^+/H^+ antiporter AtNHX or high affinity K^+ transporter (HKT) have been recognized in *Arabidopsis* (Rus et al. 2001; Yokoi et al. 2002).

2.1.4 Other Abiotic Stresses

Xenobiotics are naturally occurring compounds, but at higher concentrations, they prove very venomous to plants. No particular mechanisms are possessed by most of the plants to combat with the inordinate uptake of heavy metals from the soil. Membrane integrity, enzyme activity and nutrient uptake are impaired due to accretion of heavy metals. Activation of Ca^{2+}-dependent systems, antioxidative

glutathione pool and iron-mediated processes are troubled by metals lacking redox capacity including mercury, cadmium and lead (Pinto et al. 2003). It is presumed that the O_3-mediated ROS production preceding oxidative burst during avirulent pathogen attack mimics the signal transduction pathways (Rao and Davies 2001).

DNA damage, photosynthetic apparatus impairment and membrane lipid disintegrity are some of the drastic effects of UV-B radiation on plant life. Certain morphological adaptations in response to high light environments, especially to enhanced UV-B radiation, include thickening of leaves, stunted growth, development of trichomes, flavonoid biosynthesis and reduced stomatal densities (Gitz and Liu-Gitz 2003). Up- and downregulation of certain genes under UV-B, through which the plants perceive UV-B-specific signal has been presumed earlier, but till now, no such receptor molecule has been recognized (Tippman et al. 2006).

2.2 Biotic Stress

Besides environmental stresses, plants are continuously subjected to latent enemies including pathogenic agents like viruses, bacteria, fungi, nematodes and insects. Nature of attack determines the effect of pathogen. Nutrients from a living plant are mainly pulled up by biotrophic pathogens, while necrotrophic or hemibiotrophic pathogens stimulate cell death in host cells for nutrient uptake (Spallek et al., 2018). Soluble carbohydrates present in the infected parts of the plant get their amounts changed during infection with pathogens. Biotrophs usually manipulate the whole host metabolism in their favour, by making them to accumulate higher concentrations of carbohydrates, resulting in intracellular osmotic stress (Brodmann et al. 2002; Abood and Lösel 2003). The stimulation of pathogen stress responses per se is a cause of stress to the plant, and in turn, disease and defence mechanisms also result in reduced yield (Heil and Bostock 2002). In mutants having constitutively activated defences and elevated resistance to pathogens, the cost of fitness is more apparent. Ultimately, toxin production by necrotrophic and hemibiotrophic pathogens can result in elevated stress level and at long last to cell death (Navarre and Wolpert 1999).

Different types of stresses including various biotic and abiotic stresses can be summarized in an illustration as depicted in Fig. 1.

2.3 Stress Combinations

Combinations of two or more stresses, like salinity and drought, salinity and heat and of drought together with extreme temperature or high light intensity, under natural circumstances are globally common to various agricultural spheres and may affect crop productivity (Suzuki et al. 2014). Present-day climate prediction model points to a stepwise gain in ambient temperature, in near future, along with the

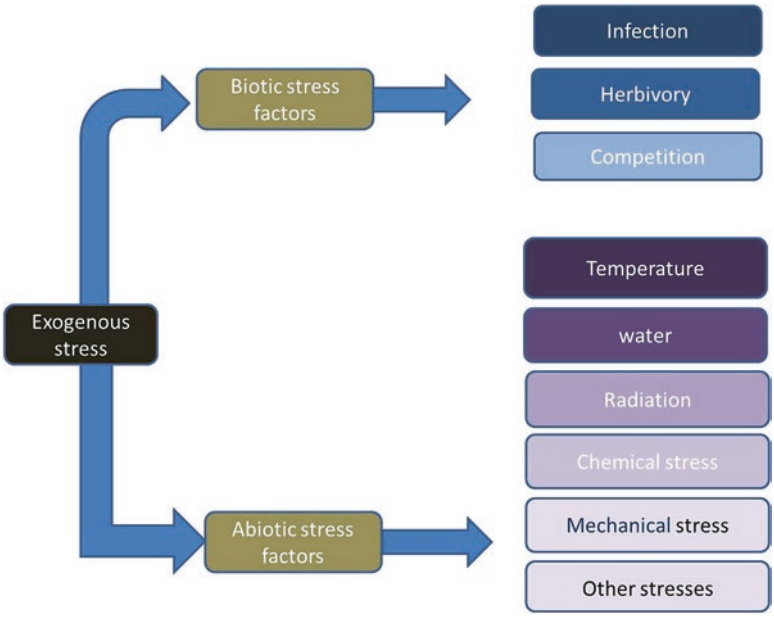

Fig. 1 Stress factors which potentially affect plants survival

increase in the amplitude and frequency of heat stress (Ahuja et al. 2010; Mittler and Blumwald 2010; Mittler et al. 2012; Li et al. 2013). Furthermore, higher temperatures along with the other weather calamities, for instance, severe droughts, are likely to impact global crop production drastically (IPCC 2008). Besides abiotic stresses, infection by pathogens (including viruses, bacteria, fungi and nematodes) and herbivore pest attack to plant world is always a menace (Atkinson and Urwin 2012). Climate changes may inevitably affect habitat range of pathogens and pests. Additionally, defence mechanisms of plants have been shown to get weaker under various abiotic stress conditions in addition to increased susceptibility to various pathogen infestations (Amtmann et al. 2008; Goel et al. 2008; Mittler and Blumwald 2010; Atkinson and Urwin 2012). Hence, major field crops are likely to get exposed to wider range of biotic and abiotic stress conditions and also to their combinations. Combination of drought and heat stress response is unique at molecular level and in no way can directly be extrapolated from individual responses to drought or heat stress (Rizhsky et al. 2002, 2004). Various stress combination responses in plants involving drought, salt, extreme temperature, high light, O_3, CO_2, heavy metals, UV-B, soil compaction and biotic stresses have been revealed (Mittler 2006; Mittler and Blumwald 2010; Alameda et al. 2012; Atkinson and Urwin 2012; Kasurinen et al. 2012; Srivastava et al. 2012; Perez-Lopez et al. 2013; Rivero et al. 2013). Moreover, the co-occurrence of various biotic and abiotic stress responses has

revealed a high degree of complexity, as there are different signalling response pathways that may interact and inhibit each another (Mittler 2006; Atkinson and Urwin 2012; Prasch and Sonnewald 2013; Rasmussen et al. 2013). Abiotic stresses, especially osmotic and ionic stresses, are responsible for the decrease in yield especially in arid and semiarid regions. It is estimated that 45% of the world's agricultural land experience drought and 19.5% of the irrigated land are affected by salinity. These problems will be further catalysed by global climate change (Koyro. et al. 2012).

3 Family Brassicaceae

The Brassicaceae (or cruciferae/mustard family) is considerably a large angiosperm family of dicots belonging to order brassicales with 10–19 tribes comprising of 338–360 genera and 3709 species with a cosmopolitan distribution (Al-Shehbaz, Beilstein and Kellogg 2006; Al-Shehbaz 1973; Appel and Al-Shehbaz 2003; Al-Shehbaz et al. 2006). The International Code of Botanical Nomenclature (ICBN) vide Art 18.5 (Veinna Code) has approved both cruciferae and Brassicaceae as authenticately published and, hence, approved names for the family. The largest genera are *Draba* (365 species) followed by *Lepidium* (230 species), *Erysimum* (225 species), *Cardamine* (200 species) and *Alysum* (195 species). The family comprises of several plant species of great scientific (Hall, Sytsma and Iltis 2002; Koch and Mummenhoff 2006), agronomic and economic significance including model species (e.g. *Arabidopsis* and *Brassica*), developing model systems (e.g. *Brassica* and *Cradamine*) as well as various cultivated plant species (e.g. cauliflower, horseradish, cabbage, turnip, etc.) (Bailey et al. 2006). *Arabidopsis* and *Brassica* species that are very well-known model plants from this very family have revolutionised the whole field of plant biology.

3.1 General Description

Mostly herbaceous plants with annual, biennial and perennial lifespans are included in this family, with only 5% of the plant species typically woody (1–9 m tall lianas) such as *Zilla spinosa*, *Dendralyssum* spp., *Vella* spp. (Al-Shehbaz 1984; Franzke et al. 2011). The leaves are characteristically alternate (rarely opposite), sometimes arranged in basal rosettes, and may be coriaceous and evergreen, which are exstipulate and often pinnately incised. Crushed leaves have a prominent pungent taste. Traits of interest also include presence of thick leaves or leaves with waxes with trichomes. Interestingly, flowers of the family are very uniform throughout. With four diagonally opposite petals in the form of cross, the family name, cruciferae draws its origin. Other important features include tetradynamous condition of stamens (Fig. 2), nectary types, superior ovary,

Fig. 3 Fruit types in Brassicaceae

Fig. 2 A cutaway view of a field mustard flower reveals tetradynamous condition

ebracteate racemose inflorescence, entomogamy pollination and highly variable fruits with variable seed size (Fig. 3). This is the only family having silique or silicules type of fruits. More than 96 glucosinolates have been reported in Brassicaceae giving orders and flavours to crucifers.

3.2 Distribution of Brassicaceae

Brassicaceae is cosmopolitan family, widespread in the southwest Asia, western North America and Mediterranean region. Different taxa of this family are widely distributed in the temperate regions of the Northern Hemisphere and Southern Hemisphere (*Draba*, *Lepidium*, *Cradamine*) and in southern regions as well (e.g. South African Genera: *Heliophila*, *Silicularia*, *Brachycarpa*, *Chamira*, *Schlechteria*) (Koch and Kiefer 2006). However, in mountainous regions and in alpine tropics, limited distribution of Brassicaceae has been seen.

3.3 Characters of Family Brassicaceae

Morphological attributes in the Brassicaceae are extremely homoplasious, virtually making it out of question to employ them alone in establishing phylogenetic relationships on a family-wide footing or even in occasions within genera (Mummenhoff et al. 1997), as amply demonstrated by numerous studies (e.g. Al-Shehbaz 1984; Price et al. 1994; Appel and Al-Shehbaz 2003; Koch et al. 2003; Mitchell-Olds et al. 2005).In the delimitation of taxa at all taxonomic levels, distinctly at ranks of genus and tribe, fruit morphology and seed embryo type (position of the radicle with respect to cotyledons) have considerate importance, while vegetative, floral and trichome characters have often been treated distantly significant. Brassicaceae once thought to be solely stenopalynous (with uniform pollen) with only tricolpate pollen (Erdtman 1972). However, exploratory surveys as conducted by, for example, Rollins et al. (Rollins and Banerjee 1979), showed the existence of several genera with 4–11 colpate pollens also. O'Kane and Al-Shehbaz 2003 later on demonstrated this group with 'polycolpate' pollen to form a monophyletic clade. Floral morphology has received little attention in establishing monophyletic groups in majority of the family. First emphasized by Prantl 1891, trichome morphology, though employed a little less in some of the earlier studies (Rollins and Banerjee 1975, 1976), seems to be much more useful in the delineation of closely related genera (e.g. Al-Shehbaz et al. 1999) and also takes hold of a significant assurance in the delimitation of monophyletic groups. Even though in most major clades of the family, both simple and branched trichomes are found to occur (Beilstein et al. 2006, Bailey pers. com.), the trichome subtypes can serve as a blue chip.

3.4 Origin and Classification

The origin of the Brassicaceae was put forth by Hayek (Hayek 1911), followed by Schulz (1936) and Janchen (1942). They strongly had the belief in a New World origin of the family from the capparaceous subfamily Cleomoideae via the 'basal'

mustard tribe Theylopodieae (Stanleyeae). The German school views were followed by Al-Shehbaz (1973, 1985), Hauser and Crovello (1982) and Takhtajan (1980). However, Dvořák (Dvořák et al. 1973) suggested an Old World origin from the Cleomaceae through the tribe Hesperiidae, but his suggestions were not welcomed. As explicitly demonstrated bimolecular studies (Hall et al. 2002; Koch et al. 2003) and references therein (Mitchell-Olds et al. 2005, Beilstein et al., 2006), Brassicaceae evolved in the Old World and is sister to the Cleomaceae. In spite of the fact Schulz's 1936 classification of the Brassicaceae has been altered and criticized by some people (Janchen 1942; Al-Shehbaz 1973, 1984), its widely use is flourishing on to the present time.

3.5 Economic Importance

The family Brassicaceae serves as a good source of oils, vegetables, weeds, and ornamentals of huge economic importance. Various useful esculent plants such as the colewort, cauliflower, mustard rape, savoy cabbage colza, kale, kohlrabi, broccoli, brussels sprout, cabbage, turnip, etc. belong to the genus *Brassica* along with the other remarkable plants. Chinese cabbage including *Brassica chinensis* (pakchoi) and *Brassica pekinensis* (petsai) are grown as vegetable or salad plants. Locally known as shagsoo, *Christolea* is used as vegetable in combination with milk in addition to the edible *Meeacarpea* species. Species of *Cardamom*, *Nasturtium* and *Lepidium* serve the purpose of salad and garnishing. Seeds of numerous genera of the family are also very important. Mustards (*Brassica nigra* and *Brassica juncea*) are utilized as condiments. The spicy flavour of seeds of *Cardaria draba* makes them more useful than pepper (Jafri 1973). Seeds of *Brassica campestris*, *B. juncea, B. nigra* and *B. pekinensis* are crushed in preparation of edible oils. In different regions, *Camelina sativa*, *Eruca sativa* and *Sinapis alba* which are odiferous are also cultivated as oil plants along with the seeds of *Capsella* which contain about 15–20% oil. Illumination purpose is served by the seeds of *Thlasplarvense* containing 2% oil. The residue left over after oil extraction being rich in proteins is incorporated into animal fodder, in addition to the commercial value of oil and fat. The seeds of *Conringia orientalis* contain fatty oil and its young sprouts are also comestible. Fresh leaves of *Lepidium sativum* are used as salad, and its seeds contain 5% fatty oil, making it worthy for illumination. The leaves of *Physorrhynchus brahuicus* are used as vegetable and are also browsed by camel and goats. Leaves and roots of *Crambe Kotschyana* are also edible, and plant as a whole is a good fodder. *Lobularia maritima* is a pretty rich source of honey. Some species like *Arabis* are cultivated as ornamentals in rock gardens. *Parrya exscapa* grows at high altitudes laced with beautiful flowers. Different pigments from *Isatis tinctoria* serve as a dying agent besides being a honey-producing plant. *Lunnaria annixa* is an ornamental and is used for interior decoration. *Erysimum Perofskianum* seeds serve as crude material for the formulation of cardiac drugs in pharmaceutical industry.

4 Effect of Environmental Stress on Family Brassicaceae

One of the plant families Brassicaceae, also known as Cruciferae, includes some important and diverse genera of cabbage, broccoli, cauliflower, brussels sprouts, kale, etc. which are consumed globally (Podsedek 2007). Resemblance of genetic make-up between *Brassica* and *Arabidopsis* has made it a substitute model plant in science (Abdel-Farid et al. 2007) and has augmented the significance of *Arabidopsis* in plant research. These Brassicaceae vegetables contain high phenolics and glucosinolate levels thereby making this family a good source of antioxidants (Moreno et al. 2006; Bruce and Pickett 2007; Jahangir et al. 2008). These compounds are known to have a preventive role against different types of diseases (Byers and Perry 1992; Verhoeven et al. 1997; Kushad et al. 1999; Moreno et al. 2006). However, presence of some polyphenols, glucosinolates, *S*-methylcysteine sulfoxide, tannins and erucic acid, in Brassicaceae vegetables, reflected its anti-nutritional effects (Griffiths et al. 1998; Lotito and Frei 2006).

Plants are frequently exposed to various biotic and abiotic factors during their growth and development (Zhao et al. 2007) where they respond through activation of their defence system (Hayat et al. 2007). With the result a significant change occurs in their plant metabolome both within and between the subspecies (Eason et al. 2007; Schonhof et al. 2007; Singh et al. 2007; Jahangir et al. 2008). In *Brassica* plants, these different biotic and abiotic stress factors largely affect the primary and secondary metabolism, increasing the metabolite level production, e.g. amino acids, sugars, indoles, phenolics and glucosinolates (Fig. 4) (Moreno et al. 2006; Bellostas et al. 2007; Gols et al. 2007; Petersen et al. 2007).

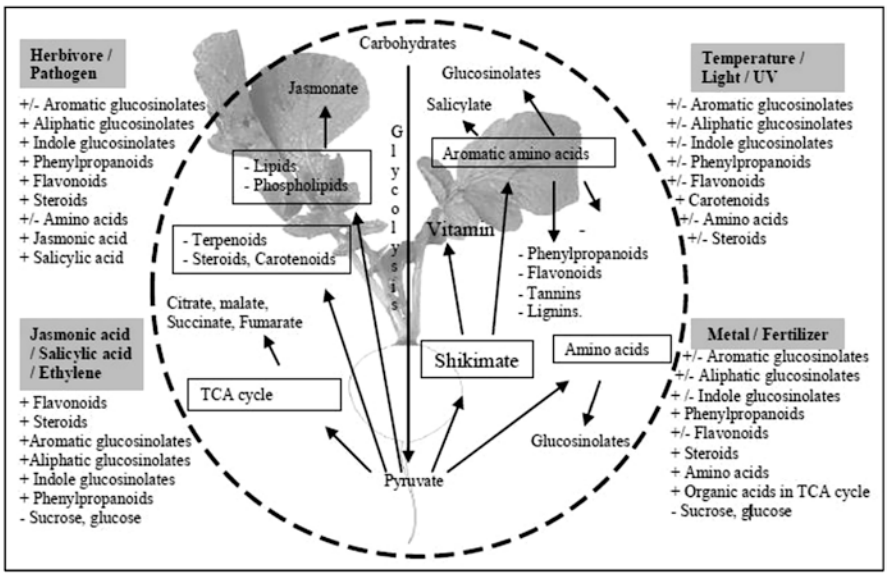

Fig. 4 Diagrammatic representation of biosynthetic pathway for the production of different metabolites during stress

Enhancement of some specific secondary metabolite production during these biotic and/or abiotic stresses has resulted (Sudha and Ravishankar 2002) which activate a number of signal pathways like salicylic acid (SA), jasmonic acid (JA), ethylene or abscisic acid pathways. These pathways are known to have a well-defined role in the plant defence responses (Sudha and Ravishankar 2002; Bruce and Pickett 2007; Zhao et al. 2007).

During biotic stress, the resistance of different plants to their pathogens gets altered in a complex manner (Fig. 4) (Bruce and Pickett 2007). In one such case, *Pieris brassicae* caterpillars, feeding on lower leaves of Brussels sprouts (*Brassica oleracea* var. *gemmifera*), lead to production of some volatile compounds from their upper leaves (Mattiacci et al. 2001). Another such example, where *Arabidopsis* triggers a defence reaction against the pathogen *Alternaria brassicicola*, was inefficient but successfully controlled damage by two bacterial leaf pathogens (*Xanthomonas campestris* pv armoraciae and *Pseudomonas syringae* pv tomato) (Bruce and Pickett 2007).

A plant responds differently to different types of stresses. Since the metabolomic pool of plant is composed of a variety of constitutive and induced metabolites, these responses can be rather more specific (Bouchereau et al. 1996; Chinnusamy et al. 2004; Bruce and Pickett 2007; Pedras et al. 2008). The biotic stress triggers a cascade of signals due to the simultaneous elicitation of several responses. In addition to this, *Arabidopsis* plants are infected by many pathogens at the same time, as observed in the case of a specialist parasitic wasp of *Pieris rapae* caterpillars, *Cotesia rubecula*, which is attracted to *P. rapae* caterpillar due to production of some specific metabolites. These volatiles are produced as a result of outbreak by *P. rapae* involving several major biosynthetic pathways like terpenoids and green leaf volatiles (Van Poecke et al. 2001). Even though being a model plant, *Arabidopsis* is not used as a food source. Nowadays emphasis is given to study alterations in metabolomic profiles in *Brassica* under different environmental stresses, because *Brassica* and *Arabidopsis* more or less share the same genetic composition in addition of serving as the most important global food crop.

Here we will briefly discuss on the interactions between different environmental stresses and *Brassica* species with reference to different metabolites.

4.1 Effect of Stress on Primary Metabolites

Plant metabolite production is largely affected by various abiotic. Drought stress in *Brassica napus* leaves lead to a distinctive linear increase of amino acids, followed by a reduction in concentration upon rehydration of the plants (Good and Zaplachinski 1994). The same stress in cabbage has been proved to increase sugar contents in these plants. Significant sugars levels were detected in drought stresses seedlings as compared to their control ones (Sasaki et al. 1998).

Similarly, metal exposure caused rapid increase in the levels of photosynthetic pigments, proteins, free amino acids and sugar content compared to the unstressed

plants (Singh and Sinha 2005). Cadmium stresses were found to generate ROS production thereby causing oxidative damage in *Arabidopsis* plants resulting in a significant decrease of chlorophyll content (Zawoznik et al. 2007). In *Brassica pekinensis* plants, increased total free amino acid content was observed after exposure to copper stress, where free amino acids are reported to play a role in the detoxification of the copper stress (Xiong et al. 2006). Metal stress is also known to accumulate low-molecular compounds with chelating properties in Brassica (Seth et al. 2008).

Temperature stress also disturbs the metabolite content of plants. After heat stress in Brassicaceae, carotenoids were observed to decrease (Gebczynski and Lisiewska 2006). Kale leaves, a good source of amino acids, after processing were testified to lose 12–14% of amino acid contents as compared to unprocessed ones (Lisiewska et al. 2008). Also a large decrease in the ascorbic acid content was reported after boiling of kale, brussels sprouts, broccoli and white cauliflower (Gebczynski and Lisiewska 2006; Sikora et al. 2008), whereas UV light exposure of broccoli (*Brassica oleracea* var. italica) caused an increased levels of ascorbic acid (Lemoine et al. 2007; Schonhof et al. 2007).

As in case of different abiotic stresses, biotic stress also leads to change in different metabolites in plants. The amino acid content in wildly grown Brassica species are generally lower than cultivated varieties due to diverse factors faced during their cultivation (Cole 1997) as the plants are exposed to different stress conditions. Herbivory and/or pathogen attack triggers a cascade of well-co-ordinated signalling system (Mewis et al. 2005). It is observed that a decrease in the levels of glucose, sucrose, and amino acids are observed after methyl jasmonate (MeJA) elicitation of *Brassica rapa* leaves (Liang et al. 2006). Similarly, infestation by aphids also resulted in increased production of both primary as well as some secondary metabolites (Cole 1997).

4.2 Effect of Stress on Secondary Metabolites

Brassicaceae family is known for some metabolites like glucosinolates, which are derived from amino acid biosynthesis (e.g. methionine, tryptophan, phenylalanine, etc.) (Chen and Andreasson 2001; Bellostas et al. 2007; Podsedek 2007). These compounds are having benefits for human health including anti-carcinogenic, cholesterol-reducing and other pharmacological effects (Moreno et al. 2006; Cieslik et al. 2007; Song and Thornalley 2007) with some known anti-nutritional effects as well (Griffiths et al. 1998). In addition of having nutritional value, they are well known to have an important role in plant defence response mechanisms, which are getting induced after wounding and/or pathogen attack (Doughty et al. 1991; Cole 1997), insect herbivory (Gols et al. 2007; Martin and Muller 2007; Burow et al. 2008), exposure to salt stress (Lopez-Berenguer et al. 2008), other environmental stresses (Rosa et al. 1997; Vallejo et al. 2003) or by plant signalling molecules (Kliebenstein et al. 2002; Mikkelsen et al. 2003), viz. SA, JA and MeJA (Bodnaryk

1994; Mithen 2001). During environmental stress such as drought, secondary metabolism was not restricted, with the result these glucosinolates are also reported to increase under deprived water conditions in mature rapeseed (Lopez-Berenguer et al. 2008; Bouchereau et al. 1996).

Brassicaceae is attributed for a group of naturally occurring plant steroidal compounds, brassinosteroids (BRs), with a broad range of biological activities and the capacity to make these Brassica plants to confer resistance against a wide range of both biotic and abiotic stresses (Krishna 2003), viz. low and high temperatures, water stress, salt stress, pathogen attack (Krishna 2003; Kagale et al. 2007) and heavy metal stress (Janeczko et al. 2005). These steroid compounds not only function as the precursors of brassinosteroids and membrane constituents but are recognised to have an important role in plant development as well (Fujioka and Yokota 2003).

A crosstalk between BRs and different plant hormones is now very much evident (Krishna 2003) For example, 24-epibrassinolide, a brassinosteroid, in *B. napus* increases its tolerance to several environmental stresses such as temperature or drought (Dhaubhadel et al. 1999). Similarly in *A. thaliana* and *B. napus* seedlings, these compounds make them overcome cold stress, besides from making them salt tolerant during their early growth and development (Kagale et al. 2007). In *B. napus*, SA causes induction of steroid sulfotransferase gene, thereby accumulating higher levels of antimicrobial protein, hence responding to pathogen infection by modifying steroid-dependent growth and developmental processes (Rouleau et al. 1999). During Cd stress in radish seedlings, brassinosteroids were able to keep the membrane intact, thus checking ROS production by increasing levels of antioxidant enzyme activities (Anuradha and Rao 2007).

In Brassicaceae plants another kind of compound known as phytoalexins is induced under defence response when subjected to diverse environmental conditions, which can function as signal molecules including poly- and oligosaccharides, proteins, polypeptides, fatty acids (Smith 1996) and jasmonate among others (Liang et al. 2006). Brassicaceae phytoalexins are generally tryptophan derivative compounds in the family Brassicaceae with different chemical as well as biological structures (Pedras et al. 2003b). One of the phytoalexin, Brassinin, with antimicrobial activity, is produced by a variety of Brassica species in response to stress (Pedras and Ahiahonu 2005; Pedras et al. 2007). In addition to these, during plant defence another phytoalexin, camalexin, was found to accumulate in higher concentrations in the tissues where bacterial growth is restricted (Soylu 2006). Previous research proved that biotic and abiotic stresses lead to an enhanced production of different phytoalexins. In continuation with this research, copper chloride sprayed on leaves of oilseed, canola and rapeseed (*B. rapa*) caused accumulation of different types of phytoalexins like spirobrassinin, cyclobrassinin, rutalexin, rapalexin A and B, brassinin, brassilexin and brassianal C, apart from the phytoanticipins, indolyl-3-acetonitrile, caulilexin C and arvelexin (Pedras et al. 2003a, 2008).

Mostly the vegetables from the Brassicaceae family are consumed both raw and after processing them (Kusznierewicz et al. 2008) which are considered as a good source of phenolics. However, the polyphenol content in Brassicaceae not only dif-

fers with the varietal difference but also gets altered when subjected to any abiotic or biotic stress during their growth and development (Dixon and Paiva 1995; Grace and Logan 2000; Podsedek 2007; Sousa et al. 2008).

5 Tissue Culture

Tissue culture is an in vitro culture of cells, tissues, organs or whole plant or plant parts (leaf, stem, root, flower, anther, ovule, etc.) under controlled aseptic conditions. These controlled aseptic conditions include optimum temperature, pH, nutrients and proper light without any microbial infection (Hussain et al. 2012). In plant tissue culture, plant cells and tissues are grown in vitro on artificial nutrient media under aseptic and controlled environment. The technique is based on the totipotency of cells that is the ability of plant cells to express its full genome by cell division, growth and metabolism (Haberlandt 1902; Hussain et al. 2012), by using an artificial nutrient medium composed of micronutrients, macronutrients, vitamins, carbon source (sucrose), plant hormones, gelling agent (for solid medium) and other organic components required for normal growth and development of plant (Murashige and Skoog 1962). The most commonly used nutrient medium for in vitro propagation of most of the plant spp. is Murashige and Skoog medium (MS medium). The pH of the nutrient should be medium 5.4–5.8 for the optimum plant growth and plant hormone activity.

The type and concentration of plant hormones/plant growth regulators (PGR's) are the key determinants of the developmental pathway of plant tissues in culture medium (Hussain et al. 2012). The commonly used PGRs are auxins, cytokinins and gibberellins. Generally the cytokinins lead to cell division and shoot formation. High ratio of auxin to cytokinin leads to root proliferation, while high ratio of cytokinin to auxin results in shoot formation, and the equal ratio of both the auxin and cytokinin leads to the formation of callus (undifferentiated mass of cells) (Rout 2004). In vitro, maximum root formation was recorded in *Stevia rebaudiana* cultured in a medium supplemented with 0.5 mg/l NAA (Rafiq et al. 2007) and maximum shoot proliferation in black pepper present in a medium containing 0.5 mg/l BA (Hussain et al. 2011). Gibberellins are generally used for cell elongation and enhanced plant growth. *Phalaenopsis* orchids showed a maximum shoot length when cultured in a medium supplemented with 0.5 mg/l GA_3 (Hussain et al. 2012). Remarkable achievements that have been made so far in the field of plant tissue culture are shown in Table 1.

Nowadays, many plant species representing either annuals or perennials, monocots or dicots, self-pollinated or cross pollinated or woody or herbaceous are cultured in vitro and regenerated into complete plants. Plant tissue culture provides enormous opportunities for plant propagation, and production of plants with desirable agronomic traits including greater yield, disease resistance, salt resistance, frost tolerance, drought tolerance, herbicide resistance and engineering of plants with desirable characters. Moreover, in plant tissue culture, the cell and tissue cul-

Table 1 Various achievements in plant tissue culture

S. No.	Achievements	Name of researcher/ research group/institute	Year
1.	Embryos cultured from several cruciferous species	Hannig	1904
2.	First discovered plant growth hormone- indole acetic acid	Went	1926
3.	First added coconut milk for cell division in *Datura*	Overbeek	1941
4.	Raised whole plants of *Lupinus* by shoot-tip culture	Ball	1946
5.	First to break callus tissues into single cells	Muir	1954
6.	Regenerated embryos from cell suspension and callus clumps of *Daucus carota*	Reinert and Steward	1959
7.	First isolated protoplast by enzymatic degradation of cell wall	Cocking	1960
8.	Filtered cell suspension and isolated single cells by plating	Bergmann	1960
9.	Developed test tube fertilization technique	Kanta and Maheshwari	1960
10.	First produced haploid plants from anther culture	Guha and Maheshwari	1964
11.	Achieved successful protoplast fusion	Power et al.	1970
12.	Introduced biotransformation in plant tissue cultures	Reinhard	1974
13.	Integrated Ti plasmid DNA from *Agrobacterium tumefaciens* in plants	Chilton et al.	1977
14.	Carried out somatic hybridization of potato and tomato resulting in pomato	Melchers et al.	1978
15.	Carried out intergeneric cytoplasmic hybridization in grape and radish	Pelletier et al.	1983
16.	Developed transgenic tobacco by transformation with *Agrobacterium*	Horsh et al.	1984

tures grown in vitro are extensively used for the production secondary metabolites which are the source of various drugs and pharmaceuticals having a great and significant effect on various animal diseases (Basavaraju 2011). Plant tissue culture includes various techniques like micropropagation, embryo culture, anther culture, protoplast culture and somatic hybridization (Basavaraju 2005). All these techniques are summarized below.

5.1 Micropropagation

Micropropagation is the multiplication of plants through in vitro tissue culture by using a plant part, i.e. explant (leaf, bud, stem, apical meristem, root, floral part, etc.) from a healthy plant, and growing it into whole plant on an appropriate nutrient medium under the controlled conditions of temperature, light and humidity (Basavaraju 2011; Hussain et al. 2012) (Fig. 5). The process involves four major stages that are summarized as under.

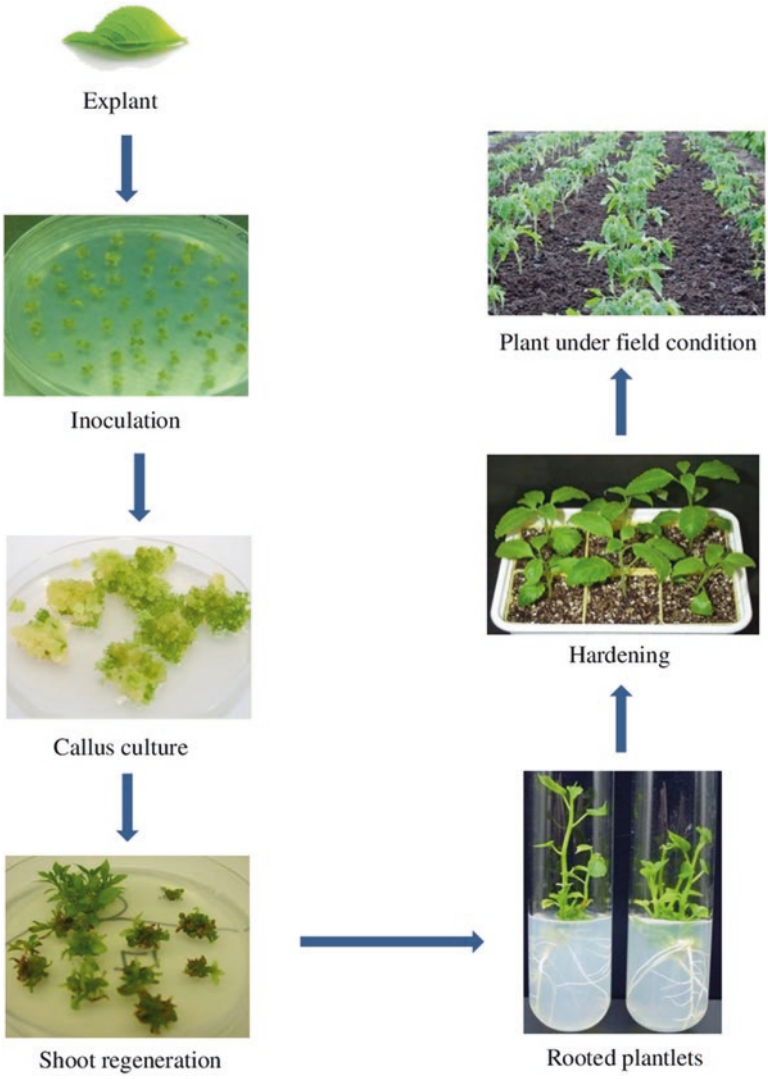

Fig. 5 Flowchart representing micropropagation

I Stage: Initiation Stage

This stage involves the preparation, surface sterilization and inoculation of explant on a nutrient medium. The explant can be obtained from plants cultivated under natural environmental conditions or from ex vitro-grown plants. However, ex vitro-raised plants should be used to reduce the probabilities of contamination and increase the success rate in in vitro culture. Surface sterilization involves the sterilization of explant in chemical by using chemical solutions/disinfectants (sodium hypochlorite, mercuric chloride ($HgCl_2$), calcium hypochlorite, ethanol) and is an

important step to remove various contaminants without causing any damage to plant cells (Husain and Anis 2009; Marana et al. 2009; Tilkat et al. 2009).

II Stage: Multiplication Stage
This stage refers to increase the mass/no. of cells by repeated subculturing of callus/propagules. The subculturing on new medium of same composition is repeated until a desired mass of cells is obtained (Saini and Jaiwal 2002).

III Stage: Rooting Stage
In this stage the callus is transferred to a rooting medium containing plant hormones for induction of rooting and development of a strong root. However, in many cases the rooting takes place on the same culture medium used for callus multiplication.

IV Stage: Acclimatization Stage
This stage involves the hardening of in vitro raised plant. The in vitro raised plant is transferred to a pot containing an appropriate nutrient substrate (sand, soil, compost, peat, etc.) covered with transparent polythene bag having many small holes. In this way the plant is gradually subjected from low to high light intensity and from high to low humidity and hardened in the greenhouse condition. After hardening the plant is ready to grow under natural environmental conditions.

Micropropagation being more advantageous over other conventional methods of propagation leads to rapid multiplication of productive plants at large scale under controlled conditions within a short period of time irrespective of season. Through micropropagation, superior quality plants with high yield, better stress tolerance and disease resistance capacities are obtained (Brown and Thorpe 1995). Virus-free banana plants were obtained by culturing of apical meristem of banana plants devoid of brome mosaic virus (BMV) and banana bunchy top virus (BBTV) (El-Dougdoug and El-Shamy 2011). Micropropagation also meets the increased demand of indoor foliage plants and flowers as now many floriculture companies have set up tissue culture laboratories for the production of foliage plantlets and other high-value ornamentals like rose, lilies, chrysanthemums, gerbera, carnation, gladioli, anthuriums, orchids, spathiphyllum and syngonium.

5.2 Embryo Culture

Embryo culture involves the in vitro growth of immature embryos, seeds or ovules on a nutrient medium to produce successful hybrids (interspecific or intergeneric) of recombinant desirable traits. It reduces the seed dormancy period and breeding cycle of plants by culturing the directly excised embryos. Through in vitro embryo culture, intraspecific hybrids of Jatropha have been produced (Mohan et al. 2011). In vitro embryo culture also leads to the conservation of critically endangered species. *Khaya grandifoliola*, an economically important plant with high timber and medicinal value, has been propagated in vitro by culturing the embryos of its mature seeds (Okere and Adegey 2011). It also leads to the propagation of elite plants from natural forest population. It also produces the interspecific as well as intergeneric hybrids

(rice, cotton, wheat, barley, maize, tomato, legumes, barley rye, wheat rye, etc.) of recombined desirable genes for high yield, resistance to bacterial, fungal, nematodes, pests and diseases in crops like rice, maize, brassica, tomato, etc. (Chawla 2009).

5.3 Anther Culture

Anther culture is the in vitro culturing of anthers for the production of haploid plants with desirable traits. Haploidy was first reported by Guha and Maheswari (1964) in *Datura innoxia*. Haploids being important sources of homozygous lines are used for the production of hybrid lines high-yielding varieties with insect and disease resistance in many crop plants including wheat, rice, rye, brassica, tobacco, etc. Anther culture being a more efficient method develops a pure homozygous line in a time period of less than 2 years compared to the conventional breeding programme which takes about 6–8 years (Chawla 2009).

5.4 Protoplast Culture

Protoplast culture is the in vitro culturing of isolated protoplasts by removing their cell wall and growing them on a nutrient medium. The protoplast in the culture reforms its cell wall and forms callus, and this callus regenerates into a plant by morphogenesis. Different methods are used for protoplast culture.

5.4.1 Agar Plating Method

This method involves the culturing on protoplast on a solid culture medium (Nagata and Takabe 1971). In this method, large number of protoplasts can be handled at the same time. After cell wall regeneration, the agar blocks containing the cultured protoplasts are transferred to a fresh medium containing osmoticum at lower concentration as fresh medium cannot be added to the solid medium. This method was further modified into different techniques as multi-drop method (Potrykus et al. 1979), micro vessel (Button 1978) and feeder technique (Raveh et al. 1973).

5.4.2 Microculture Technique

This technique involves culturing of a small drop of nutrient medium containing one or more protoplasts on a microscopic slide. The method was developed by Jones et al. (1960). This technique is used for the culturing of protoplasts of Petunia and tobacco (Durand et al. 1973).

5.4.3 Hanging Drop Method

This method was developed by Kao et al. (1970) and used by Bawa and Torrey (1971). In this method, a drop of protoplast suspension is placed on a Petri plate, sealed with the parafilm and incubated at 25–30 at low light intensity. The plates are kept in an inverted position. After regeneration of cell wall and cell division initiation, a fresh nutrient medium is added to plates for the formation of cell suspension (Tomar and Dantu 2010).

5.4.4 Multi-Drop Array Technique

This is a modified technique of hanging drop method in which a small plant material is used for screening of large number of nutritional and hormonal factors (Potrykus et al. 1979).

5.4.5 Suspension Culture

It involves the culturing of protoplasts in a liquid medium in Erlenmeyer flask with continuous shaking to provide aeration for sufficient growth. The rpm for shaking should be standardized; otherwise the shaking at fast speed can lead to protoplast burst. For better growth, Ficoll should be added to the culture medium (Vasil 1976).

5.5 Somatic Hybridization

It involves the fusion of two isolated protoplasts of different genotypes and selection of somatic hybrids with desired traits. The process goes through protoplast isolation, protoplast fusion, somatic hybrid selection and regeneration of complete plants from desired somatic hybrids (Fig. 6). The methods used for protoplast fusion are polyethylene glycol, high Ca2+ and electric field (Tomar and Dantu 2010). It is an efficient method for the transfer of genes of desired traits from one species to other and leads to the development of unique hybrid plants by overcoming the sexual incompatibility barrier (Hussain et al. 2012). The technique is used in horticulture to produce the hybrids with high yield and better disease resistance. Motomura et al. (1997) produced successful hybrids by fusion of protoplasts of citrus and other citrinae species. Mostageer and Elshihy (2003) produced salt-tolerant somatic hybrids by fusion of protoplasts of rice and ditch reed. Moreover, in Brassicaceae somatic hybridization is extensively utilized for the production of desired intergeneric hybrid plants (Hussain et al. 2012). By somatic hybridization, the crop plants of more than 400 plant species belonging to 146 genera and 50 families have been obtained including fruits, vegetables, legumes, cereals, medicinal plants and other important crop plants (Basavaraju 2011).

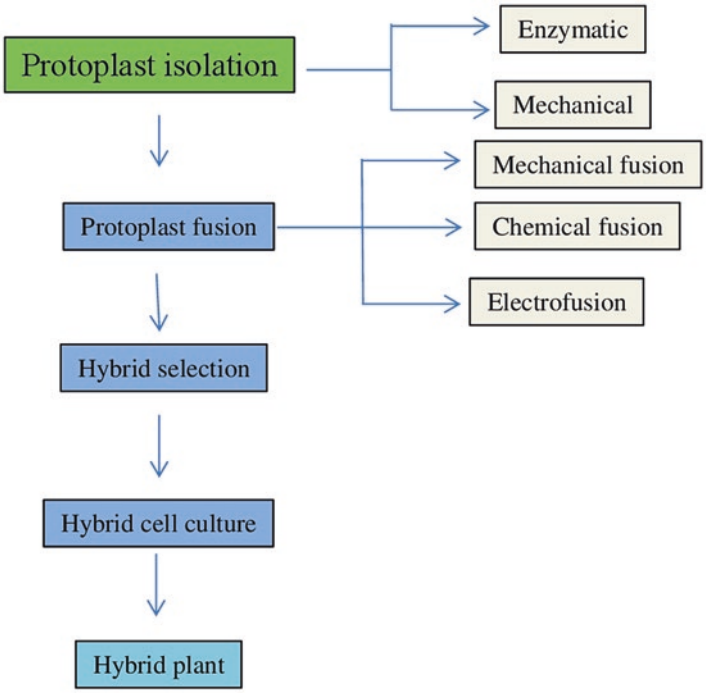

Fig. 6 Schematic representation of somatic hybridization

5.6 Applications of Tissue Culture

In vitro plant tissue culture as an emerging technology and an indispensable tool has great demand and applicability in agriculture and various industries. It leads to the development of biotechnological tools used for crop improvement for sustainable agriculture. Tissue culture produces disease-free crop plant and the demand of the growing world population (Chatenet et al. 2001). Plant tissue culture approach is used in genetic engineering for introgression of genes of stress response and selection of stress-tolerant plants by in vitro selective pressure in culture conditions (Pérez-Clemente and Gómez-Cadenas 2012). In vitro plant tissue culture is extensively used in agriculture and biotechnology.

5.6.1 Haploid Production

In vitro tissue culture technique enables the production of haploids/homozygous plants through protoplast, pollen and anther culture in a short period of time. These haploids are further converted to homozygous diploids by spontaneous or induced

chromosome doubling resulting in the production of double haploids of a pure breeding new cultivar (Basu et al. 2011). Sudherson et al. (2008) developed haploid plants of sturt's desert pea by pollen culture. The technique overcoming the constraints of embryo non-viability and seed dormancy leads to development of inbred lines in plant breeding programmes. The technique has been remarkably used in genetic transformation for the production of haploid plants with disease resistance and high stress tolerance. It leads to production of drought-tolerant double haploid inbred wheat (Chauhan and Khurana 2011).

5.6.2 Genetic Transformation

Genetic transformation involves the transfer of genes of desirable trait into a host plant and recovery of transgenic plant (Hinchee et al. 1994). It can be vector (indirect gene transfer) or vectorless (direct gene transfer) method (Sasson 1993). Vector-mediated Agrobacterium-mediated transformation is most commonly used for the expression of desired genes in plants. Through *Agrobacterium tumefaciens*-mediated transformation, Franklin and Lakshmi (2003) transferred various agronomic traits in *Solanum melongena* using root explants. Direct gene transfer in shoot apices via particle bombardment method has led to the successful production of transgenic plants of Jatropha (Purkayastha et al. 2010). By using marker-free transformation, potato virus Y (PVY)-resistant potato plants were developed by Bukovinszki et al. (2007). By using a multi-auto-transformation (MAT) system, *Petunia hybrida* transgenic plants resistant to *Botrytis cinerea* were produced (Khan et al. 2011).

5.6.3 Germplasm Conservation

The increasing rate of disappearance of plant species worldwide has increased the demand of germplasm conservation. Germplasm conservation is the in vitro cell, organ and tissue culture for the conservation of endangered genotypes (Sengar et al. 2010). It is the preservation of vegetative tissues of crop plants in the form of clones to keep their genetic background and to prevent the loss of conserved inheritance due to natural calamities (Tyagi et al. 2007). The plants producing no seeds or having recalcitrant seeds can be preserved by cryopreservation for maintenance of gene bank. Cryopreservation is the in vitro preservation of cells or tissues in liquid nitrogen with their ability to regenerate into complete plants or reform new colonies (Harding 2004). The fidelity of regenerated plants can be assessed at phenotypic, cytological, histological, biochemical and molecular levels, by various approaches. Cryobionomics is used for the study of fidelity of cryopreserved plant materials (Harding 2010).

5.6.4 Secondary Metabolite Production

Plant tissue culture has been extensively used for the in vitro production of secondary metabolites. Plant tissue culture having high potential for the production of bioactive plant metabolites proved to be an alternative of medicinal compound production from plants (Ramachandra and Ravishankar 2002). Cell suspension cultures and hairy root cultures are used for the production of secondary metabolites. In cell suspension culture, the plant cells are cultured at large scale in bioreactors for the extraction of secondary metabolites. Cell suspension culture is developed by culturing friable callus into a liquid medium maintained under suitable conditions of agitation, aeration, light and temperature (Chattopadhyay et al. 2002). It leads to the continuous secondary metabolites under controlled conditions independent of natural climatic conditions (Karuppusamy 2009). In various cell cultures, a large number of flavonoids, terpenoids, alkaloids, steroids, phenolics, saponins and anticancer drugs are produced (Yesil-Celiktas et al. 2010; Vijayasree et al. 2010). Various secondary metabolites produced by cell suspension culture of different plants are given in Table 2.

In recent decades, hairy root cultures induced by *Agrobacterium rhizogenes* has been used commonly used for the production of secondary metabolites synthesized in plant roots (Palazon et al. 1997). The root cultures being highly stable are used extensively for secondary metabolite production (Giri and Narasu 2000; Pistelli et al. 2010). Hairy root cultures supplemented with optimal nutrient medium lead to the high production of secondary metabolites (Hu and Du 2006). Various hairy root culture produced secondary metabolites of various plants are given in Table 3.

Table 2 Secondary metabolites produced in cell suspension culture

Secondary metabolite	Plant	Reference
Sennosides	*Cassia senna*	Shrivastava et al. (2006)
Vasine	*Adhatoda vasica*	Shalaka and Sandhya (2009)
Azadirachtin	*Azadirachta indica*	Sujanya et al. (2008)
Podophyllotoxin	*Podophyllum hexandrum*	Chattopadhyay et al. (2002)
Stilbenes	*Cayratia trifoliata*	Roat and Ramawat (2009)
Cathin	*Brucea javanica*	Wagiah et al. (2008)
Taxane chinensis	*Paclitaxel Taxus*	Wang et al. (1999)
Sterols	*Hyssopus officinalis*	Skrzypek and Wysokinsku (2003)
Artemisinin	*Artemisia annua*	Baldi and Dixit (2008)
Vincristine Ajmalicine	*Catharanthus roseus*	Lee-parsons and Rogce (2006) Zhao et al. (2001)
Capsaicin	*Capsicum annum*	Umamaheswai and Lalitha (2007)
Berberin	*Coscinium fenestratum*	Khan et al. (2008)

Table 3 Secondary metabolites produced in hairy root culture

Secondary metabolite	Plant	Reference
Rosmarinic acid	*Agastache rugosa*	Lee et al. (2007)
Plumbagin	*Plumbago zeylanica*	Verma et al. (2002)
Deoursin	*Angelica gigas*	Xu et al. (2008)
Withanolide A	*Withania somnifera*	Murthy et al. (2008)
Tropane	*Brugmansia candida*	Marconi et al. (2008)
Rutin	*Fagopyrum esculentum*	Lee et al. (2007)
Glucoside	*Gentiana macrophylla*	Tiwari et al. (2007)
Vincamine	*Vinca major*	Tanaka et al. (2004)
Glycoside	*Panax ginseng*	Jeong and park (2007)
Glycyrrhizin	*Glycyrrhiza glabra*	Mehrotra et al. (2008)
Flavonolignan	*Silybum marianum*	Alikaridis et al. (2000)
Asiaticoside	*Centella asiatica*	Kim et al. (2007)
Anthraquinone	*Rubia akane*	Park and lee (2009)
Resveratrol	*Arachis hypogaea*	Kim et al. (2008)
Silymarin	*Silybum marianum*	Rahnama et al. (2008)

5.7 *Tissue Culture and Stress-Tolerant Plants: Family Brassicaceae*

Nowadays, agriculture production is at a major risk because of the threats imposed by different biotic and abiotic stresses. Therefore, it is the peak time to think and execute our efforts to make stress-tolerant plants for increased crop productivity. For developing stress-tolerant crops, conventional breeding methods are being employed to integrate genes of interest into different crops to induce stress tolerance. However, these breeding methods have limited scope and have failed to provide desirable results (Purohit et al. 1998). On the other hand, tissue culture based in vitro selection has emerged as a possible and cost-effective technique for developing stress-tolerant plants. Plant genetic transformation is a widely used practice for transfer of genes within or between different plant species for the development of stress-resistant crop plants, and considerable efforts have been put together to make this goal achievable by using this technique (Borsani et al. 2003; Yamaguchi and Blumwald 2005). Both abiotic and biotic stress-tolerant crop plants can be acquired by subjecting them to specific agents such as NaCl (salt tolerance), PEG or mannitol (drought tolerance) and different types of phytotoxin or pathogen itself (disease resistance) in their culture media. Those explants which are capable of growing pleasantly in such unfavourable environments and survive in the long run are selected.

5.7.1 Development of Abiotic Stress-Tolerant Plants Through In Vitro Selection

Major abiotic stresses like salinity, drought, water logging, heat, frost and mineral toxicities limit the productivity of most important commercial crops. Development of in vitro salt and drought-tolerant crops has been reported in a wide range of plant species including cereals, vegetables, fruits and other commercially important plant species (Table 4).

Table 4 Screening and in vitro selection of some economically important plants for various abiotic stresses (salt, drought, low temperature, metal and ultraviolet) tolerance

Plant species	Stress	References
Arachis hypogaea (groundnut)	Drought	Purushotham et al. (1998)
Beta vulgaris (sugar beet)	Ultraviolet	Levall and Bornman (1993)
Brassica juncea (Indian mustard)	Salt	Jain et al. (1990)
Brassica juncea (Indian mustard)	Drought	Gangopadhyay et al. (1997)
Brassica napus (rapeseed)	Salt	Rahman et al. (1995)
Brassica oleracea (cauliflower)	Salt	Elavumoottil et al. (2003)
Brassica sp.	Zn + Mn–	Rout et al. (1999)
Capsicum annuum L. (chilli pepper)	Drought	Santos-Diaz and Ochoa-Alejo (1994)
Chrysanthemum morifolium (chrysanthemum)	Salt	Hossain et al. (2007)
Citrus aurantium (sour orange)	Salt	Koc et al. (2009)
Citrus limon (lemon)	Salt	Piqueras et al. (1996)
Citrus sinensis (shamouti orange)	Salt	Ben-Hayyim and goffer (1989)
Cocos nucifera (coconut)	Drought	Karunaratne et al. (1991)
Cymbopogon martinii (Palma rosa)	Salt	Patnaik and Debata (1997a)
Cynodon transvaalensis × *C. dactylon* (Bermuda grass)	Salt	Lu et al. (2007)
Daucus carota (carrot)	Drought	Fallon and Phillips(1989)
Dendrocalamus strictus (bamboo)	Salt	Singh et al. (2003)
Diplachne fusca (kallar grass)	Salt	Nanakorn et al. (2003)
Echinochloa colona (jungle rice)	Cr and Ni –	Samantaray et al. (2001)
Fragaria ×*ananassa* (strawberry)	Salt	Dziadczyk et al. (2003)
Glycine max (soya bean)	Salt	Liu and Staden (2000)
Helianthus annuus (sunflower)	Salt	Davenport et al. (2003)
Hordeum vulgare (barley)	Salt	Ye et al. (1987)
Ipomoea batatas (sweet potato)	Salt	He et al. (2009)
Linum usitatissimum (flax)	Salt	McHughen (1987)
Lycopersicon esculentum (tomato)	Salt	Kripkyy et al. (2001)
Lycopersicon peruvianum (wild tomato)	Salt	Hassan and Wilkins (1988)
Medicago sativa (alfalfa)	Salt	McCoy (1987), Safarnejad et al. (1996)

(continued)

Table 4 (continued)

Plant species	Stress	References
Morus sp. (mulberry)	Salt	Vijayan et al. (2003)
Nicotiana tabacum (tobacco)	Salt	Rout et al. (2008)
Nicotiana tabacum (tobacco)	Cu –	Rout and Sahoo (2007)
Oryza sativa (rice)	Salt	Binh and Heszky(1990), Basu et al. (1997), Shankhdhar et al. (2000) and lee et al. (2003)
Oryza sativa (rice)	Drought/ chillin/Al	Adkins et al. (1995), Bertin and Bouharmont (1997), Jan et al. (1997), Roy and Mandal (2005) and Biswas et al. (2002)
Prunus avium (colt cherry)	Drought	Ochatt and power (1989)
Saccharum sp. (sugarcane)	Salt	Gandonou et al. (2006)
Saccharum sp. (sugarcane)	Drought	Errabii et al. (2006)
Setaria italica (foxtail millet)	Zn –	Samantaray et al. (1999)
Solanum tuberosum (potato)	Salt	Sabbah and Tal (1990), Ochatt et al. (1999) and Queiros et al. (2007)
Solanum tuberosum (potato)	Drought	Sabbah and Tal (1990)
Sorghum bicolor (sorghum)	Drought	Smith et al. (1985), Duncan et al. (1995)
Tagetes minuta (Mexican marigold)	Drought	Mohamed et al. (2000)
Trifolium pratense (red clover)	Cold –	Nelke et al. (1999)
Triticum aestivum (wheat)	Salt	Vajrabhaya et al. (1989), Karadimova and Djambova (1993), Barakat and Abdel-Latif (1996) and Zair et al. (2003)
Triticum aestivum (wheat)	Frost/drought	Dorffling et al. (1993), Barakat and Abdel-Latif (1996), Barakat and Abdel-Latif (1995) and El-Haris and Barakat (1998)
Triticum durum (durum wheat)	Drought	Hasissou and Bouharmont (1994)
Vigna radiata (mungbean)	Salt	Hassan et al. (2008)

Development of Salt-Tolerant Plants

Since the time of human origin on earth, soil salinity existed, but the problem has intensified after agricultural practices such as irrigation and poor drainage systems. In agricultural fields salinity is one of the severe constraints to the crop yield in many regions, and the situation has now become a global concern. Salinity affects around 20% of the irrigated land in the world (Yamaguchi and Blumwald 2005). Furthermore, it has been projected that within next 25 years, salinization in agricultural fields will reduce 30% of the land available for cultivation and up to 50% by the year 2050 (Wang et al. 2003).

Higher salt content in soil or in solutions affects several physiological and biochemical processes, which results in an ion imbalance, mineral deficiency, osmotic stress, ion toxicity and oxidative stress, ultimately damaging several cellular components, including DNA, proteins and lipids in plants (Zhu 2002), restricting growth and development of some important crop plants. The crop protection against salt stress has become a global challenge. Plant responds to salinity by two ways: (a) a quick osmotic phase, which begins instantly once the salt concentration around the roots increases to a threshold level, resulting in a significant reduction in shoot growth, and (b) a slower ion-specific phase, which starts with salt accumulation to toxic levels in old leaves after which they die (Munns and Tester 2008).

In order to overcome this salt stress problem, salt-tolerant crops can be developed that can tolerate high levels of salinity which would be a useful solution for this global problem (Yamaguchi and Blumwald 2005). For developing salt-tolerant plants, different strategies are in progress. In vitro selection procedure and Agrobacterium-mediated transformation are emerging tools for development of such tolerant plants (Hossain et al. 2007). The first report for salt-tolerant crop was *Nicotiana sylvestris* (Zenk 1974). After that series of attempts have been made to make salt-tolerant crops using in vitro techniques (Nabors et al. 1980; Watad et al. 1983; Kumar and Sharma 1989; Vajrabhaya et al. 1989; Binh et al. 1992; Karadimova and Djambova 1993; Olmos et al. 1994; Tal 1994; Barakat and Abdel-Latif 1996; Winicov 1996; Patnaik and Debata 1997a; Patnaik and Debata 1997b; Singh et al. 2003; Zair et al. 2003; Gandonou et al. 2006; Hossain et al. 2007). Different types of systems (callus, suspension cultures, somatic embryos, shoot cultures, etc.) are being used to screen plants in their ability to withstand high levels of salt (NaCl) in media (Woodward and Bennett 2005). In most of the salt studies, NaCl was used; however, researchers have worked with other Cl^- and SO_{2-} salts including KCl, $Na2SO_4$ and $MgSO_4$ during in vitro screening to do a comparative analysis. When *Nicotiana tabacum* was grown in seawater, synthetic seawater, mannitol, NaCl and other Cl^- and SO_2^- salts, a diverse response was observed (Chen et al. 1980).

Development of Drought-Tolerant Plants from Family Brassicaceae

Major losses to the agricultural productivity in arid and semiarid areas are mostly caused due to another type of abiotic stress referred to as drought or water stress. For that reason, drought-tolerant plants can be obtained by in vitro cultures assuming that there is a deep relation between cellular and in vivo plant responses (Mohamed et al. 2000). Previous research has shown that cells exhibiting increased tolerance to water or drought stress has been produced using in vitro selection (Bressan et al. 1981; Harms and Oertli 1985; Sabbah and Tal 1990; Borkird et al. 1991; Barakat and Abdel-Latif 1995; El-Haris and Barakat 1998; Mohamed et al. 2000; Hassan et al. 2004; Errabii et al. 2006). Several researchers have used different osmotic stress agents like polyethylene glycol (PEG), sucrose, mannitol or sorbitol for in vitro selection. But, PEG has been found to be more effective to stimulate water stress in plants. It is a high molecular weight compound, lowering the osmotic

potential of nutrient solutions without being taken up or being phytotoxic (Hassan et al. 2004). Like salt-tolerant plants, the drought-tolerant plants are also characterized by accumulation of higher levels of compatible solutes mainly proline as well as increased anti-oxidative enzyme activities.

In addition to salt and drought stress, other reports for the development of economically important crops tolerant to other abiotic stresses (metal, chilling, UV and frost) have also been raised through in vitro selection (Table 4).

5.7.2 Development of Biotic Stress-Tolerant Plants Through In Vitro Selection

Annually the yield of many economically important crops is reduced due to their susceptibility to different biotic stresses like various fungal, bacterial and viral pathogens. Pathogen control by using different chemicals is difficult, costly and laborious (Bezier et al. 2002). Also these chemicals being non-biodegradable can cause heavy environmental pollution, reducing soil productivity and a bigger threat to flora and fauna of different water bodies. Therefore, the need of the hour is to develop some novel techniques to produce disease-tolerant plants. One of the effective approaches is in vitro selection for developing stress-tolerant lines (Jayashankar et al. 2000; Ganesan and Jayabalan 2006). In vitro selection through enhanced expression of pathogenesis-related (PR) proteins, antifungal peptides or biosynthesis of phytoalexins is an important tool for desirable plant selection (Ganesan and Jayabalan 2006; Kumar et al. 2008). This technology is having an upper hand over transgenic approach for developing improved disease-tolerant crops (Jayashankar et al. 2000). Developing pathogen resistance through in vitro selection can be carried out using organogenic or embryogenic calli, shoots, somatic embryos or cell suspensions. By exposing these cultures to different toxins produced by various plant pathogens, tolerant plants can be raised (Kumar et al. 2008).

First report for developing in vitro selection for disease resistance in tobacco for *Pseudomonas syringae* was first reported by Carlson (1973). Since then, different plant lines resistant to multiple fungal, bacterial and viral pathogens have been developed for many species (Gengenbach et al. 1977; Behnke 1979; Brettell et al. 1980; Hartman et al. 1984; Chawla and Wenzel 1987; Hammerschlag 1988; Vidhyasekaran et al. 1990; Jayasankar and Litz 1998; Jayashankar et al. 2000; Fuime and Fuime 2003; Krause et al. 2003; Gayatri et al. 2005; Ganesan and Jayabalan 2006; Kumar et al. 2008). Most commonly pathogen culture filtrate and phytotoxins are used by researchers for in vitro selection and regeneration of disease-resistant crop plants (Kumar et al. 2008). Selection by means of in vitro methods in which host-specific phytotoxin is added such as fusaric acid and pathogen produced non-specific phytotoxins, i.e. deoxynivalenol (DON), crude pathogen culture filtrate or sometimes the pathogen itself to the growing media has been reported to increase the rate of resistant plants compared to the tissue cultures raised without selection (Gayatri et al. 2005).

Using phytotoxin during in vitro selection has also been carried out by other several workers. Cell suspension cultures of 'Peter Pears', a cultivar of *Gladiolus* × *grandiflorus* (Hort.), susceptible to the fungus *Fusarium oxysporum* f. sp. gladioli (Mass.), have been selected against fusaric acid, one of the toxins produced by this pathogen (Remotti et al. 1997). Similarly, two barley genotype calli were used for selection of resistance using fusaric acid (Chawla and Wenzel 1987). Resistant lemon 'mal secco' were produced by screening embryogenic cultures of nucellar origin against a partially purified phytotoxin produced by *Phoma tracheifila* (Gentile et al. 1992, 1993). Through in vitro selection *Triticum aestivum* L., tolerant plantlets to *Fusarium graminearum* were successfully screened using deoxynivalenol (Yang et al. 1998). Tobacco plants resistant to tobacco mosaic virus were selected in vitro using callus lines infected with tobacco mosaic viruses itself (Table 5).

Table 5 Screening and in vitro selection of some economically important plants for biotic stress

Plant species	Resistant against pathogen	References
Arachis hypogaea (groundnut)	*Cercosporidium personatum*	Venkatachalam and Jayabalan (1996)
Brassica napus (rapeseed)	*Phoma lingam*	Sacristan (1982)
Carthamus tinctorius (safflower)	*Alternaria carthami*	Kumar et al. (2008)
Citrus limon (lemon)	*Phoma tracheiphila*	Gentile et al. (1992, 1993)
Curcuma longa (turmeric)	*Pythium graminicolum*	Gayatri et al. (2005)
Gladiolus grandiflorus (gladiolus)	*Fusarium oxysporum*	Remotti et al. (1997)
Glycine max (soya bean)	*Septoria glycines*	Song et al. (1994)
Gossypium hirsutum (cotton)	*Fusarium oxysporum, Alternaria macrospora*	Ganesan and Jayabalan (2006)
Hordeum vulgare (barley)	*Fusarium* sp.	Chawla and Wenzel (1987)
Linum usitatissimum (flax)	*Fusarium oxysporum*	Krause et al. (2003)
Lycopersicon esculentum (tomato)	*Pyrenochaeta lycopersici*	Fuime and Fuime (2003)
Mangifera indica (mango)	*Colletotrichum gloeosporioides*	Jayasankar and Litz (1998)
Medicago sativa (alfalfa)	*Fusarium oxysporum*	Hartman et al. (1984)
Musa sp. (banana)	*Fusarium* sp.	Matsumoto et al. (1995)
Nicotiana tabacum (tobacco)	*Pseudomonas syringae, Pseudomonas and Alternaria, Tobacco mosaic virus* (TMV)	Carlson (1973) Thanutong et al. (1983) Toyoda et al. (1989)
Oryza sativa (rice)	*Helminthosporium oryzae*	Vidhyasekaran et al. (1990)
Prunus persica (peach)	*Xanthomonas campestris*	Hammerschlag (1988)
Saccharum sp. (sugarcane)	*Colletotrichum falcatum*	Sengar et al. (2009)
Solanum tuberosum (potato)	*Phytophthora infestans*	Behnke (1979)
Triticum aestivum (wheat)	*Fusarium* sp., *Fusarium graminearum*	Maier and Oettler (1992) Yang et al. (1998)
Vitis vinifera (grapes)	*Elsinoe ampelina*	Jayashankar et al. (2000)
Zea mays (maize)	*Helminthosporium maydis*	Gengenbach et al. (1977)

6 Conclusion

The plant growth development and yield are highly affected by environmental conditions and different pathogens. Among plants, most of the commercially important crops are known to be vulnerable to most of these stresses. *Brassicaceae* being one of the plant families, severely affected by both biotic and abiotic stress, needs to be protected against these stresses by using tissue culture technique. Through tissue culture techniques, various biotic and abiotic stress-tolerant lines of *Brassicaceae* with improved tolerance to both types of stresses along with better yield have been developed in a small space within a short period of time. These lines are further field-tested for confirmation of their genetic stability under field conditions. Therefore, in vitro selection technique along with molecular approaches provides new opportunities for improving stress tolerance in family *Brassicaceae* for environmental sustainability.

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Somatic Embryogenesis, In Vitro Selection and Plantlet Regeneration for *Citrus* Improvement

Krishan Kumar, Manav Indra Singh Gill, and Satbir Singh Gosal

Abstract Citrus is the most widely cultivated fruit crop across the globe. The commercially cultivated varieties and rootstocks are the adapted genotypes and need improvement for one or few traits. Such targeted improvement though the conventional breeding approaches is hindered by the complex biological attributes of *Citrus* such as polyembryony and sterility and sometimes sexual incompatibility with the donor sources. The cell- and tissue culture-based techniques such as somatic embryogenesis and in vitro selection are vital for the improvement of *Citrus*. The somatic embryogenesis facilitates improvement of *Citrus* through somatic hybridization and genetic transformation. Somatic hybridization has been used for the genetic improvement of both scion and rootstocks. Somatic hybridization-mediated scion improvement involves production of superior allotetraploids that can be used for crossing with diploid elite varieties to develop seedless triploids. The development of cybrids by combining cytoplasmic genome from Satsuma mandarin and nuclear genome from desired varieties is also an attractive approach. In the rootstock improvement, somatic hybridization targets packaging of resistance to different biotic and abiotic stresses in a single rootstock. The allotetraploid somatic hybrids reduce tree size and can improve adaptability of the composite trees by imparting resistance to different stresses. The in vitro selection has been used for improvement against biotic (mal secco, *Phytophthora*-induced diseases and canker) and abiotic stresses (salt, cold and Al toxicity) in *Citrus*. Techniques like shoot-tip grafting have shown promise for restoring the true yield potential of citrus cultivars by cleansing them from infectious virus and virus-like diseases.

Keywords *Citrus* · Somatic embryogenesis · Crop improvement · In vitro selection · Micropropagation · Shoot-tip grafting

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1 Introduction

Globally, *Citrus* is a very important fruit crop with total world production of over 135 million tons (FAO 2014). It is grown across six different continents with the highest acreage concentrated between 40° north and south of the equator. The modern *Citrus* trees are composed of scion and rootstock. Sweet orange, mandarin, grapefruit, lime and lemon are the major group of cultivated scion varieties. From each group, only a few varieties are preferentially cultivated over the world. For instance, among sweet oranges, Valencia Late, Hamlin and Pera are the main cultivars in the top orange-producing countries like Brazil and the USA. In India, the *Citrus* production is primarily based on the mandarins (Nagpur mandarin and Kinnow mandarin) and sweet oranges (Mosambi and Sathgudi). The improvement is required for one or few traits in the scion varieties. For instance, seedlessness is the most desirable breeding trait for fresh and juice purpose *Citrus* varieties. In case of lemons, resistance to mal secco and seedlessness are important breeding traits (Gulsen et al. 2007). Different rootstocks are being used to suit to the different agroclimatic conditions. In India, rough lemon and Rangpur lime are the most widely used rootstocks (Sonkar et al. 2002; Kumar et al. 2010). The Brazilian *Citrus* industry thrives on Rangpur lime (Tazima et al. 2013). The US *Citrus* growers use trifoliate hybrids like Carrizo citrange and Swingle citrumelo as rootstocks. In China, Trifoliate orange is the most regularly used rootstock (Guo et al. 2002). However, none of the cultivated rootstock contains all the desired attributes. For instance, rough lemon and Rangpur lime are susceptible to *Phytophthora*-induced diseases and blight (Mendes et al. 2001). Trifoliate orange and citranges have resistance to *Phytophthora* but are sensitive to salts. Sour orange is resistant to *Phytophthora* (Mourao Filho et al. 2008) and *Citrus* blight (Mendes et al. 2001) but is susceptible to *Citrus tristeza* virus.

The genetic improvement through conventional breeding is impaired by various biological barriers such as polyembryony, incompatibility, sterility and long juvenile periods (Gong and Liu 2013). The conventional breeding is also of limited value when the resistance is not present in sexually compatible genera. For instance, the resistance against greening is present in *Murraya paniculata*, a remote genera, which is sexually incompatible with *Citrus* (Guo and Deng 1998). The cell-based techniques like somatic hybridization and genetic transformation are the attractive alternates to the conventional breeding for crop improvement. The technique of somatic hybridization is helpful in scion improvement through production of superior allotetraploids and cybrids (Grosser and Gmitter 2011). Somatic hybridization technique also allows the transfer of genes from the sexually incompatible donors and, therefore, is capable of overcoming the species and genera barriers. Somatic embryogenesis is a prerequisite for regeneration of somatic hybrids and cybrids. It also facilitates the regeneration of genetic transformants derived from embryogenic cultures. Somaclonal variation and in vitro mutagenesis are the other important tissue culture-based techniques that facilitate improvement in one or few traits of a genotype without disturbing the rest of the genome (Predieri 2001). These techniques

can be helpful in identifying variants and mutants tolerant to various biotic and abiotic stresses. The regenerative technique of in vitro shoot tip grafting can cleanse the planting material of virus and virus like diseases. The techniques of somatic embryogenesis, in vitro selection and plant regeneration; factors affecting their success and application in the improvement of *Citrus*, have been discussed in the chapter.

2 Somatic Embryogenesis

Somatic embryogenesis is the process by which a bipolar structure, resembling a zygotic embryo, is produced from a somatic cell (von Arnold et al. 2002). Somatic embryo is developed and differentiated from the somatic cell without a vascular connection from the original tissue (von Arnold et al. 2002). In terms of developmental pattern, somatic and zygotic embryos follow similar pathway and typically pass through globular, heart, torpedo and cotyledonary stages in dicot plants (Mordhorst et al. 1997). The process of embryogenesis is under gene regulation. To date, several genes related to somatic embryogenesis have been revealed; notable among them are *Citrin*, a globulin protein (Koltunow et al. 1996); two *SERK* genes, *CitSERK1* and CitSERK1-*like* (Shimada et al. 2005; Ge et al. 2010); *CsHpt1* gene encoding for a phospho-transmitter protein (Maul et al. 2006); and 11 candidate genes spanning over an 80 Kb region (Wang et al. 2017). The ovular origin explants (ovule and nucellus) in *Citrus* are the most responsive for somatic embryogenesis. It has been observed that the proteins expressing in the polyembryonic seeds are also present in abundance in the somatic embryos (Koltunow et al. 1996). The nucellar cells which tend to form nucellar embryos develop as embryo initial cells at the time of anthesis, indicating that the genes regulating the nucellar embryogenesis start expressing at this stage (Koltunow 1993). Wang et al. (2017) recently have provided a deep insight about the process of apomixes through genetic, genomic and transcriptomic analysis of polyembryonic and monoembryonic *Citrus* types. The phenotype and genotype segregation pattern of the F_1 population individuals derived from a cross between monoembryonic (pummelo) and polyembryonic (Fairchild mandarin) varieties revealed that polyembryony is governed by a single dominant gene. It was found that an 80 Kb region containing 11 candidate genes on chromosome 4 primarily controls the polyembryony and the *CitRWP* gene expressing in the ovule was directly correlated with the polyembryony. The co-segregation of a miniature inverted-repeat transposable element (MITE) insertion (in *CitRWP* gene) with the polyembryonic phenotypes indicates dominant gene mutation model for polyembryony and predicts the evolution of polyembryony from monoembryony in *Citrus*. Of the other genes, the *AGL2*, *EDA24* and *ACR4* genes that control zygotic embryogenesis are also found expressing at high level in polyembryonic *Citrus* species (Wang et al. 2017), which indicates that the genes controlling the sexual and asexual embryogenesis are common. The process of somatic embryogenesis can be divided into two parts: induction of somatic embryos and maturation and germination of somatic embryos.

2.1 Induction of Somatic Embryos

Somatic embryogenesis has been achieved by two approaches: direct embryogenesis and indirect embryogenesis. Under direct somatic embryogenesis, the somatic embryos are directly initiated from the explant without an intermediate callus phase, whereas the indirect somatic embryogenesis involves an intermediate callus phase and proembryogenic masses (PEM) preceding somatic embryo formation (Guan et al. 2016). However, majority of the studies in *Citrus* deal with indirect somatic embryogenesis. In this mode, the callus is initiated from the cultured explants. The calli which are set to form embryos are called embryogenic calli and can be distinguished morphologically from the counterpart non-embryogenic calli. The embryogenic callus in *Citrus* is generally friable and creamy white in colour (Carimi et al. 1995; Carra et al. 2006; Chiancone et al. 2006; Benelli et al. 2010). The embryos in such callus are formed usually from the peripheral callus layers but sometimes also develop internally from callus masses (Benelli et al. 2010). The non-embryogenic calli are of compact nature and green in colour (Chiancone et al. 2006). Even after induction of somatic embryogenesis, the transitional competency varies in *Citrus*. Moiseeva et al. (2006) while studying somatic embryogenesis in Tarocco sweet orange (*C. sinensis*) found that the embryogenic cells exhibit highest amenability to form globular embryoids (64%) and further morphogenic transition to the heart (40%) and ultimate somatic embryo (20%) declined progressively. Various factors such as genotype, explant, nutrient media, growth regulators, carbohydrates and growth adjuvants influence induction of somatic embryos. All these factors are covered in detail in subsequent subsections.

2.1.1 Genotype

The response for somatic embryogenesis seems to be under the influence of genotype. The style-derived calli of *Citrus aurantium*, *C. paradisi* cv. Marsh Seedless, *C. deliciosa* cv. Tardivo di Ciaculli and *C. sinensis* (cultivars Brasiliano, Sanguinello and Valencia) differentiated into somatic embryos on culture medium containing 13.3 μM BAP, whereas *C. deliciosa* cv. Avana and *C. paradisi* cv. Star Ruby did not initiate somatic embryos under similar set of conditions (Carimi et al. 1995). Likewise, Tomaz et al. (2001) reported the response of calli of *Citrus* genotypes for somatic embryogenesis. The genotypes could be divided in two groups in terms of their response to the carbohydrate. The Valencia, Caipira sweet oranges and a callus line of Cleopatra mandarin I were highly embryogenic, while the Rangpur lime and Cleopatra mandarin II exhibited poor embryogenic response (Tomaz et al. 2001). The genotypic differences for induction of somatic embryogenesis are not confined to the genus *Citrus* but are also apparent in other genera of the subfamily Aurantioideae. Jumin and Nito (1996) studied the somatic embryogenesis from the protoplast in six different genera *Fortunella polyandra*, *Atalantia bilocularis*, *Hesperethusa crenulata*, *Glycosmis pentaphylla*, *Triphasia trifolia* and *Murraya koenigii* related to *Citrus*. The *F. polyandra* showed the higher embryogenic response, while the *A. bilocularis* showed the lowest embryogenic potential.

2.1.2 Explant

The explants constitute the second most important factor in realizing the potential of a *Citrus* genotype for somatic embryogenesis. The ovular origin explants (ovule and nucellus) perform better than the non-ovular-type explants for somatic embryogenesis (Fiore et al. 2002; Savita et al. 2015). In the polyembryonic species, the epicotyls derived from nucellar seedlings have also proved to be an ideal option for somatic embryogenesis (Gill et al. 1994; Gill et al. 1995). In the monoembryonic species, the use of floral organs like style, stigma and ovary can be an ideal option (Carimi et al. 1999; Fiore et al. 2002; Cardoso et al. 2012). The ovular explants usually induce direct somatic embryogenesis (Gmitter and Moore 1986; Carimi et al. 1998), while the non-ovular explants undergo indirect somatic embryogenesis with an intermediate callus phase (Carimi et al. 1998). Embryogenesis response in the ovular explants and zygotic embryos usually has been reported to increase with ovule age (Moore 1985; Perez et al. 1998; Kiong et al. 2008). Contrary to ovular origin explants, the success of somatic embryogenesis was more in juicy vesicles (a non-ovular explant), when excised from the young (10 days old) fruits (Nito and Iwamasa 1990). The somatic embryogenesis response of a genotype is dependent upon the explant. Carimi et al. (1998) used thin cell layers of style, stigma and ovary for *C. limon*, *C. medica*, *C deliciosa*, *C. tarvida*, *C. sinensis* and *C. madurensis*. The maximum embryogenic potential for *C. limon* and *C. medica* was obtained from the stigma- and style-derived thin cell layers, while the ovary-derived thin cell layers were the best explants for *C. deliciosa* and *C. tarvida*. In *C. madurensis*, the stigma- and ovary-derived thin cell layers were the suitable explants for somatic embryogenesis, while the *C. sinensis* responded only for stigma-derived thin cell layers. The type of explant also decides the normalcy of developed embryos. The use of anthers as explants leads to as much as 30% abnormal embryos (Benelli et al. 2010). The explant origin also affects the clonal fidelity of the regenerated plantlets. Somatic embryo-derived plantlets from the ovary show as much as 20% variation, whereas those derived from the nucellus were true to type (Savita et al. 2015).

2.1.3 Nutrient Medium

The basal nutrient medium supplies all the necessary mineral elements required for the growth of the cells or tissues. The MS (Murashige and Skoog 1962), MT (Murashige and Tucker 1969), WPM (Woody Plant Medium) (Lloyd and McCown 1980) and N_6 (Chu 1978) are the commonly used medium for *Citrus* somatic embryogenesis. Koc and Can (1992) found Murashige and Tucker (MT) medium superior over Murashige and Skoog (MS) medium for initiation of somatic embryos from the hypocotylar callus of *C. aurantium*. Song et al. (1991) found that out of KNO_3 and NH_4NO_3, the NH_4^+ source of nitrogen was more important for induction of somatic embryos in *C. grandis*. Cardoso et al. (2012) found that N_6 medium was more responsive than WPM for induction of somatic embryogenesis in pistil explants of sweet orange cv. 'Tobias'. The N_6 medium though has lower nitrogen content than the WPM but has higher nitrate to $NH4^+$ ratio 4.3:1 versus 2.1:1.

However, in their study, N_6 medium contained the galactose and lactose as carbon source, while the WPM possessed the sucrose as energy source. The percent embryogenesis response was improved, when the callus induced on WPM medium was subcultured to N_6 medium for induction of embryos.

2.1.4 Growth Regulators

The somatic embryogenesis system of *Citrus* differs from the model plant, *Daucus carota*. In the model plant, the presence of auxins especially 2,4-D in the medium is essential for the expression of somatic embryogenesis, whereas the reduced auxin concentration promotes embryogenesis and embryo development in *Citrus* (Kochba et al. 1978). Cytokinins alone or in combination with auxins have been used for somatic embryogenesis in *Citrus*. The cytokinin activity is a property of two types of substances: N^6-substituted adenines like BA or BAP (Shaw 1994) and the synthetic phenylurea derivatives like CPPU, 2,3- MDPU and 3,4- MDPU (Shudo 1994). The embryogenesis has been demonstrated with both types of cytokinins. The adenine-derived cytokinin BAP has been used at 13.3 μM (Carimi et al. 1995; Carimi et al. 1998; Carimi et al. 1999) or 4 mg l^{-1} (Savita et al. 2015) for inducing somatic embryogenesis in *Citrus.* The type of cytokinin and its concentration required for somatic embryogenesis vary with the species. Carra et al. (2006) evaluated both BAP (adenine-derived cytokinin) and diphenylurea derivatives N-(2-chloro-4-pyridyl)-N-phenylurea (4-CPPU), N-phenyl-N′-benzothiazol-6-ylurea (PBU) and N,N′-bis-(2,3-methilendioxyphenyl)urea (2,3-MDPU) at 4 and 12 μM for somatic embryogenesis in *C. limon*, *C. madurensis* and *C. myrtifolia*. PBU (4 μM) was able to induce somatic embryogenesis in all the three species, while for the best response, the requirement varied as per the genotype. BAP at 4 and 12 μM, respectively, was the best for *C. madurensis* and *C. myrtifolia*. The best embryogenic response in *C. limon* was observed with 4 μM PBU. The phenylurea derivatives exhibit higher cytokinin activity and induce higher per cent embryogenesis and number of somatic embryos per explant in Calamondin (Siragusa et al. 2007). However, the use of phenylurea derivatives specially PBU, CPPU and 2,3 MDPU also induces the somaclonal variation in the regenerated plants (Siragusa et al. 2007). The auxins NAA (10 mg l^{-1}) + kinetin (0.5–1 mg l^{-1}) along with 10× MS vitamins have been used for inducing somatic embryogenesis response in *C. reticulata* cv. Local Sangtra (Gill et al. 1995). Ling and Iwamasa (1997) induced somatic embryogenesis from the immature seeds in wild relatives of *Citrus* (*Aegle marmelos*, *Atalantia ceylanica*, *Citropsis gabunensis*, *Clausena excavata*, *Glycosmis pentaphylla*, *Microcitrus australasica*, *Murraya paniculata* and *Severinia buxifolia*) on MT medium supplemented with 400 mg l^{-1} malt extract and plant hormones (0.05 mg l^{-1} 2,4-D and 0.05 mg l^{-1} BA). However, Nito and Iwamasa (1990) induced somatic embryos in the juice vesicle-derived callus on medium containing 1 mg l^{-1} NAA alone, which probably reflects the different requirement of explants for somatic embryogenesis. Fiore et al. (2002) supplemented the medium with 2,4-D and CPPU at the concentration of 0, 0.4, 4 and 12 μM for expression of

somatic embryogenesis from the style and stigma thin cell layer (tcl)-derived callus. In general, 2,4-D at either concentration inhibited the somatic embryogenesis, while the CPPU supported somatic embryogenesis with best results at 4.0 μM. Similar were the observations of Savita et al. (2015), and they observed induction of somatic embryogenesis only with BA 4 mg l^{-1}. The inhibitory impacts of the 2,4-D could be due to the osmotic stress induced by this growth regulator (Pan et al. 2010). The essentiality of the cytokinins for expression of somatic embryogenesis was proven by the study of Jimenez et al. (2001), where they recorded a significantly higher level of endogenous cytokinins in the calli cultured on glycerol in comparison with sucrose. The glycerol is known to stimulate somatic embryogenesis in *Citrus*. Growth inhibitors, namely, ABA and ethephon, improved the embryogenic response of the ovular calli of Shamouti orange in a concentration-dependent manner. The ABA (0.04–20 μM) and ethylene (0.1 mg l^{-1}) improved embryo development. The gibberellic acid inhibits the somatic embryogenesis, while growth retardants like alar and CCC (0.01–10.0 mg l^{-1}) promote the embryogenesis in *Citrus*. The promotive role of the growth retardants is probable due to the gibberellic acid antagonism.

2.1.5 Carbohydrates Type

The exogenous supply of the carbohydrates in the nutrient medium serves as energy and carbon source (George 1993). Carbohydrate is also an important component of the medium for inducing embryogenesis in *Citrus*. The carbohydrate compounds mainly sucrose (Perez et al. 1998; Ricci et al. 2002; Kayim and Koc 2006; Gill et al. 1995), maltose (Perez et al. 1998; Tomaz et al. 2001), lactose (Kochba et al. 1982; Perez et al. 1998; Ricci et al. 2002; Kayim and Koc 2006), galactose (Kochba et al. 1982; Tomaz et al. 2001; Ricci et al. 2002; Kayim and Koc 2006), mannitol (Kayim and Koc 2006), sorbitol (Kayim and Koc 2006) and glycerol (Kayim and Koc 2006; Wu et al. 2009) have been tested to produce somatic embryos in *Citrus*. There are limited reports where sucrose has promoted the differentiation of somatic embryos. In general, in the growth regulator-free media, the substitution of sucrose with other carbohydrate sources especially glycerol (Kayim and Koc 2006; Wu et al. 2009), galactose or galactose-releasing carbohydrates (Perez et al. 1998; Tomaz et al. 2001) and maltose (Perez et al. 1998; Tomaz et al. 2001) has induced somatic embryogenesis. The type and concentration of these carbohydrates for eliciting the embryogenesis response are dependent upon the genotype. Glycerol in the range of 2–5% has been found to be effective for induction of somatic embryogenesis (Kayim and Koc 2006; Wu et al. 2009). Galactose has been reported to be toxic to some species like sugarcane (Maretzki and Thom 1978), but it stimulates somatic embryogenesis in *Citrus* (Cabasson et al. 1995). Lactose, a galactose-releasing sugar at 15 mM for Satsuma mandarin (Kunitake et al. 1991), 37 mM for Cravo mandarin (Ricci et al. 2002), 37–75 mM for Valencia sweet orange (Tomaz et al. 2001; Ricci et al. 2002), 75 mM for Ponkan and Kinnow mandarin (Ricci et al. 2002) and 110 mm for Cleopatra (Tomaz et al. 2001) in the medium has induced

somatic embryogenesis. The galactose itself at the concentration of 110 mM in the medium induced maximum embryogenesis in 'Itaborai' sweet orange. Maltose at 0.5% for sweet orange cvs. Pineapple and Salustiana (Perez et al. 1998) and at 150 mM (approximately 5.1%) for Valencia sweet orange (Tomaz et al. 2001) proved effective for induction of somatic embryos. The stimulatory impact of glycerol on somatic embryogenesis is due to their involvement in cell metabolism. Glycerol is involved in the polyamine metabolism (Wu et al. 2009) and also regulates the level of cytokinins in the cultured calli (Jimenez et al. 2001). Glycerol mediates effective utilization of polyamines and their conversion to spermidine and spermine, which regulate the somatic embryogenesis (Wu et al. 2009). Further, Wu et al. (2009) presented experimental proofs for this by inhibiting the somatic embryogenesis through addition of 5–10 mM alpha-difluoromethylornithine (DFMO), a potent inhibitor of enzymes involved in polyamine biosynthesis. The DFMO significantly reduced the formation of the heart and torpedo and completely inhibited the cotyledonary-stage embryos. The process of embryogenesis could be restored only by external addition of polyamine and putrescine (Wu et al. 2009). Glycerol in the medium increases the level of endogenous cytokinins, which regulate the process of somatic embryogenesis (Jimenez et al. 2001). Vu et al. (1993) observed higher activity of enzymes like phosphoenolpyruvate carboxylase (PEPCase) and sucrose phosphate synthase (SPS) in the nucellar tissues in glycerol-supplemented medium, while the activity of these enzymes was quite low in sucrose-containing medium, but their direct relationship with the somatic embryogenesis could not be established. The glycerol in the medium prolongs the lag period of growth for the cells, while this period was less in presence of sucrose in the medium (Ben-Hayyim and Neumann 1983). The proembryogenic masses release certain glycoproteins of 53–57 KDa in the sucrose-containing medium, which probably arrest the cell transition to globular embryo (Gavish et al. 1992). The exogenous addition of such glycoproteins in glycerol-containing medium also blocked the transition of proembryos to globular embryos (Gavish et al. 1992). The promotive role of galactose and galactose releasing other carbohydrates in somatic embryogenesis is attributed to their property of either inhibiting biosynthesis of auxins (Kochba et al. 1978) or modifying the endogenous auxin balance of the callus (Cabasson et al. 1995). Galactose induces low callus growth compared to sucrose and also ensures the supply of the phosphorus during second week of cycle, an integral component of ATP, probably required during the process of somatic embryogenesis (Cabasson et al. 1995). Maltose on the other hand influences somatic embryogenesis in *Citrus* physiologically (Kochba et al. 1978).

2.1.6 Growth Adjuvants

Malt extract, yeast extract and casein hydrolysate are the common growth adjuvants, which have been tested for inducing somatic embryogenesis in *Citrus*. Malt extract had a better potential over other complex substances, viz. coconut milk and yeast extract to establish the somatic embryogenesis in *C. junos* (Song et al. 1991).

Ghazvini and Shirani (2002) evaluated the effect of different concentrations of malt extract for induction of somatic embryos from the unfertilized ovules of Mexican lime (*C. aurantifolia*). Malt extract at a level of 300 mg l^{-1} in the medium increased embryo formation while at higher concentration (1000 mg l^{-1}) inhibited embryogenesis and produced embryogenic callus only. Rojanasiriwongse et al. (2004) reported that a combination of malt extract (500 mg l^{-1}) and casein hydrolysate (1000 mg l^{-1}) in the medium was optimum for initiation of somatic embryos from the immature seeds of *C. reticulata* cv. Shogun. The malt extract 500 mg l^{-1} with 146 mM sucrose has been used for induction of somatic embryogenesis in different *Citrus* species (Carimi et al. 1995; Carimi et al. 1998; Carimi et al. 1999). Besides, the effect of solidifying agents on somatic embryogenesis has also been investigated. Jin et al. (2007) reported enhanced embryogenesis in *C. unshiu* in the medium containing lactose and with increased concentrations of agar.

2.2 Embryo Germination/Conversion

For germination like the true zygotic embryo, somatic embryos also need precise cultural conditions and composition of the medium for initiation and development of shoots and roots leading to the complete plantlet (embling) formation. In a few instances, the somatic embryogenesis induction medium was equally suitable for the germination of embryoids as well. Gill et al. (1994) germinated the somatic embryos of 'Kinnow' mandarin on MS medium supplemented with BAP, NAA and malt extract on which they had earlier produced the somatic embryos. Tomaz et al. (2001) achieved the further development and germination of the nucellar callus-originated somatic embryos of Valencia, Caipira (*C. sinensis*), Cleopatra (*C. reshi*) and Rangpur lime (*C. limonia*) on a medium containing different carbohydrate sources. Malt extract, a compound earlier described for its role in stimulation of somatic embryo production in *Citrus*, has also played a crucial role in germination of somatic embryos. The lone supplementation of malt extract (500 mg l^{-1}) in the medium was sufficient to achieve germination in somatic embryos of 11 different genotypes of *Citrus* (Rodriguez et al. 1999). The conversion of developed embryos of *C. madurensis* into complete plantlets was achieved by culturing them on BP (Barba and Patena) or MS medium containing 20 g sugar and 100 ml coconut water/litre (Patena et al. 2005). Somatic embryos usually develop into small plants on culture medium lacking plant growth regulators, but exogenous application of these also stimulates the germination of somatic embryos in *Citrus*. The plantlets have been formed from the somatic embryos in the medium containing GA_3 at a small range of 3.0 μM (Kayim and Koc 2006) to 1.0 mg l^{-1} (Beloualy 1991; Kunitake et al. 1991). Deng et al. (1991) converted the embryoids of *C. reticulata* and *C. sinensis* into complete plantlets, after culturing them on MT medium supplemented with glycerol (0.5%) and ABA (10 mg l^{-1}). Auxins also influence the development of plants from the embryoids. Pasquale et al. (1994) transformed somatic embryos

of three lemon cultivars on to MS medium containing 0.27 μM NAA along with malt extract to finally produce the plantlets.

2.3 *Application of Somatic Embryogenesis in* Citrus *Crop Improvement*

The somatic embryogenesis facilitates the regeneration of somatic hybrids and genetic transformants derived from embryogenic cultures (Fig. 1). Both these applications are described below.

2.3.1 Somatic Hybridization

The ability of the protoplast to regenerate via somatic embryogenesis has opened the new opportunities to improve *Citrus* through somatic hybridization. Somatic hybrids are the products of protoplast fusion from two different individuals and combine their nuclear and cytoplasmic genomes. The successful isolation of protoplasts and their differentiation into somatic embryos were demonstrated in *Citrus*

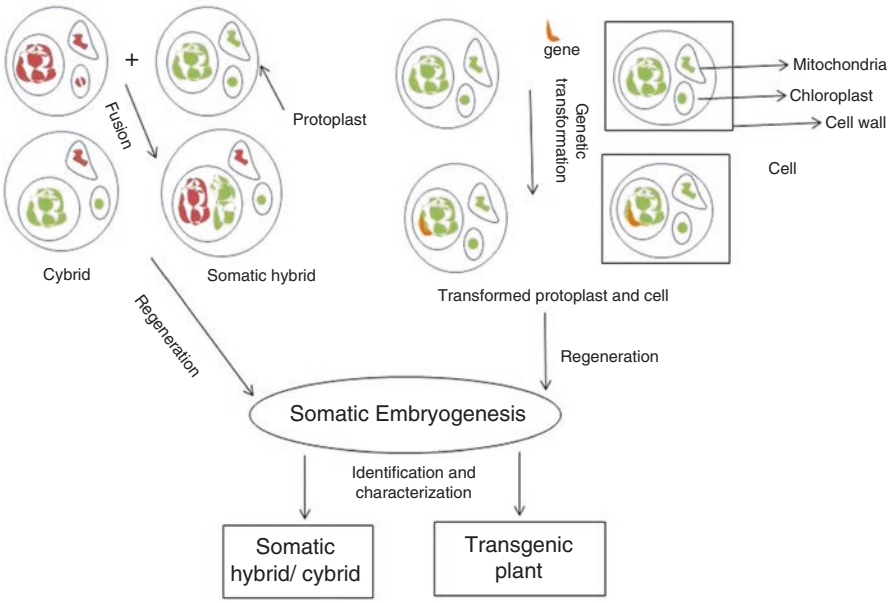

Fig. 1 Symbolic presentation for the role of somatic embryogenesis during regeneration of somatic hybrid/cybrid and genetically transformed protoplast/cells

as early as 1975 (Vardi et al. 1975). Subsequently, the first somatic hybrid was developed between *Citrus sinensis* and *Poncirus trifoliata* by Ohgawara and coworkers in 1985 (Omar et al. 2016). *Citrus* is a model plant for somatic hybridization. The technique involved in the process of somatic hybridization is briefly described below.

(i) *Protoplast Isolation*

For protoplast isolation, the embryogenic callus, cell suspension culture and leaves have been used. The use of callus and cell suspension culture in log phase of growth results in good protoplast yields (Grosser 1994). The leaves source in case of polyembryonic *Citrus* has been the nucellar seedlings, while in case of monoembryonic varieties, the budded plants maintained in the greenhouse are the ideal source (Omar et al. 2016).

(ii) *Protoplast Fusion*

The isolated protoplasts have been induced to fuse through the chemical polyethylene glycol (PEG) and electrical means or by a combination of both, i.e. electrochemical fusion. PEG-induced fusion requires no special equipment, and cost involved is less (Grosser and Gmitter 2011); the electrofusion is more convenient and causes no toxicity (Guo et al. 1998), while the electrochemical fusion combines the best features of both the fusion systems (Olivares-Fuster et al. 2005). The systems based on embryogenic callus protoplast + embryogenic callus protoplast (Ling and Iwamasa 1994; Ollitrault et al. 1996) and embryogenic callus protoplast + leaf protoplast (Grosser et al. 2000; Guo et al. 2013) have been used for protoplast fusions. However, the latter scheme is more routinely used. The fusion of intact protoplasts is called as symmetric fusion; however, selective fusion of nuclear genome from one parent and cytoplasmic genome from the other parent is called asymmetric fusion. The asymmetric fusion in *Citrus* has been achieved by fusing irradiated protoplasts whose nuclei have been destroyed and recipient protoplasts whose organelle genomes usually have been metabolically inhibited by the aid of iodoacetate (IOA). Therefore, the heterokaryons combine vital cytoplasm from donor parent with intact nucleus (from recipient parent) and form cybrids (de Bona et al. 2009).

(iii) *Regeneration of the Somatic Hybrids Through Somatic Embryogenesis*

After the fusion of the protoplasts, the regeneration of the fused and unfused protoplasts is facilitated by the process of somatic embryogenesis. In general, the fusion scheme based on embryogenic callus/cell suspension culture-derived protoplasts + mesophyll protoplasts is used in *Citrus*. The mesophyll-derived protoplasts lack totipotency and are incapable to form somatic embryos at their own.

Therefore, theoretically, following fusion, the developed embryoid could be from embryogenic unfused protoplasts, homofused embryogenic protoplasts and heterofused protoplasts. As a byproduct of symmetric fusion, somatic cybrids have also been produced, which have the nuclear genome from the mesophyll protoplast, mitochondrial genome from embryogenic parent and chloroplast genome from either of the parent. Generally, the somatic hybrids exhibit hybrid vigour and have regenerative advantage over both embryogenic and mesophyll protoplasts for embryoid formation, which has also been shown in planned somatic hybridization experiment (Guo and Grosser 2005). It is hypothesized that the less regenerative potential of the embryogenic protoplast could be due to build-up of cytological mutations over time and complementation of mesophyll protoplast overcomes such mutations in the fused product. However, in rare cases, the researchers have also obtained mesophyll-parent-type autotetraploid cybrids through this system (Grosser et al. 1992; Louzada et al. 1992).

(iv) *Identification and Characterization of Somatic Hybrids*

Following regeneration, the next step is identification and characterization of the somatic hybrids. Somatic hybrids sometimes can be identified by intermediate morphology. However, the somatic hybrids and cybrids are identified by a series of steps sequentially: ploidy evaluation through flow cytometry and chromosomal counts and nuclear and cytoplasmic genome characterization through molecular markers (Guo et al. 2013). The ploidy analysis gives a clue about the tetraploid or diploid status of the fused products, the nuclear genome characterization identifies the somatic hybrids and cybrids, and the mitochondrial genome and chloroplast genome characterization gives information about the cytoplasmic contributors in the somatic hybrid or cybrid. Somatic hybridization-aided *Citrus* breeding is being practised in a number of countries like the USA (Grosser et al. 2000), France (Ollitrault et al. 1996), Japan (Ohgawara and Kobayashi 1991; Oiyama et al. 1991), China (Deng et al. 1996; Deng et al. 2000; Guo et al. 2007), Italy (Tusa et al. 1996), Brazil (Mourao Filho et al. 2008), Spain (Aleza et al. 2016), Turkey and Costa Rica. To date, more than 500 parental combinations have been used in *Citrus* to generate somatic hybrids, of which more than 300 have been raised at CREC, University of Florida, USA (For review, refer Grosser et al. 2000; Grosser and Gmitter 2005; Omar et al. 2016). Additionally, more than 40 somatic cybrids have also been produced as a byproduct of somatic hybridization (Guo et al. 2013). The role of protoplast fusion, in scion and rootstock improvement, is briefly described below.

Scion Improvement

Initially, somatic hybridization was viewed as a direct approach to cultivar improvement. However, except few combinations (Guo et al. 2004), the real potential of the approach has been in the development of superior breeding parents (Grosser and Gmitter 2011). Seedlessness is the primary trait that is desired in the fresh and processing purpose *Citrus* varieties. The somatic hybridization is being used in the

development of desired tetraploid combinations. Such tetraploid parents when crossed with the diploids can be useful in the development of triploid seedless hybrids coupled with the introduction of other desirable attributes from the somatic hybrids. The interploid crossing can be implemented either in diploid x tetraploid or in tetraploid x diploid fashion. In the interploid approach, when tetraploid parent is used as pollen parent, the viable embryos are not recovered probably due to unfavourable embryo: endosperm ratio. This imbalance results in endosperm failure and subsequently leads to embryo abortion (Grosser and Gmitter 2011). The technique of embryo culture has been used to rescue the embryos before abortion (Viloria et al. 2004; Viloria et al. 2005); however, the technique is laborious. The problem of embryo abortion can be overcome to some extent by using tetraploid parents as female parent. The University of Florida-Citrus Research and Education Center (CREC), Lake Alfred, has used several tetraploid somatic hybrids as breeding parents in the interploid breeding program for the varietal improvement of mandarins, grapefruits, limes and lemons with different objectives (Table 1).

The use of tetraploid monoembryonic parent as female parent can enhance the breeding efficiency by direct recovery of the hybrid individuals. The somatic hybrid between 'Succari' sweet orange + 'Hirado Buntan Pink' pummelo is one such example of monoembryonic tetraploid and is being used as female parent at CREC, University of Florida (Omar et al. 2016). A triploid mandarin hybrid, C-4-15-9, an outcome of cross between LB8–9 and a somatic hybrid Nova mandarin hybrid + Succari, has been released for commercial cultivation out of this breeding endeavour (Omar et al. 2016). A direct approach for the production of triploids has also been tested in *Citrus* at CIRAD (Ollitrault and Dambier 1997; Ollitrault et al. 2000). This approach involves fusion of protoplasts of diploid and haploid origin to get the triploids. However, this can be realized only with the availability of haploid lines. At CIRAD, gynogenesis induced two haploid Clementine lines H_1 and H_2 are being used for this purpose. The haploid lines have been used to produce triploids between 11 diploid cultivars from mandarins, sweet oranges, grapefruit, lime and kumquat. The schemes, however, in addition to triploids, also yield to products with 4X or higher ploidy level. The availability of stable haploid lines can enhance the success rate in this scheme.

The Satsuma mandarins are male sterile by virtue of cytoplasmic male sterility (Yamamoto et al. 1997). The CMS sterility is under the control of mitochondrial gene in higher plants (Kumar and Cocking 1987). The Satsuma-derived 'seedless-

Table 1 Use of allotetraploids for the variety improvement in *Citrus*

Group	Objectives for genetic improvement
Mandarin	Seedless, easy peeling, varied maturity periods, good external and internal colour and flavour and good shelf life
Acid lime	Seedless, cold hardiness resistance to diseases like canker and witches broom
Grapefruit/ pummelo	Seedless, extended maturity seasons, reduction or elimination of furanocoumarins, improved resistance to diseases like canker and witches broom

ness' is viewed as an alternative to the interploid crossing approach for seedless induction in elite cultivars at diploid level (Cai et al. 2009). This is possible by developing cybrids between Satsuma and elite cultivars with mitochondrial genome from Satsuma and nuclear genome from the elite diploid cultivars. The cybrids so produced can induce seedlessness in a single step without disturbing the nuclear genome of the elite cultivar. Yamamoto and Kobayashi (1995) produced a cybrid plant between Satsuma mandarin and Washington Navel orange, and the resultant cybrid was seedless. But the exact reason for male sterility could not be ascertained as the Washington Navel itself is also male sterile. To facilitate the easy identification of cybrids during plant regeneration, Guo and his group are using *gfp* expressing transgenic Satsuma cv. G_1, as embryogenic parent. Since the cybrids lack nuclear genome from the embryogenic parent; So, the regenerated cybrid plants are free of the trans *gfp* gene. The cybrids between Satsuma mandarin cv. G1 + HBP pummelo were also developed through this approach and have been reported to be male sterile (Zheng et al. 2012). By learning from the success of this experiment, G1 Satsuma mandarin has been used for induction of seedlessness in *C. sinensis*, *C. grandis*, *C. reticulata* and *C. maxima*, as reviewed by Guo et al. (2013).

Rootstock Improvement

The major objective in rootstock breeding through somatic hybridization is to package resistance to different biotic and abiotic stresses, to control tree size, wide adaptability and good yields (Grosser and Gmitter 2011). The complementary somatic hybridization and wide somatic hybridization have been used for achieving this. In the complementary hybridization, fusion is induced between the sexually compatible genotypes *Citrus* + *Citrus* and *Citrus* + sexual compatible donors like *Poncirus*. Of the different combinations, certain potential somatic hybrids which include sour orange + Palestine lime, sour orange + Carrizo citrange, Succari sweet orange + Hirado Buntan sdl pummelo, Hamlin sweet orange + rough lemon, sour orange + Flying Dragon, Valencia + Femminello lemon and Red Marsh grapefruit + Argentine Trifoliate orange produce polyembryonic seeds (Grosser and Chandler 2003). The somatic hybrids: sour orange + Palestine lime and sour orange + Rangpur lime have been reported to be promising as they reduce tree size and also positively influence the yields of the budded varieties (Grosser and Gmitter 2011).

The wide somatic hybridization involves combining the protoplasts of *Citrus* with distant sexually incompatible donors. The wild relatives harbour resistance to various important biotic stresses mainly greening (*Murraya paniculata*), *Phytophthora* (*Murraya*, *Citropsis*, *Atlantia*), *Citrus* nematodes (*Murraya*, *Severinia*, *Citropsis*) and abiotic stresses mainly drought (*Feronia*) and wet soils (*Atlantia*) (Grosser et al. 1996). Wide somatic hybridization approach has been used to harness the resistance from these distant relatives. Few of the potential wide somatic hybrid examples include *Citrus* + Murraya (Guo and Deng 1998), Succari sweet orange + *Atlantia ceylanica* and Nova tangelo + *Citropsis gilletiana* (Grosser et al. 2000). The sour orange was a premier rootstock in the past, but its use has

been abandoned due to its high vulnerability to *Citrus tristeza* virus (CTV). Molecular studies of Nicolosi et al. (2000) had revealed that sour orange is a natural hybrid between a pummelo and a mandarin. This has also been confirmed through genome sequence studies of Wu et al. (2014). Professor Grosser and his group at CREC, University of Florida, are involved in resynthesizing sour orange that exhibits resistance to CTV as well as possesses other good features (Grosser and Chandler 2003; Ananthakrishnan et al. 2006). The assumption for this development is that sour orange during hybridization could not be an outcome of a best possible mandarin and pummelos combination. The somatic hybrids are now being produced by combining well-adapted mandarins (*C. amblycarpa*), 'Murcott' tangor and 'Succari' sweet orange with improved pummelo clones that exhibit resistance to *Tristeza*, *Phytophthora nicotianae* and *P. palmivora* and capable of growing well in calcareous soils. The tetraploid somatic hybrids have also opened the doors to start breeding at tetraploid level. The researchers at CREC, University of Florida, are using the term 'tetrazyg' for the sexual hybrids obtained at the tetraploid level (Grosser and Gmitter 2011). Two somatic hybrids, 'Nova' mandarin + 'Hirado Buntan' pummelo (zygotic) and sour orange + Rangpur, which predominantly produce zygotic seeds, are being cross-pollinated with potentially good somatic hybrids like sour orange + 'Carrizo', 'Cleopatra' + trifoliate orange and sour orange + 'Palestine' sweet lime.

2.3.2 Somatic Embryogenesis and Genetic Transformation

Genetic transformation is an alternative approach for targeted genetic improvement of *Citrus* by altering a single trait without disturbing the integrity of the original cultivar. For genetic transformation experiments in *Citrus*, the epicotyl explants are routinely used. However, this explant is not applicable for monoembryonic and seedless varieties where sufficient number of nucellar seedlings cannot be raised to supply the desired number of explants. Somatic embryos can be proved very useful in such varieties as these can be induced from a range of explants. Since the somatic embryos are usually of single-cell origin (Omar et al. 2016), which eliminates the probability of chimeras formation post-transformation (Polito et al. 1989). The attractive feature of the most of the embryogenic cultures is that the plants derived from them are predominantly normal and devoid of any phenotypic and genotypic variations as only the normal cells participate in the embryogenesis process (Vasil 1999). The examples of genetic transformation where embryogenic cultures have been used are described in Table 2.

3 In Vitro Selection

It is the in vitro technique of selecting the positive variants against a target selection pressure. The variability already existing in the explants or induced through cell/tissue culture cycle (somaclonal variation) and through mutagens (in vitro

Table 2 Genetic transformation in *Citrus* using embryogenic cultures

Genotype	Explant	Transgene	Reference
Sweet orange cv. Hamlin	Protoplasts	*Gus*	Niedz et al. (2003)
Sweet orange cv. Valencia	Embryogenic callus	*Bar* gene	Li et al. (2003)
Sweet orange cv. Valencia	Protoplasts	Sense copy of cDNA of PME gene	Guo et al. (2005)
Sweet oranges, Mandarins and Nasnaran	Embryogenic callus	*hptII*	Dutt and Grosser (2010)
Satsuma mandarin cv. Miyagawa Wase	Embryogenic callus	*hptII*	Al Bachchu et al. (2011)

mutagenesis) is used for this purpose. The selection pressure allows the preferential survival/growth of the desirable variant cells/tissues. The technique is based on the assumption that positive variant selected at in vitro level would also express the trait at whole-plant level (Perez-Clemente and Gomez-Cadenas 2012). Below are the described advantages over conventional field and greenhouse screening techniques (Predieri 2001; Perez-Clemente and Gomez-Cadenas 2012):

(i) Allows screening of large number of cells, tissues or regenerated plants for a target stress
(ii) Overcomes the space constraints usually experienced under field evaluations
(iii) Offers controlled and uniform conditions for screening
(iv) Maintains high phytosanitary conditions throughout the selection process
(v) Provides ample opportunity to deeply understand the basis of improved trait

The section has been divided into different sub-sections: creation of genetic variability, in vitro selection and plant regeneration followed by in vivo selection for different stresses.

3.1 Creation of Genetic Variability

Genetic variability is the primary requirement for in vitro selection. In vitro selection-based improvement system relies upon the somaclonal variation and the in vitro mutagenesis for the genetic variability.

3.1.1 Somaclonal Variation

The somaclonal variation has been defined as the genetic variation which appeared among the plants regenerated through tissue culture cycle (Larkin and Scowcroft 1981). Tissue culture is a rapid method of clonal propagation, but variants also emerge through this mode of propagation. The variations appearing among the tissue-cultured plants could be due to the pre-existing spontaneous mutations

(expressed under in vitro*)* or generated de novo under the influence of triggering agents (Skirvin et al. 1994; Krishna et al. 2016). The mutations develop in the multicellular tissues spontaneously, which remains hidden in nature, but can be recovered under tissue culture cycle. The triggering factors for inducing variations include state of the explant, explant wounding, exposure to the sterilants during sterilization, imbalances of the medial components and culture environment. These triggering agents lead to oxidative stress, due to which the level of peroxidants or reactive oxygen species (ROS) such as super oxides, hydrogen peroxide, hydroxyl, peroxyl and alkyl radicals is elevated. The reactive oxygen species cause hyper- and hypomethylation of the DNA (Wachsman 1997), induces changes in chromosome number and structure (Czene and Harms-Ringdahl 1995) and even can induce the nucleotide-based substitutions (Skirvin et al. 1994).

The type of explant and mode of regeneration largely determine the degree and frequency of variation (Sahijram et al. 2003). Highly differentiated explants like roots, leaves and stems are more prone to variations during regeneration than the explants with pre-existing meristems like shoot tips and axillary buds (Duncan 1996). Similarly, the indirect shoot organogenesis from the highly differentiated explant through an intermediate callus phase is more vulnerable to induced variations than that of direct shoot organogenesis from such tissues (Pijut et al. 2012). It seems that the dedifferentiation of the already differentiated tissue imposes a great degree of stress. The callus or protoplast regenerated plantlets are the result of this extreme degree of stress. Apart from the explant, the growth regulators alone or in combinations also mimic the role of mutagens. The application of several growth regulators such as 2,4-dichlorophenoxy acetic acid (2,4-D), naphthalene acetic acid (NAA), BAP (6-benzylaminopurine) and synthetic phenylurea derivatives 4-CPPU, PBU and 2,3-MDPU in the culture medium induces genetic variability in the tissue-cultured plants (Krishna et al. 2016). Even the type of cytokinin influences the degree of somaclonal variation (Siragusa et al. 2007). The somatic embryo-derived Calamondin plantlets induced on adenine-type cytokinin (BAP) were normal, whereas those produced on the synthetic phenylurea derivatives (4-CPPU, PBU and 2,3-MDPU) exhibited 3.7% to 7.4% somaclonal variation. The synthetic phenylurea derivatives inhibit cytokinin oxidase, which oxidizes the excess cytokinins. The inhibition of cytokinin oxidase results in accumulation of endogenous level of cytokinin, which leads to build-up of reactive oxygen species (ROS) (Siragusa et al. 2007). The ROS as described earlier are responsible for inducing somaclonal variations. The somaclonal variation is of two types: epigenetic and stable. The epigenetic variation, also known as developmental variation, is temporary in nature, reversible and non-heritable (Kaeppler et al. 2000). The epigenetic variations occur due to alterations in the methylation pattern of DNA, in the amino acid patterns of the histone tail or in both, resulting in suboptimal or overexpression of the certain genes under in vitro conditions (Smulders and de Klerk 2011). Such variations are temporary in nature, and plantlets can revert back to normalcy just after removal of the stress or may continue to express for few more cycles post-stress removal. The stable variation on the other hand is heritable in nature. Such variations are either already present in the explants of multicellular origin and

expressed under in vitro conditions or induced de novo (by change in genomic constitution). This type of variations is of interest to plant biotechnologists as it is stable in nature and is passed to the progenies. The somaclonal variation can be detected by a number of tools like morphological differences, cytogenetical analysis for the determination of numerical and structural variation in the chromosomes (Clarindo et al. 2012; Currais et al. 2013; Abreu et al. 2014), biochemical markers (Vujovic et al. 2010; Kar et al. 2014), molecular DNA markers (Krishna and Singh 2007; Pathak and Dhawan 2012; Hossain et al. 2013) or their combinations (Dey et al. 2015).

3.1.2 In Vitro Mutagenesis

Mutagens have been widely used to improve one or few traits in *Citrus* under field conditions (Latado et al. 2012). These have been used to develop seedless/low-seeded version of the elite *Citrus* varieties (Latado et al. 2012) and for improving the disease resistance (Gulsen et al. 2007). The Rio Red and Star Ruby grapefruits, Jincheng 7 and 9 sweet oranges, seedless Tango mandarin and Ktudiken seedless lemon are among the classical examples of seedless or low-seeded *Citrus* varieties developed through the use of mutagens (Latado et al. 2012). In general, the multicellular tissues like budwood or seeds have been used for mutagen treatment. This form of mutation breeding is termed as in vivo mutation breeding. The mutagen treatment of multicellular tissues leads to the development of a mosaic of mutated cells and unmutated cells. In the competition for growth and survival, the wild unmutated cells are in advantageous position (Penna et al. 2012). Three to four cycles of vegetative propagation (M1 V3 to M1 V4) are required to dissociate the stable mutants from the unmutated cells in the mosaic tissue (Gulsen et al. 2007). However, the treatment of totipotent single cell or few cell aggregates or the explants arising from single-cell regenerants with the mutagens leads to stable mutants, and the step of recovering the chimeras can be skipped (Broertjes and Van Harten 1988). The combination of in vitro culture system with mutagens is called as in vitro mutagenesis. The use of undifferentiated cells/tissues also adds somaclonal variation effect to the mutagenesis. Both physical (gamma rays, X-rays, UV rays and thermal neutrons) and chemical [alkylating agents, ethyl methane sulphonate (EMS), methyl methane sulphonate (MMS), methyl-nitroso-urea (MNH) etc.)] mutagens are being used for mutation induction. The two categories of mutagens have their own advantages over each other. The advantages of using physical mutagens are accurate dosimetry, reasonable reproducibility and uniform penetration of multicellular tissues. The chemical mutagens on the other hand cause high mutation rate with predominant point mutations (Jain 2005). The preferred physical and chemical mutagens are gamma rays and EMS, respectively (Table 3). Gamma rays induce breaks in the genomic DNA, and mutation is produced by the repair mechanism of DNA. EMS causes mutation by adding ethyl group to N or O at the 7th and 6th positions of guanine base, respectively. The altered guanine shows high affinity to bind with thymine instead of cytosine. Thus, GC is

Table 3 In vitro mutagen sensitivity assay in *Citrus*

Species/genotype	Substrate	Mutagen-sensitive dose/LD_{50} dose	Reference
C. aurantium	Seeds	Gamma rays – 155.6 Gy	Tallon et al. (2015)
C. jambhiri cv. rough lemon	Calli	Gamma rays – 20 Gy	Kumar et al. (2010)
C. jambhiri cv. rough lemon	Calli	0.1% EMS and MMS for 3 h	Kumar et al. (2010)
C. jambhiri cv. rough lemon	Seeds	Gamma rays – 62.0 Gy	Kaur and Rattanpal (2010)
C. jambhiri cv. rough lemon	Seeds	0.64% EMS	Kaur and Rattanpal (2010)
C. limon	Protoplasts	Gamma rays – 200 Gy	Helaly and El-Hosieny (2011)
C. macrophylla	Nodal segments	Gamma rays – 29.2 Gy (LD_{50})	Tallon et al. (2015)
C. macrophylla	Seeds	Gamma rays – 129 Gy	Tallon et al. (2015)
C. reticulata	Epicotyls	Gamma rays −16.2–20.7 Gy	Gonzaga et al. (2011)
Citrus sinensis cv. Pera sweet orange	Protoplasts	Gamma rays – 37.5 Gy (LD_{50})	Goldman and Ando (1990)
C. sinensis	Seeds	27 Gy (LD_{50})	Ling et al. (2008)
C. sinensis	Cell suspension culture	1.5% EMS for 1 h (lethal dose)	Ge et al. (2015)
Citrus sinensis cv. Shamouti orange	Callus	Gamma rays – 240 Gy	Spiegel-Roy and Kochba (1973)
Citrus sinensis cv. Shamouti orange	Nucellus Embryogenic callus Cotyledons	Gamma rays – 20–40 Gy 120 Gy 40 Gy	Tulmann Neto et al. (1994)
Citrus sinensis cv. Shamouti orange	Protoplasts	X-rays – 340 Gy	Vardi et al. (1975)
C. sinensis cv. Shamouti	Protoplasts	0.3% EMS for 1 h	Vardi et al. (1975)

subsequently converted into TA. The basic steps of in vitro mutagenesis are mutagen sensitivity assay and determination of optimum dose or LD_{50} dose, mutant isolation by in vitro screening and regeneration of the mutants.

Mutagen Sensitivity Assay and Estimation of LD_{50} Dose

For mutagen application, the first step is to determine the optimum dose of a particular mutagen. This has been determined by assessing the sensitivity of the tissue/cells to the mutagens. The dose where 50% or higher reduction in the physiological response (growth, survival or regeneration) compared to control is observed, is considered to be the appropriate dose. In case of ionizing radiations, the radiosensitivity is measured in

terms of absorbed radiations, the unit of which is Gy (Gray, which is equivalent to 1 J Kg^{-1} or 100 rads). For radiations, Heinze and Schmidt (1995) advocated the use of $LD_{50} \pm 10\%$ dose of the ionizing radiations or the dose which renders only 20% tissue survival after treatment. The physiological response and radiosensitivity of the treated material are determined by the water content of the tissues, as ionizing radiations target water molecules (Britt 1996). A wide range of dose between 20 to 240 Gy for gamma rays and 340 Gy for X-rays, depending upon the explant and species, has been used in *Citrus* (Table 3). Kumar et al. (2010) treated the 60-day-old rough lemon callus with gamma rays (0, 10, 20, 30, 40, 50 and 60 Gy) and observed the effect for survival and regeneration. The gamma rays induced mortality of the callus and also caused a reduction in its regeneration potential (Fig. 2). The callus turns brown from green following gamma ray treatment. The LD_{50} dose of gamma rays was between 20 and 30 Gy (Table 3). The optimum dose of the chemical mutagen is estimated by varying the concentration and duration of exposure (Novak 1991). The chemical mutagen EMS, for instance, has been used in the range of 0.1–1.5% to induce mutations in *Citrus* (Table 3).

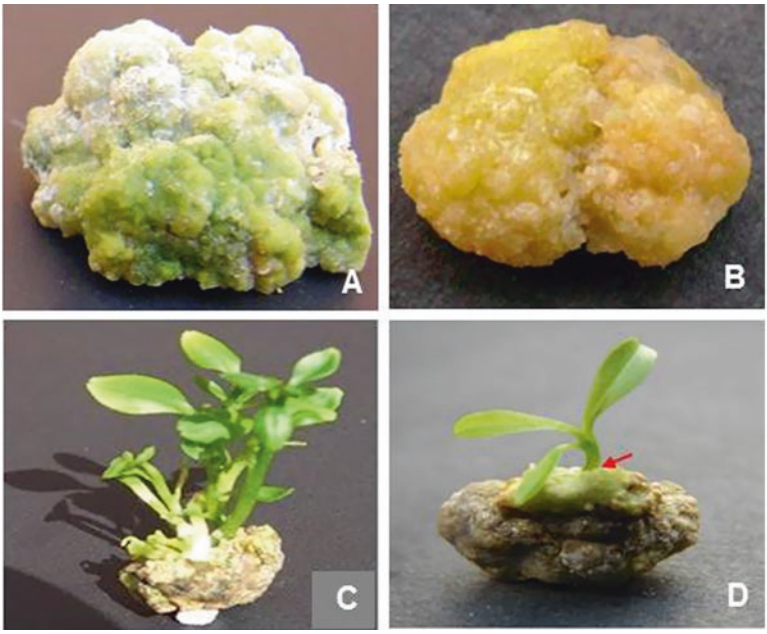

Fig. 2 Determination of optimum dose of gamma rays for mutagenesis in rough lemon callus (**a**) Control (untreated) 60-day-old callus just before subculturing (**b**) Callus browning, 15 days post-gamma ray treatment (**c**) Shoot regeneration from untreated (control) callus on MS + NAA (0.5 mg l^{-1}) + BA (3.0 mg l^{-1}) + kinetin (0.5 mg l^{-1}) (**d**) Shoot regeneration from 20 Gy gamma ray-treated callus on MS + NAA (0.5 mg l^{-1}) + BA (3.0 mg l^{-1}) + kinetin (0.5 mg l^{-1})

3.2 Selection Agents and In Vitro Screening

At this step, the induced variability is subjected against the selection pressure to find out the positive variants or mutants. The in vitro selection has been used to isolate the variants tolerant to various biotic stresses mainly mal secco toxin (*Phoma tracheiphila*), canker pathogen (*Xanthomonas citri* subsp. *citri*) and *Phytophthora* and among the abiotic stresses like salt, cold and heavy metals like Al (Table 4). For in vitro selection, the callus or other target culture has to be grown in the presence of a selective agent. The use of protoplasts, single or few cell aggregates, increases the possibility of stable mutations as such explants get in full contact with the mutagens as well as in the selection agent (Ge et al. 2015). The optimum level of the selection agent is either standardized initially with the control calli/cell suspension culture (Deng et al. 1995; Kumar et al. 2010) or its level is increased step by step to an extreme, where no culture survives (Savita et al. 2011). In the former strategy, the cultures are grown in the absence and presence of different levels of selection agent, and optimum level is determined by comparing the growth and regeneration pattern. Kumar et al. (2010) subjected the young calli to 0, 25, 50, 75 and 100 mM NaCl in the regeneration medium. The regeneration at 25 mM NaCl was reduced tenfold compared to control, and 25 mM NaCl was taken as optimum for selecting the variants. Similarly, Yacoubi and Rochdi (2011) established the threshold concentration of salt by growing the callus on callus induction medium containing 0, 4, 6, 8 and 9 g l^{-1} NaCl. At 8 g l^{-1} NaCl, the growth of the callus was reduced to 50% of the control. For the second strategy, the calli are initially grown in the presence of lower levels of selection agent, and the surviving calli are then maintained on the medium with same as well as elevated level of selection pressure. In Citrus, most of the variants against biotic stress (except tolerance to *Phytophthora*), have been selected by growing the cultures in the presence of a predetermined concentration of cultural filtrate. The salt-tolerant somaclones have been selected in the presence of 2.0–11.0 g l^{-1} NaCl (Table 4).

The stable expression of the selected trait is desired in the somaclones. This has usually been checked by studying the stability of the trait independent of stress (Yacoubi and Rochdi 2011). This has been done by usually culturing selected somaclone on a medium devoid of selection agent for few cycles and retransferring them on the medium with the same level of selection pressure, at which these were initially selected. The salt-tolerant cell lines are able to maintain similar callus growth rate in a medium containing or devoid of NaCl (Ben Hayyim and Kochba 1982; Yacoubi and Rochdi 2011). Ben-Hayyim and Kochba (1983) attributed the salt tolerance ability of somaclones to the partial avoidance to NaCl. The tolerance to a stress in a somaclonal variant and mutant must be governed by the genes. Few studies have revealed the basis of tolerance or resistance to stresses in the somaclones. Yacoubi and Rochdi (2011) observed that the salt-tolerant lines maintained the endogenous K^+ and also adjusted the excess Na^+

Table 4 Isolation of variants for tolerance to various biotic and abiotic stresses in *Citrus*

Species/ genotypes	Culture system	Selection type and its level	Result	Selection exercised at	Reference
C. limon cv. Villafranca	Cell suspension culture	*Phoma tracheiphila* toxin	Var. 1.117 stably resistant	In vitro	Nadel and Spiegel-Roy (1987)
Citrus limon cv. Femminello Siracusano	Protoplasts	0.5 µM *Phoma tracheiphila* toxin	One protoclone (Siraculsano S1) and one somaclone (Siracucano S2)	In vitro and in vivo	Deng et al. (1995)
C. jambhiri cv. rough lemon	Callus	*Phytophthora* culture filtrate toxin (5–100%) by elevating gradually	Lines with tolerance up to 50% CF	In vitro and in vivo	Savita et al. (2011)
C. sinensis cv. Bingtang	Cell suspension treated with 1.5% EMS for 1 h	*Xanthomonas citri* sub sp. *citri* (10%)	One tolerant somaclone DG-2	In vitro and in vivo	Ge et al. (2015)
Citrus aurantium cv. Amargo	Callus	400–2000 µM Al	Two lines with tolerance to 400–1000 µM Al regenerated	In vitro	Toan et al. (2004)
C aurantium	Callus	100 mM NaCl	Tolerant cell lines	In vitro	Koc et al. (2009)
C. sinensis cv. Pineapple	Callus	400–2000 µM Al	One line tolerant to 400 µM Al	In vitro	Toan et al. (2004)
Citrus sinensis cv. Shamouti	Callus	0.2 M NaCl for four passages	R-10, a salt-tolerant line	In vitro	Ben-Hayyim and Kochba (1982)
C. sinensis cv. Shamouti	Nucellar callus	85 mM NaCl for ten passages	R13 and R14 regenerated	In vitro	Spiegel-Roy and Saad (1997)
C. limon	Embryogenic cells	10 g/l NaCl	Salt-tolerant cell lines	In vitro	Piqueras and Hellin (1992)
Poncirus trifoliata x *C. sinensis* cv. Troyer	Callus	8 g/l NaCl level	Salt-tolerant cell lines	In vitro	Yacoubi and Rochdi (2011)
Troyer citrange	Unfertilized ovules treated with 0.1% EMS	45 mM NaCl	PG2128 a salt-tolerant plant	In vitro	Garcia-Agustin and Primo-Millo (1995)
C. sinensis cv. Jincheng	Cell suspension culture irradiated with gamma rays	Hydroxyproline	Cell line more cold tolerant than control cells	In vitro	Lin et al. (1999)

by compartmentalizing it to the vacuoles. Contrarily, the salt sensitive lines experienced the build-up of Na+ in the cytosol, which is considered injurious to the cells. In response to the increased vacuolar Na^+, the level of proline and soluble sugars also increased in the salt-tolerant cell lines compared to salt-sensitive and control calli. In *C. limon* cv. Femminello Siracusano, protoclone tolerant to *Phoma tracheiphila* was found expressing pathogenesis-related proteins of 34 and 45 KDa molecular weight (Deng et al. 1995).

3.3 Plantlet Production and In Vivo Evaluation

The ultimate aim of in vitro selection is production of plants which are capable of growing in the presence of biotic and abiotic stress against which they are initially selected. In few studies, the somaclones selected at in vitro level have also been tested under greenhouse and field conditions. Gentile et al. (1992) obtained a lemon somaclone of 'Femminello Continella' for tolerance to *Phoma tracheiphila*. The somaclone was found to be disease-free even after 3 years of plantation in the disease-prone area (Deng et al. 1995). For the development of canker tolerant somaclones, Ge et al. (2015) used a two-step selection process for elimination of false positive somaclones. They incubated the identified somaclones in the presence of 10% crude extract of the pathogen, followed by inoculation of detached leaves with bacterial pathogen. By this assay, the number of tolerant somaclones was reduced from 271 to seven. Out of these seven somaclones, one somaclone DG-2 was found tolerant to canker under in vivo conditions. Savita et al. (2011) obtained the *Phytophthora parasitica*-tolerant callus lines by exposing calli to the fungal cultural filtrate of 5 to 50% concentration. When the tolerant calli were cultured for regeneration in the presence of cultural filtrate, the calli could regenerate at or lower than 20% CF. Of the total regenerated plants, 81% exhibited resistance to *Phytophthora* at in vivo level. The findings of these studies allude to the need for testing the tolerance of somaclones against the biotic and abiotic stresses at in vivo level.

4 Plant Regeneration

The direct plantlet regeneration in citrus has been achieved with primary aims of clonal perpetuation of the plants and development of protocol necessary for genetic transformation and for producing the virus-free plantlets. The direct plant regeneration here has been dealt under two subheads: micropropagation and shoot-tip grafting. The former is primarily used for clonal propagation while the latter to make the mother stocks of scion varieties free of virus and virus-like diseases.

4.1 Micropropagation

In citrus, the technique involves five major steps, namely, in vitro shoot organogenesis, in vitro shoot proliferation/elongation, in vitro rooting, acclimatization of plantlets and finally transfer to soil (Fig. 3). The first step is the most important step and has been described in detail here. The in vitro shoot organogenesis response in *Citrus* seems to be governed by a variety of factors including genotype, explant type and medium composition. The differential response exists among various citrus genotypes for in vitro shoot organogenesis (Bordon et al. 2000). The *Poncirus* hybrids like Troyer and Carrizo citrange have high regeneration potential, *C. sinensis* exhibit intermediate and the rootstocks like *C. limonia*, *C. macrophylla* and *C. aurantium* fall in recalcitrant category (Bordon et al. 2000; Costa et al. 2004). Usually, the juvenile explants (epicotyl, hypocotyl, leaf petiole and root segments) and, to a limited extent, mature explants have been used for in vitro regeneration. Among the different juvenile explants, the epicotyl explants have been found to be superior for in vitro regeneration (Garcia-Luis et al. 2006; Saini et al. 2010; Rattanpal et al. 2011). The type of cut and orientation of their placement on the culture medium also influence the adventitious bud production in epicotyl explants (Saini et al. 2010; Rattanpal et al. 2011). A thorough longitudinal cut induced more

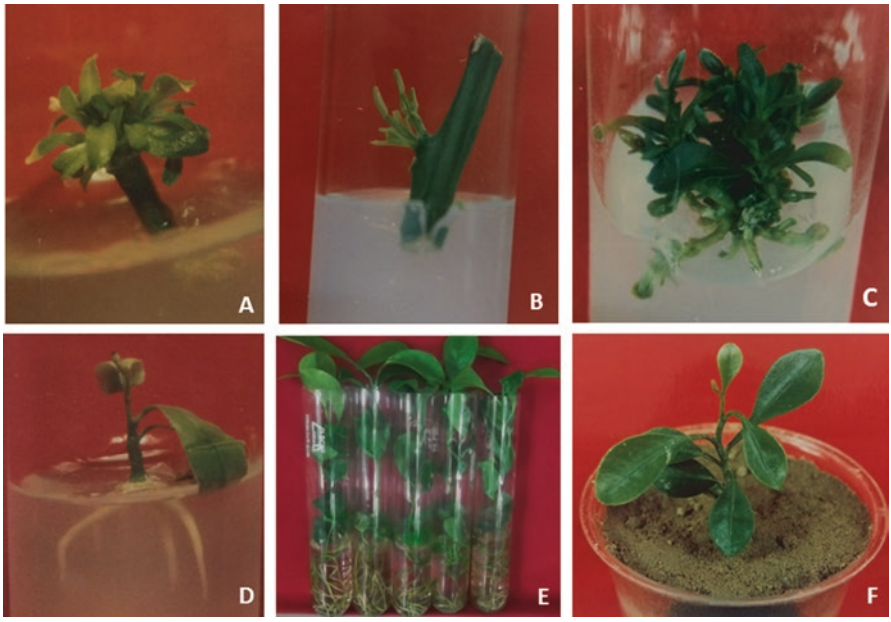

Fig. 3 Micropropagation of *Citrus* (**a**) Shoot regeneration from cultured epicotyl segments (**b**) Shoot regeneration from cultured nodal segments (**c**) In vitro shoot proliferation (**d**) Induction of rooting in vitro (**e**) Acclimatization of rooted plantlets (**f**) Transfer of acclimatized plants to soil in pots

number of adventitious buds over transverse cut in epicotylar explants of *Citrus jambhiri* (Rattanpal et al. 2011). Similarly, the number of formed adventitious buds in this rootstock were more when the epicotyl segments were cultured in the horizontal position as compared to their vertical or inverted placement on the medium (Saini et al. 2010). Nevertheless, the plantlets raised through the juvenile explants tend to retain juvenility for many years, while those raised from the mature tissues of field-grown plants lack such juvenility (Tallon et al. 2013). Due to this advantage of adult tissues, the regeneration protocols based on mature tissues are under investigation in *Citrus* (Tallon et al. 2013; Navarro-Garcia et al. 2016). Like juvenile explants, the in vitro regeneration potential also differs among the mature explants. Nodal segments were found to be superior over internodal segments for in vitro regeneration response. Even within the nodal segments, the positional gradient for in vitro response is known to exist, and those excised from the apical zone were found to be the most responsive (Navarro-Garcia et al. 2016). The medium composition is critical in realizing the in vitro shoot organogenesis potential. The juvenile epicotylar segment in most of the *Citrus* species proves self-reliant for inducing adventitious shoot buds, and the external application of BA rather inhibits the production of shoot buds (Goh et al. 1995). Exceptionally in certain species like *C. jambhiri*, the addition of BA in the medium has induced high frequency of adventitious buds (Saini et al. 2010). Unlike juvenile epicotyl explants, the adult tissue need external supply of BA alone on in combination with auxins or gibbereliins for initiating the shoot regeneration response. The optimal BA concentration for initiating the adventitious shoot buds in the nodal segments of *C. aurantium* and *C. macrophylla* was 2 and 3 mg l^{-1}, respectively (Tallon et al. 2013), whereas, in the nodal segments of different lemon cultivars, it was achieved, when BA was supplemented with GA_3 (both at the rate of 1 mg l^{-1}) (Navarro-Garcia et al. 2016). The adventitious buds induced in the epicotylar explants are very small (<1 cm) and need to pass through an elongation phase. The elongation has been facilitated by the use of GA_3 (1 mg l^{-1}) alone (Rattanpal et al. 2011) or with supplementation of the cytokinin BA (Saini et al. 2010). Rooting in the cultured shoots has been induced with the help of NAA and IBA (both at 1 mg l^{-1}) (Saini et al. 2010). The acclimatized plants show high rate of survival (85–100%) in the field (Saini et al. 2010).

4.2 Shoot-Tip Grafting

Citrus trees are affected by a number of virus and virus-like diseases, which have serious implications on tree health, yield and longevity. Many a viruses like *Citrus tristeza* virus (CTV) have caused destruction of citrus industry in many parts of the world, while the others like citrus variegated chlorosis (CVC) in Brazil and the 'witches' broom disease of limes in Oman and Iran are causing substantial damage annually (Roistacher 2004). The spread of viral diseases is determined by the status of initial planting material, transmitting vectors and prevailing weather conditions. So, the use of healthy and virus-free planting material can check the spread up of

such diseases to a great extent. Shoot-tip grafting, a tissue culture-based technique, offers to produce healthy planting material and is also useful for recovering the healthy plantlets from the already infected sources. The resulting plants also do not have the juvenile characters. The technique was initially attempted by Murashige et al. (1972), who were able to recover a few exocortis-free citrus plants by using this technique. The technique was further standardized by Navarro et al. (1975), who named it as shoot-tip grafting (STG) (Juarez et al. 2015).The technique can effectively eliminate all the graft-transmissible virus and virus-like diseases in citrus with the success rate varying from 60 (tatterleaf, psorosis) to 100% (citrus viroids) (Carimi et al. 2001). The technique is now being used around the world for sanitizing infected citrus cultivars, for cleaning the exchanged material from virus and virus-like diseases and for producing the certified planting material. The technique has high impacts on the citrus industry as about hundreds of millions of plants propagated from the STG-derived budwood have been planted worldwide (Juarez et al. 2015). The basis of virus elimination through this technique is that the extreme shoot tip has actively dividing cells, which are devoid of vascular connection with the main plant body, due to which even systemically spread virus does not enter these cells (Juarez et al. 2015). The original technique involves the aseptic excision of the shoot tips of 0.1–0.2 mm size from decontaminated shoot tips and micro-grafting it on the rootstock seedling growing in vitro. For grafting, the 2–3-week-old rootstock seedlings (which are 3–5 cm tall) growing in vitro are decapitated at a height of 1.0–1.5 cm, and an inverted T-incision is made; the cotyledonary leaves are stripped off; and the root is cut back to 4–6 cm length (Navarro 1992). The shoot tip is placed inside the inverted T incision of the rootstock, so that its cut surface comes in contact with the cortex of the rootstock. Histological analysis has shown that callus is completely developed at graft union after 5 days of grafting, the vascular differentiation is initiated after 7 days of grafting and the vascular connection between the rootstock and the shoot tip is complete after 11 days of grafting, while 3–4 small leaves emerge from the grafted shoot tips after 4–6 weeks of grafting (Juarez et al. 2015). The technique has been complemented with other strategies like thermotherapy (Navarro 1992; Sharma et al. 2008) and chemotherapy (Sharma et al. 2007). Apart from clonal propagation, STG has also been used for facilitating regeneration of somatic hybrids, irradiated shoots, haploid and tetraploid cells/tissues incapable of regeneration following routine procedure, and for production of transgenic plants from transgenic shoots difficult to root in vitro (Juarez et al. 2015).

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In Vitro Androgenesis for Accelerated Breeding in Rice

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Abstract In vitro androgenesis by culturing male gametophyte is the preferred route for obtaining haploids in rice. Androgenesis is used for the production of haploids through anther/microspore culture, and further the developed haploids are converted to doubled haploids by spontaneous diploidization of chromosome number during tissue culture phase. Anther culture for the production of haploids in rice is being practised since the 1980s, and it has been well integrated into *japonica* rice breeding programmes. But it has not been exploited, to its full potential, in *indica* rice breeding, mainly due to its recalcitrant nature. Haploid plants are used in traditional plant breeding, plant biotechnology and molecular genetics for accelerated breeding thereby increasing the crop yield. Production of doubled haploid plants in breeding programmes shortens the period for development of new variety. There is a need for improving anther culture success for haploid production in *indica* rice. This chapter is focused on anther and microspore culture in rice, factors affecting anther culture, QTLs for anther culturability and applied aspect of doubled haploids in rice.

Keywords Anther culture · Doubled haploids · Microspore culture · Albino plants · Androgenesis · Accelerated breeding · Rice

1 Introduction

Rice (*Oryza sativa* L.; $2n = 2x = 24$) is an important cereal and a source of calories for one third of the world population. The genus *Oryza* contains 24 species, only two of which are cultivated, i.e. *Oryza sativa* (Watanabe 1997) cultivated in south-east Asian countries and Japan and *Oryza glaberrima* cultivated in West Africa. It is the staple food in Asia where 60% of the world's population lives and more than 90% of the world's rice is grown and consumed. Rice is planted over 150 million

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hectares annually, which covers about 10% of the world's arable land. Several biotic and abiotic stresses continue to threaten rice productivity. We need 25% more rice to meet the growing need of human population. Furthermore, the increased rice production has to meet with less land, less water, less chemicals and less labour. In order to meet the increasing demands of entire production to consumption chain, considerable efforts are being directed toward improvement of important traits using different types of conventional and unconventional approaches. Among these the doubled haploid technique has been viewed as a very useful technique for crop improvement. Haploid plants are sporophytes carrying the gametic chromosome number and are seed sterile; doubled haploid (DH) fertile plants are produced through spontaneous or colchicine-induced chromosome doubling of a haploid, whereas dihaploid plants ($2n = 2x$) are haploid plants obtained from an autotetraploid ($4x$) (Kasha and Maluszynski 2003). Doubled haploids (DH) have been recognized as a valuable tool in plant breeding since it offers potential to expedite crop breeding programmes by shortening breeding cycle of varieties through fixation of desirable gene combinations (recombinants) in the immediate generation. Homozygous lines can be produced in 2–3 generations unlike 6–7 generations through conventional breeding. Thus, varieties can be evolved in the shortest period with less effort. An ideal situation would be the rapid fixation of heterotic gene combinations from an F_1 generation to develop homozygous lines as good as heterotic F_1 hybrids. Thus, with DH approach, true-breeding lines are produced in the immediately succeeding generation. In doubled haploid population, selection efficiency is increased, as in anther culture the number of plants required to obtain the desired recombinants are less than the conventional breeding (Martinez et al. 1996). Production of DHs is an alternative approach for the development of inbred line through several cycles of inbreeding. The DH approach is increasingly being used for rapid development of mapping populations and construction of genetic linkage maps for traits of interest because they can be multiplied and reproduced without genetic change occurring over time (Semagn et al. 2006). These features enable the accurate measurement of quantitative traits and the reduction in the environmental component of the total phenotypic variance (Lu et al. 1996). Therefore it helps in introgression of desirable genes. Conventional methods used to achieve homozygosity consist of carrying out several selfings/backcrosses, and as such, they are time-consuming and labour-intensive procedures, whereas haploid development by in vitro *methods* includes anther/pollen culture, ovary culture and chromosome elimination from wide crosses. However, in rice, no chromosome elimination method is yet available, and androgenesis is a preferred and proven technology (Khush and Virmani 1996) for the production of haploids by culturing anthers/isolated microspores. In general, microspores divide mitotically and differentiate into multicellular male gametophytes or pollen grains. During androgenesis, the development of pollen grain is arrested and leads to the formation of haploids either by direct embryogenesis or via callus formation. Therefore, in microspore culture, there is induction of microspores to sporophytic instead of gametophytic pathway. The effectiveness of the technique depends on the efficiency of haploid plant regeneration from microspores contained within the anthers and the conversion of these

haploids to doubled haploids either spontaneously during tissue culture phase or induced thereafter. Another androgenesis method is the isolated microspore culture, but the reports on this method are rather limited. The scope of anther culture has also been expanded to gene transformation technology. Anther culture has considerable value in shortening the time required to convert the transgenic plants to homozygous breeding lines (Otani et al. 2005), and its role in producing marker-free transgenic rice has also been recognized (Zhu et al. 2007). Since the first report on production of haploids through anther culture by Guha and Maheshwari (1964) in *Datura*, the technique has been used to produce haploids in a large number of crop plants. Protocols for the production of haploids for over 250 plant species belonging to almost all families of the plant kingdom have been published (Maluszynski et al. 2003). In rice the first successful report of anther culture was made by Niizeki and Oono (1968), in *japonica* rice. Anther culture is now being used on routine basis for the production of haploids and homozygous doubled haploids in crop plants belonging to Gramineae, Brassicaceae and Solanaceae. In rice, anther culture has been well integrated into *japonica* rice breeding programmes to select for microspore-derived recombinants with improved traits such as high yield, disease resistance and better quality (Lapitan et al. 2009). The immense potential of the technique is evident from China, Korea and Japan where several varieties have been developed and released using the anther culture technique (Mishra and Rao 2016). The anther culture-derived, salt-tolerant variety PSBRc 50 'Bicol' has been released for commercial cultivation in the salt-affected areas of the Philippines (Senadhira et al. 2002). Induction of haploidy through isolated microspore culture performed by removing diploid anther tissue has also been reported in rice. Nitsch (1974) first cultured *Nicotiana* microspores that were shed naturally from cultured anthers. Eight years later, Lichter (1982) mechanically isolated microspores from *Brassica* buds prior to culturing them. Since then studies have been focused on increasing the frequency of microspore embryogenesis with responsive species and on developing protocols for recalcitrant species. Despite the progress that has been made in microspore culture, many species are still considered recalcitrant. Though many reports have been published (Cho and Zapata 1988; Cho and Zapata 1990; Datta et al. 1990; Ogawa et al. 1995; Xie et al. 1997; Raina and Irfan 1998), however, low levels of callus induction, genotypic response and plant regeneration have limited isolated microspore culture as a replacement for anther culture in DH rice plant production. Islam et al. (2013) reported that microspore isolation by homogenizer produces high yield of embryoids and green plant regeneration. Though the technique of isolated microspore culture requires extra equipment and more skills as compared to anther culture, yet it is the better method for understanding cellular, physiological, biochemical and molecular processes involved in pollen embryogenesis (Nitsch 1974; Reinert and Bajaj 1977). It has been observed that there is diffusion of nutrients through the anther walls that helps in the induction of sporophytic divisions in pollen. Anther wall also served as a filter by preventing excessive concentrations of Fe around the microspores within the anther, even when the concentrations present in the culture medium were high. This protective role of the cell wall against toxic elements such as Cd, Zn and Ni was also shown by Kramer et al. (1997) and Kupper et al. (2000).

2 Factors Affecting Anther Culture

Anther culture in rice is accomplished in two steps: the first step involves the induction of embryogenic calli from microspores, and the next step deals with the regeneration of green plants from the anther-derived calli (Fig. 1a–h). Anther culture success depends on various factors, viz. developmental stage of microspores (Chen 1976), period of cold pretreatment of anthers (Zapata et al. 1982), growing conditions of donor plants (Lee et al. 2003), composition of culture medium (Mandal and Gupta 1997), orientation of plated anthers (Mercy and Zapata 1987) and genotype of donor plant (Shen et al. 1983). Efforts have been made to optimize media requirements for androgenesis and green plant regeneration in *indica* rice (Guiderdoni et al. 1992; Lentini et al. 1995; Sathish et al. 1995; Raina and Zapata 1997).

2.1 Physiological State of the Donor Plant

Physiological state of the donor plant is an important factor which determines the success of anther culture. The release of microspores from the anthers and their subsequent divisions leading to plant regeneration depend on the conditions under which

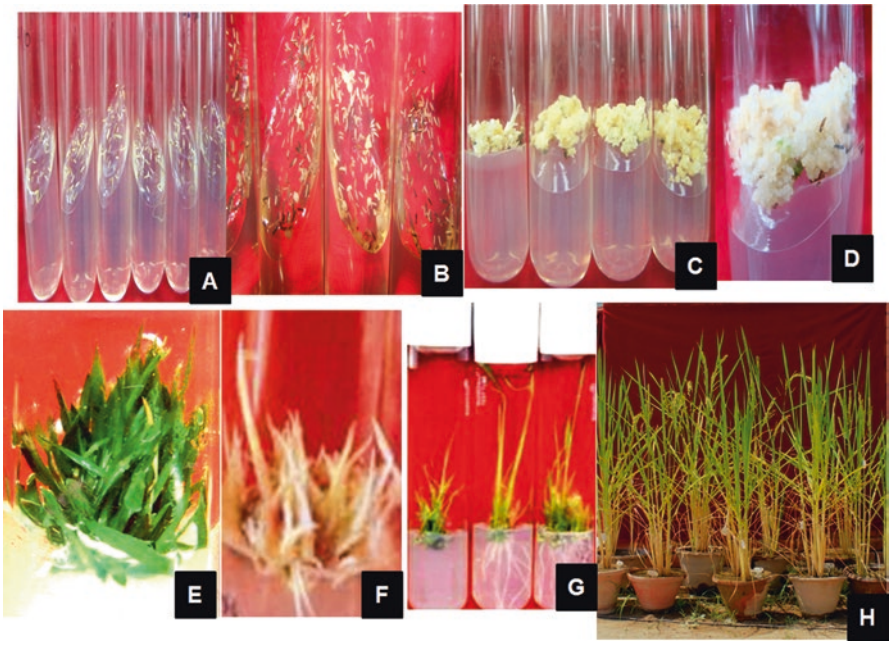

Fig. 1 Androgenesis through anther culture in rice. (**a**) In vitro anther culture. (**b**) Initiation of callus. (**c**) Proliferation of androgenic calli. (**d**) Induction of plant regeneration. (**e**) Regeneration of green plant. (**f**) Regeneration of albino plant. (**g**) Rooting of regenerated plants. (**h**) Transfer of plantlets to soil in pots

the donor plants grow. Therefore, for better anther culture response, the primary requirement is to ensure the quality of microspores which further depends on the growth conditions of donor plants. Many factors that affect plant growth, such as age of donor plant, temperature, season, light intensity, photoperiod, growing conditions and nutritional status, ultimately affect the response of anther culture. Temperature variations during booting stage of donor plants affect the development of microspores in vivo as well as androgenesis of microspores in vitro (Raina 1989). It has been observed that long days (>12 h), intense solar radiation (>18 M j/m^2) and higher day/night temperature regime of 34/24 °C at panicle emergence stage are best for increasing the efficiency of anther culture in *indica* rice. Raina (1997) and Veeraraghavan (2007) observed that anthers collected from field-grown plants exhibited better response to anther culture as compared to anthers collected from pot-grown plants placed in the greenhouse or near the field. On the other hand, Vladislavovna (2015) reported that when donor plants were grown in growth chamber, callus induction and plant regeneration were better as compared to the donor plants grown in open space. But response to callus induction and plant regeneration was not observed in all the genotypes grown in growth chamber. Similarly, high degree of success of anther culture response was observed in anthers collected from panicles of plants grown under optimized conditions of phytotron with controlled light, temperature and humidity as these enable plants to maintain a healthy growth without any disease and insect (Datta 2005). Moreover, these controlled conditions extend the time for panicle formation and provide longer duration during which the panicle at the appropriate stage of development can be selected for the inoculation of anthers; therefore, during season there is no limitation of season on the availability of anthers. It is also possible to reduce the intensity of disinfection of panicles by any surfactant.

Thermoperiod and photoperiod under which the donor plants were grown have also effect on anther culture response. *Indica* rice gave best anther culture response when donor plants were grown at low temperatures of 18–20 °C (Hu 1985) and 23.2–34.2 °C (Raina et al. 1987). Sun et al. (1992) obtained high callus induction of 42.2% when *indica* plants were grown at 26 °C under 14 h photoperiod. Therefore, mid- or short photoperiod and mid- or low temperatures were needed for high callus induction. Ling et al. (1990) observed that photoperiod-sensitive and photoperiod-insensitive genotypes respond equally to callus induction from anthers. The growth conditions of the donor plants used for pollen culture have a profound effect on the embryogenic response. It has been observed that there was significant influence of donor plants' growth conditions on callus induction and plant regeneration (Datta 2005; Goncharova 2012). However, Vladislavovna (2015) observed that different types of growth conditions of donor plants have profound effect on shoot regeneration from embryos but there was no difference in anther callus induction. Low temperature affected plant regeneration and led to spontaneous doubling of chromosomes particularly in case of abrupt stress, whereas exposure of donor plants to high temperature had an adverse effect on green plant regeneration (Huang et al. 1983). Guzman and Arias (2000) reported that callus induction and frequency of green plant regeneration were increased in anthers derived from ratooned plants than that of the main crop plant. The frequency of callus induction was raised when anthers of ratooned plants were cultured on N_6 medium containing ABA (5 and 10 mg/l)

and 2,4-D (2 mg/l). Efficiency of green plant regeneration was also increased from 30% to 70% when ABA (10 mg/l) was used in MS medium containing BAP (2 mg/l), NAA (1 mg/l) and kinetin (2 mg/l). Furthermore, Chen and Tsay (1986) reported that the frequency of callus formation was significantly high in anthers collected from primary and secondary tillers than tertiary tillers. Anther culture response is also affected by the maturity of donor plants as callus formation from the anthers of middle and lower part of panicles is high as compared to upper part (Jacquard et al. 2006). Overall it is not possible to make general recommendations about the optimal growth conditions of donor plant, as they seem to vary between genotypes. Therefore, androgenic response can be enhanced by carefully nurturing the donor plants and growing them under favourable environmental conditions. In an intervarietal breeding programme, the most common practice is to use anthers of F_1 hybrids for anther culture, thereby fixing the products of recombination between the two parental genomes at earliest. However, it is suggested that anthers from F_2 generation plants are more responsive and green plants recovery is also increased than the anthers from F_1 hybrids and parental rice varieties especially for characteristics such as disease resistance and grain quality (Shih-Wei and Zhi-Hong 1991; Meifang 1992; Bishnoi et al. 2000). In a breeding programme, 100–150 doubled haploids are sufficient for the purpose of selection (Zongxiu and Chengzhang 1992), whereas in basmati rice 1000 plants are required so as to have intact basmati rice quality traits.

2.2 Pretreatment

Pretreatment of anthers and isolated microspores prior to culture has shown to improve androgenesis in rice (Shariatpanahi et al. 2006). Pretreatments are given in the form of temperature stress, osmotic shock and sugar starvation (Bhojwani and Razdan 1996). Optimization of temperature for pretreatment of anthers of a particular genotype is very important to increase rice anther culturability (Nurhasanah et al. 2016).

2.2.1 Cold Treatment

Cold shock is the most commonly used pretreatment in rice anther culture as it delays the senescence of anther wall tissue thus allowing sufficient time for anther wall tissue to nurture developing microspores (Raina 1997). It also increased the symmetric division of pollen grains and release of amino acids and shock thermic proteins which are necessary for androgenesis (Kiviharju and Pehu 1998). As a result the degeneration of microspores gets delayed. The importance of cold pretreatment has also been reported by Chen (1976), Zapata et al. (1982), Lai and Chen (1984), Tsay et al. (1988) and Ogawa et al. (1992). Treatment of the rice anthers before culture by low temperature influences on the release of microspores from the

anther and their subsequent divisions leading to plant regeneration. In different experiments, temperature and treatment duration varied with the variety, and panicles were subjected to a range of temperatures from 4 to 12 °C for 7–30 days. In general, the optimal temperature recommended for pretreatment in many varieties of rice is 8–10 °C for 1 week, whereas a longer cold treatment resulted in a higher frequency of albinos among the regenerants (Gupta and Borthakur 1987). Herath et al. (2009) reported that cold pretreatment at 8 °C for 14 days is effective for *indica* and *japonica* rice varieties and inter-subspecific hybrids. Vladislavovna (2015) pretreated panicles of five rice hybrids at 5 °C for 7 days. Recently, it was reported that cold pretreatment at 10 °C for 7–9 days is the most effective for callus induction and plant regeneration in *indica* rice genotypes (Mishra et al. 2013; Kaushal et al. 2014).

2.2.2 Heat Shock Treatment

High temperature pretreatment disrupts the normal integrated development of somatic anther tissue (Dunwell et al. 1983) as it disrupts the cytoskeleton in microspores at the initial phase (Ferrie and Keller 1995). Heat treatment for short period has been reported for callus induction from rice anthers. Reddy et al. (1985) induced callus from rice anthers by pretreating them at 35 °C for 5 min followed by cold treatment at 10 °C for 7 days. Zapata and Torrizo (1986) reported an increase in callus induction frequency from anthers treated at 35 °C for 15 min prior to cold treatment at 10 °C for 7 days. Sathish et al. (1995) found that a heat treatment of immersing the panicles in water bath at 32 °C for 2.5 or 5 h enhanced the ability of anthers to initiate callusing. At the temperature 10 °C, frequency of callus induction was higher than that at 5 °C. The treatment of anthers by 5 °C temperature was effective for plant regeneration in comparison with 10 °C.

2.2.3 Osmotic Stress Pretreatment

Osmotic stress or loss of cellular water content often disrupts the plasmodesmatal connections between the pre-embryonic cells, making the cells physiologically isolated and allowing a greater number of cells to differentiate (Wetherell 1984). Several reports have shown that mannitol induced osmotic stress in microspore/anther culture of rice enhances androgenic calli and regenerants in *indica* and *japonica* cultivars (Cistue et al. 1994; Raina and Irfan 1998; Mandal and Maiti 1999). Osmotic stress has been used in place of cold pretreatment and also in combination with cold treatment. In the absence of cold treatment, mannitol treatment promoted androgenesis in anther culture of *indica* cultivar IR 43 from 3% to 33%, while mannitol treatment combined with the cold treatment was detrimental, and it had no promotory effect on anther culture in rice (Pande 1997; Kaushal et al. (2014).

2.2.4 Sugar Starvation

Sugar starvation is effective in inducing embryogenesis, particularly in isolated microspores for 3 days; Ogawa et al. (1995) reported that sugar starvation of anthers of *indica* rice variety IR 43 initially for 2 days enhanced androgenesis 12-fold, which is more than mannitol pretreatment. They further concluded that sugar starvation could substitute, to some extent, the cold treatment, but the cold treatment was superior to sugar starvation for the induction of androgenesis in microspore cultures of *indica* rice. Raina and Irfan (1998) also reported increase in androgenesis with sugar starvation of microspores isolated from anthers of *indica* and *japonica* rice.

2.2.5 Gamma Radiation

Pretreatment of anthers with gamma radiation has proved beneficial (Zapata and Aldemita 1989). Chen et al. (2001) reported prolific green plants regeneration by radiating rice anthers with a gamma ray dose of 20 Gy. Mkuya et al. (2005) enhanced doubled haploid production and green plant regeneration in *indica* rice line TM7–5 upon irradiation of anthers with gamma rays at 20 Gy.

2.3 Culture Medium

The nutrient medium not only provides nutrition to the microspores but also directs the pathways of embryo development. The source of carbon, macronutrient (particularly the form in which nitrogen is supplied in the medium), micronutrients and plant growth regulators may determine whether the androgenesis will be initiated or not.

2.3.1 Basal Medium

Culture medium is another important component for producing embryos/callus from cultured anthers/microspores. Nutrient requirement may differ for the induction of androgenesis and for growth of developing embryos. Chu et al. (1975) developed N6 medium with reduced $(NH_4)_2SO_4$ and increased KNO_3 specifically for anther culture of rice; this medium has subsequently been demonstrated to be most suitable (Genovesi and Magill 1979; Chu 1981; Chen et al. 1982; Tsay et al. 1982). Both B5 (Gamborg et al. 1968) and modified LS (Linsmaier and Skoog 1965) which also have high concentrations of nitrate nitrogen and reduced concentrations of ammonium nitrogen have also been found to give good results (Chaleff and Stolarz 1981; Zapata et al. 1982). Gioi and Tuan (2002) while comparing the basal medium observed that callus formation was best in N6 medium as compared to LS and MS media. Huang et al. (1978) modified N6 medium and developed He2 medium amended with half-strength ammonium, double-strength KH_2PO_4 and one fiftieth

the concentration of $MgSO_4$. Kaushal et al. (2014) proved He2 to be a superior medium for callus induction, green plant regeneration and least albino plant development, followed by B5, SK1 showing moderate level and N6 medium producing lowest frequency of callus induction and green plant regeneration. The better response of B5 and SK1 seems to be due to lower-level $NH4^+$ (1/4 or 1/2 strength compared to N6) in induction media. Higher anther response from He2 medium was also observed by Mandal and Gupta (1997) and Reddy et al. (1985), whereas Silva and Ratnayake (2009) and Rukmini et al. (2013) found N6 medium to be superior as compared to MO19 and SK1 media. Subsequently, several other media, Heh 5 (Reddy et al. 1985), potato 2 (Rout et al. 1989), RZ (Raina and Zapata 1997), etc., have been used to improve anther culture response in *indica* rice. The ammonium nitrogen level in the medium was a crucial factor (Chu et al. 1975; Chu 1981; Gosal et al. 1991; Gosal et al. 1997; Sandhu et al. 1993), and its requirement by *indica* cultivars is different from that of *japonica*. N6 medium widely used for rice anther culture was unsuitable for *indica* rice anther culture (Gosal et al. 1997). It was also suggested by Huang et al. (1978) that *indica* genotypes require low NH4+ just half of the concentration required by *japonica*. The higher concentration of ammonium ions in the N6 medium is less suitable for the tested *indica* genotypes, and hence there is poor anther response in N6 medium. Yang et al. (1980) used modified N6 medium by replacing $(NH_4)_2SO_4$ and KH_2PO_4 with NH_4HPO_4 for *indica* cultivars. Chen et al. (1986) observed a significant increase in anthers forming callus when 0.1 mM Na_2Fe EDTA was added to the induction medium; however, further increase in the amount of iron was deleterious. Several modifications have been made to improve anther culture of *indica* rice. Micronutrients also play an important and sometimes crucial role in normal plant growth and development. Copper and zinc are two important micronutrients influencing microspore embryogenesis. For callus induction from rice anther cultures generally solid medium is used. However, it has been observed that agar solidified media increased anther necrosis. Therefore, Lentini et al. (1995) used liquid media for callus induction as they observed that media solidified with agar increased anther necrosis. It has been suggested that liquid medium provides microspores and calli with greater access to nutrients and hormones while dispersing more rapidly the toxic substances released from dying or dead anthers. The tendency for the rice anthers to sink in liquid media and rapidly lose viability has been a major reason for avoidance of liquid culture conditions by many (Raina 1997). However, efforts made to keep anthers afloat and viable in liquid medium, by adding substances such as Ficoll that increases buoyancy, have been reasonably successful. Embedding anthers in agarose was also found better than culturing on semi-solid or liquid media (Gill et al. 2000).

2.3.2 Hormones

The effect of plant growth regulators has been widely investigated in rice anther culture. The type and concentration of growth regulators as well as their interactive presence can be the deciding factors that would influence pollen embryogenesis

(Ball et al. 1993). It is regarded as important parameter in determining the success of induction of calli and subsequent plant regeneration. The combinations of hormone type and concentration of hormones can greatly affect the development of microspores and impact the morphogenetic process leading to the production of plants (Trejo-Tapia et al. 2002). Ball et al. (1993) stated that the type and concentration of growth regulators as well as their interactive presence can be the deciding factors that would influence pollen embryogenesis. Numerous detailed and extensive studies were undertaken to analyse a broad-spectrum type of auxins and cytokinin for culture initiation. Both auxin and cytokinin are crucial constituents in rice anther culture medium and control the dedifferentiation and redifferentiation in the in vitro cultures. The auxin 2,4-D is the most widely used growth regulator irrespective of the explants in all cereal crop species. Earlier studies have proved the 2,4-D as potent auxin for in vitro development of rice microspores, and hence it was always included in the culture medium either singly or in combination with a cytokinin and/or with other auxins (Niizeki and Oono 1968; Guha et al. 1970; Iyer and Raina 1972; Wang et al. 1974). Among the synthetic auxins 2,4-dichloro phenoxyacetic acid (2,4-D) and naphthalene acetic acid (NAA) were commonly used for callus induction from rice anthers (Zhu et al. 1998). Other auxins such as phenylacetic acid (PAA), picloram and dicamba have also been used either alone or in combination with 2,4-D for their usefulness in in vitro anther response (Lentini et al. 1995). The callus induction from anthers was better when 2,4-D is used with picloram in comparison to use of 2,4-D with NAA. The effect of dicamba was similar to 2,4-D with respect to callus induction. Incorporation of PAA in the callus induction medium was detrimental for anther response and inhibited callus induction frequencies by about 10 times even in the most responsive genotypes and plant regeneration by as much as 20 times (Lentini et al. 1995).

Nevertheless, prolonged exposures of cell cultures to high concentration of 2,4-D affect the frequency of regenerated plants and cause chromosomal abnormalities (Deambrogio and Dale 1980; Ziauddin and Kasha 1990). Superiority of 2,4-D for callus induction and green plant regeneration has also been demonstrated by Zhu et al. (1998) and Bregitzer et al. (1995). Besides, 4-amino-3,5,6-trichloro picolinic acid (picloram) has also been used as an alternate synthetic auxin in rice anther culture. Surprisingly, regeneration from cultures derived from picloram-containing medium was almost twice as efficient as regeneration from cultures induced on 2,4-D (Barro et al. 1999). Mondoza and Kaeppler (2002) and Sharma et al. (2005) reported that a significant increase in the frequency of plant regeneration was caused by picloram that was confirmed for embryo-derived callus cultures. Chen et al. (1991) reported that callus-forming ability from anthers of rice was high in medium supplemented with 2,4-D, but the regeneration ability from these calli was quite low as compared to calli formed on medium supplemented with NAA. However, replacement of 2,4-D with picloram in the callus induction medium appeared to have a beneficial effect on green plant regeneration. It has also been observed that NAA promotes the formation of roots and sometimes completes plants, while 2,4-D inhibits the organogenesis of calli (Cornejo-Martin and Primo-Millo 1981). A combination of auxins [2,4-D (2,4-dichlorophenoxyacetic acid), NAA (naphthalene acetic acid), IAA (indole acetic acid) and cytokinin [BA (benzyladenin), kin. (kinetin),

zea (zeatin)] has been widely used in rice anther culture media (Niizeki and Oono 1968; Guha et al. 1970; Iyer and Raina 1972). Application of higher dose of auxins significantly increase the callus induction efficiency, however such calli are less embryogenic and poor in green plant regeneration. Therefore, optimum level of auxin in the callus induction media is required. Ling et al. (1990) reported that hormone requirement is genotype specific, and different genotypes respond differently under different hormone concentrations. Combination of both auxins 2,4-D (0.5 mg/L) and NAA (2.5 mg/L) with kinetin (0.5 mg/L) has been used effectively to induce callus from *indica* varieties and hybrids (Shahnewaz et al. 2003; Shahnewaz and Bari 2004). The regeneration frequency of calli produced in the presence of both auxins was also reported to be higher (Raina 1997).

2.3.3 Sucrose

A carbohydrate source is essential in anther culture because of its osmotic and nutritional effects (Bishnoi et al. 2000). Sugars in a culture medium function both as a major carbohydrate source and an osmotic regulator. Both functions are critical for embryoid or callus formation (Last and Brettell 1990). Sucrose doubles the osmolality of the medium as it is rapidly hydrolyzed to glucose and fructose. Sometimes sucrose is toxic to androgenesis due to sensitivity of microspores to fructose. Although glucose seems to help initiate or promote the early stages of development in anther culture, it becomes inhibitory, unless it is rapidly metabolized by anthers (Raina and Zapata 1997). High sugar concentrations have been found beneficial for the induction of sporophytic growth from rice microspores (Lentini et al. 1995). But Wang et al. (1978) reported increase in albino plant regeneration by using high (6%) concentration of sucrose in the culture medium. However, maltose (4–5%) has been shown to be a superior source of carbohydrate than sucrose. Bishnoi et al. (2000) substituted sucrose with maltose and reported increase in androgenesis in all the media compositions used. With the addition of maltose to the induction medium, the plant regeneration rate was increased, while albino plant regeneration frequency was minimized (Raina and Irfan 1998). Certain sugar alcohols such as mannitol (Raina and Irfan 1998) and sorbitol (Kishore and Reddy 1986) had beneficial effects on rice anther culture.

2.3.4 Nitrogen Source

Nitrogen can be supplied to the culture medium in an inorganic or organic form. The inorganic nitrogen is usually introduced in the form of nitrate or ammonium ions, while nitrogen in the organic form can be supplied as vitamins and amino acid supplements. The ratio of NO_3^- to NH_4^+ has been observed to be an important determinant for success of anther culture in *indica* rice (Grimes and Hodges 1990). N6 medium containing high KNO_3 and NH_4 SO_4 has proved to be very efficient for *japonica* rice anther culture. The *indica* cultivars require even lower levels of NH_4^+ ions. The MSN and SK-1 media which are derived from N6 medium have been used

frequently in *indica* rice anther culture. SK-1 medium has inorganic nitrogen in the form of nitrate ions and without ammonium ions. A comparative study proved that SK-1 was overall a better medium for *indica* rice anther culture because green plant regeneration was higher from callus induced on this medium than from callus induced on MSN. However, Raina et al. (1989) reported that callus induction from anther culture was better on MSN medium than SK-1 medium. Further Raina and Zapata (1997) have reported RZ medium having combination of both nitrate and ammonium ions at appropriate concentrations that induced better callus induction and green plant regeneration. Anther culture on He2 medium and a modified SK-1 medium which had lower NH_4^+ had beneficial effects on callus induction and subsequent regeneration of green plants from induced callus in *indica* rice (Lentini et al. 1995). Organic nitrogen supplements such as casein hydrolysate (CH) which is a source of calcium and several micronutrient, vitamins and amino acids added to the medium have been particularly beneficial for positive anther culture response (Roy and Mandal 2005) although a few reports suggest otherwise (Lentini et al. 1995; Raina and Zapata 1997).

2.3.5 Organic Substances

Various natural extracts such as yeast extract (YE) and coconut water (CW) have also been found to be beneficial for increased callus formation and subsequent plant regeneration in anther culture (Lentini et al. 1995; Raina and Zapata 1997). There are reports describing both the positive and negative effects of casein hydrolysate on callus proliferation from rice microspores (Lentini et al.1995; Gosal et al. 1997).

2.3.6 Amino Acids

On the other hand, amino acids such as glutamine and alanin have proved to be useful for callus formation and green plant regeneration from microspore cultures in *indica* rice varieties (Ogawa et al. 1995). Putrescine can improve androgenesis and enhance embryo or callus formation from microspores by inhibiting early senescence of cultured anthers. Putrescine is a naturally occurring low molecular weight polycation that is an obligate requirement for cell growth and sustenance. It has been implicated in many important cellular processes such as cell division, protein synthesis, DNA replication and response to abiotic stress (Kakkar and Sawhney 2002).

2.3.7 Ethylene Inhibitors

Low response in *indica* rice is due to early senescence of cultured anthers, and senescence is accelerated with in vitro *generation* and accumulation of the ethylene in sealed culture vessels (Silva 2009). This ethylene is produced by plant cells in the presence of auxin (Yang and Hoffman 1984), sucrose (Zhou et al. 1991) or calcium

(Hepler 2005) in the callus induction medium. Studies showed that addition of silver nitrate ($AgNO_3$) in the induction medium blocks the inhibitory effect of endogenously produced ethylene from excised anthers (Ghamemi et al. 1994). Thus, the use of $AgNO_3$ in the callus induction medium enhanced both callus inductions and plant regeneration from these calli (Lentini et al. 1995). However, $AgNO_3$ has a greater effect on the induction of morphogenic callus than the subsequent regeneration of green callus transferred onto regeneration medium with $AgNO_3$. Use of maltose than sucrose in the callus induction medium may also inhibit the induction of ethylene because the in vitro generation of ethylene in excised tissue is promoted by sucrose (Zhou et al. 1991). Polyamines such as putrescine also inhibit the ethylene biosynthesis because they compete for the same substrate S-adenosyl-methionine (SAM) in their biosynthetic pathway (Dewi and Purwoko 2008), hence further delaying the senescence of cultured anthers in both *japonica* and *indica* varieties (Sasmita 2007).

2.4 Pollen Development Stage

Pollen development stage for androgenesis depends on the stage of maturity of the pollen grains at the time of culture. As compared to the uninucleate stage of pollen, anther culture response is less when pollen is at tetrad stage and reduces further after the first pollen mitosis. Furthermore, at the tetrad stage, starch deposition in the microspore begins, and there is no sporophytic development. However, upon cold pretreatment at this stage, normal development of microspore stops, and sporophytic growth starts. Hence, the most suitable stage of pollen development for anther culture in rice is the late uninucleate to early binucleate stage as well as early to mid-uninucleate stage (Jahne and Lorz 1995). However, this stage may vary from one genotype to another. Therefore, before starting large-scale anther culture programme in a particular genotype, pre-examination of pollen to determine most responsive development stage is imperative. For this, mostly an easily observable morphological trait of the plant that shows good correlation with the pollen development stage is used as a guide to identify the required stage of pollen. Usually the distance between the collar of the flag leaf and ligule of the penultimate leaf of the tiller is used to define the appropriate stage of pollen development (Bishnoi et al. 2000). Previous studies have shown that florets having anther length of less than half of the size contain anthers having microspores of mid- to late uninucleate stage (Niroula and Bimb 2009).

3 Plant Regeneration

It was observed that quality of callus plays a significant role in plant regeneration. The embryogenic calli which were milky white in colour and compact in texture had excellent regeneration ability. On the contrary, friable calli had poor plant regeneration ability or did not respond at all. These results clearly suggest that the

callus induction medium has an influence on the morphogenic competence of the induced callus, determining its regeneration capability. Growth regulators used in the regeneration medium and their effects on green plant production have been analysed to a lesser extent than in the case of callus induction. It has been reported that the cultivars that display high callusing ability show the best regeneration frequencies (Javed et al. 2012), but He et al. (1998) observed that genotypes which showed high callus induction have displayed poor regeneration ability and vice versa. The higher callus induction observed might attributed to higher doses of auxins in the callus induction medium or may be due to poor quality of callus. Hence, embryoid induction and plant regeneration are independently inherited traits (Forough-Wehr et al. 1982; Deaton et al. 1987). Therefore, media modifications should target the production of embryogenic callus with good regeneration ability rather than simply inducing prolific callusing. Therefore, the priority should be given to the frequency of green plants regeneration rather than high frequency of callus induction. Gioi and Tuan (2002) reported that rice callus induced in early stage (around 30–50 days after inoculation) offers high differentiation for green plants. Sripichitt et al. (2000) have reported that regeneration of the calli was influenced by the callus induction medium. In *indica* rice, regeneration ability of callus induced under high 2,4-D levels is poor, in comparison to callus induced on medium with lower 2,4-D levels. Sohn et al. (1997) and Daniel (2006) observed that callus formation and plant regeneration were higher in the medium containing picloram (1 mg/L) than that of 2,4-D (1 mg/L). However, Bishnoi et al. (2000) reported that medium containing low levels of 2,4-D (0.5 mg/L) is very effective for callus induction medium and appeared eventually to be the most effective for green plant regeneration also. Silva and Ratnayake (2009) reported that by increasing concentration of agarose from 0.5% to 1.0%, shoot regeneration increased over eightfold in *indica* rice varieties. On comparing basal medium, it has been observed that MS medium was better for shoot regeneration than N6 medium (Mandal and Gupta 1995; Rout et al. 2016).

4 Albino Plant Production

The occurrence of a large proportion of albinos among the regenerated plants following anther culture is the serious drawback of androgenesis. Albinism is a widely stated phenomenon in the rice anther culture especially of *indica*, which might be due to the long culture duration, culture medium and the genotype that restricts the use of anther culture in breeding programmes (Chen et al. 1991; Asaduzzaman et al. 2003). Another factor for albino plant production might be the stressful in vitro conditions which make the plant cells fight their own plastids with antibiotic-like compounds (Torp and Andersen 2009). Yamagishi (2002) observed the presence of large-scale deletions in some plastid genomes of the albino haploid plants derived from anther culture of *japonica* x *indica* hybrids, while such deletions are absent in green regenerants. Hence, the role of plastid genome is suggested in the determination of the albino phenotype. Kumari et al. (2009) studied that the basic cause of albinism in rice

is the impairment of DNA in plastids or nuclei or in both of them and absence of 23S and 16S rRNA (Zubko and Day 2002). George and Sherrington (1984) reported that the accumulation of ethylene in culture vessel might inhibit chlorophyll synthesis and chloroplast development that lead to albino plant formation. The frequency of albino plants developed was lower across the genotypes and media as compared to the frequency of green plants, and it may vary from 5% to 100% (Talebi et al. 2007). The frequency of albino formation was lowest in medium He2 (3.72%), followed by B5 (7.26%), SK1 (7.28%) and 10.65% in N6 medium (Kaushal et al. 2014). Albino plants are frequently regenerated from pollen-derived calli; therefore, the conditions which will lead to direct pollen embryogenesis may be the means to overcome albinism. Albinism could be considerably reduced by shortening the culture period (i.e. frequent subculturing). Replenishing the media to avoid depletion of some of the essential micronutrients and balancing the pH often help in the conditioning of the cultures and their development (Datta 2005). Sah (2008) reported that higher rate of albino plant production might be attributed to higher rates of 2,4-D, while Sohn et al. (1997) reported that albinism might be due to use of high concentration of picloram. Even though albinism is genetically determined, the trait is amenable for manipulation, at least to some extent, by reducing the hormone concentration and by combination of starvation and cold stresses for short period (Torp and Andersen 2009). QTLs which control the frequency of albino plants among regenerated rice plants have been identified on chromosomes 9 and 10 (Yamagishi et al. 1998).

5 Genotype

Genotype is a deciding factor in achieving the success of anther culture in rice. Studies have shown that callus induction and anther culture response to plant regeneration were highly dependent on the genotype of donor plants (Bishnoi et al. 2000; Lee and Kim 2004). The average frequency of green plant regeneration from cultured anthers of *indica* rice was only 1% in comparison with the 10% of *japonica* rice (Hu 1985). Though, most of the Basmati rice (aromatic *indica*) varieties have been reported to be closer to *japonicas* based on isoenzyme polymorphism (Glaszmann 1987). But, unlike *japonicas*, basmati rice varieties have proven recalcitrant to anther culture (Raina et al. 1987; Jain et al. 1996). Miah et al. (1985) reported that the anther culture response varied from 41% for a *japonica* cultivar to 0% for an *indica* cultivar and even among the *indica* cultivars, a considerable variation for pollen callusing and plant regeneration was noted. Grewal et al. (2011) also observed low anther response of 1.2% in *indica* cultivars while 28.1% (20-fold higher) in *japonicas*. The different genotypes hold different potential for callus induction on different media (13.72–35.79%). Silva (2010) reported that the low response of *indica* genotype is due to early senescence of cultured anthers. A number of varieties and improved breeding lines have been developed through anther culture mostly in *japonica* cultivars in China, Korea, Japan and the USA. In general, *indica* cultivars have low anther culture response (Dewi et al. 2009). The recalcitrance of *indica* cultivars relates to early

anther necrosis, poor callus proliferation, low plant regeneration and frequent occurrence of albino plant regeneration (Balachandran et al. 1999; Chen et al. 2005; Silva 2010). However, some attempts have been made to overcome low anther culturability by evaluating different culture media compositions (Ogawa et al. 1995; Shimada et al. 1999; Bishnoi et al. 2000). However, most developments have occurred in temperate *japonica* cultivars, while success with tropical *indica* types has remained poor (Brar and Khush 2003). As a result, anther culture is now used as a supplementary breeding tool in *japonica* rice (Brar and Khush 2006), but the potential of the technique for *indica* rice breeding is yet to be fully exploited in spite of an initial report of the release of a salt-tolerant *indica* variety through anther culture breeding (Senadhira et al. 2002). Gueye and Ndir (2010) reported that *O. glaberrima* genotypes produced more callus than *O. sativa* genotypes. Furthermore, all the plants regenerated in *O. glaberrima* genotypes were green in colour, but in *O. sativa* many albino plants were obtained. Previously many researchers observed the general trend of callus induction ability in different *japonica* and *indica* varieties and their hybrids, as *japonica* (28%) > *indica*/*indica*/*japonica* (14.4%) > *japonica*/*indica* (11.2%) > *indica*/*indica* (5.4%) > *indica**2/*japonica* (4.4%) > *indica* (1.2%) (Guiderdoni et al. 1992; Yan et al. 1996; Grewal et al. 2011). Therefore, anther culture response is largely species and genotype dependent.

6 Genetics and Inheritance

The studies conducted by different researchers on the *indica* and *japonica* varieties and their hybrids (Silva 2010) pointed toward a general consensus that callus induction from cultured anthers is controlled by additive genes to a large extent with the nonadditive effects being less important (Miah et al. 1985; He et al. 2006; Bagheri and Jelodar 2008). For green plant regeneration, additive, nonadditive and in some instances cytoplasmic genetic sources have been suggested to play role (Yan et al. 1996). Callus induction ability is inherited as a recessive character, while genetic control of green plant regeneration on the other hand is less well established. Moreover, Grewal et al. (2011) reported that the genes for anther culturability are partially dominant. It is also reported that the additive genetic variation component for anther culture traits is higher in *japonica* than in the *indica* varieties (Yan et al. 1996) thus indicating that the genetic determination of the trait is stronger in *japonica* types. A diallel analysis has revealed that anther culturability is a quantitative trait controlled by the nuclear genome (He et al. 2006; Bagheri and Jelodar 2008; Miah et al. 1985). Hennawy et al. (2011) observed a significant and negative better parent heterosis in 5 out of 15 hybrids for callus induction. Therefore, considerable genetic gain can be made in callus induction trait by hybridization and selection of higher responsive cultivar and recalcitrant *indica* types, but achievement in the transfer to green plant regeneration ability may not be of comparable magnitude since the gene controlling the trait shows less additive effects and relatively low heritability (Zhang and Qifeng 1993; Yan et al. 1996).

7 QTL Mapping for Anther Culturability

The genes responsible for anther culture response have been mapped to specific chromosomal regions in maize (Cowen et al. 1992; Murigenux et al. 1994; Beaumont et al. 1995) and barley (Devaux and Zivy 1994). However, only three reports (He et al. 1998; Yamagishi et al. 1998; Kwon et al. 2002) of QTL mapping for anther culturability in rice are available. Yamagishi et al. (1998) identified QTLs on chromosome 1 and chromosome 10 of rice for controlling callus formation and balance between albino and green plant regeneration capacities, respectively. He et al. (1998) identified QTLs on chromosomes 1, 6, 7, 8, 9, 10 and 12 for callus induction and green plant differentiation and a major QTL for albino plant differentiation on chromosome 9. QTLs that influence green plant regeneration have also been mapped on chromosomes 3 and 10, and molecular markers that co-segregate with these genes have been identified (Kwon et al. 2002). The biotechnological approaches that would assist in the reliable screening of germplasm for good and bad genotypes for anther culture ability by the development of molecular markers linked to QTL of tissue culture response can help to speed up the development of high responding lines with improved anther culture ability in *indica* rice through introgressive breeding. Also, for addressing the nonadditive gene effects, the components of tissue culture media had also been demonstrated to have a crucial role in coaxing an in vitro response from cultured anthers of otherwise recalcitrant genotypes. Doubled haploid populations are extremely useful in the development of molecular maps and in tagging genes for important agronomic traits.

8 Ploidy Level of Plants

The haploid set of chromosomes in microspore-derived plants spontaneously doubled under culture conditions. This spontaneous doubling is up to 72% in rice. Anther-derived plants with different ploidy levels can be readily distinguished on the basis of plant morphology, fertility data and cytogenetic characterization. Morphologically haploid plants grow slowly and have relatively lower plant height with higher number of tillers. Length and width of leaves, as well as panicles, are smaller than those from diploid plants. These plants produce flowers, but there is no seed setting. Doubled haploid plants show normal growth, with broader and thicker leaves, and have high spikelet fertility values. The higher degree of spikelet fertility in doubled haploids can be due to the elimination of meiotic irregularities by doubling of the haploid genome. Cytogenetic characterization has revealed that the anther-derived plants have different ploidy levels (Sah and Niroula 2007). Counting of chromosome numbers from the root tip is the most accurate way for identification of ploidy level. However, the ploidy level in plants is also estimated by measuring the C-value (amount of DNA in the unreplicated gametic nucleus) using flow cytometry (Ochatt et al. 2009). Ploidy is also confirmed by pollen grain size.

Diploids and polyploids are produced by natural doubling which can occur during different stages in vitro, including callus induction, callus redifferentiation and embryogenesis. High ploidy levels are associated with large pollen grains, while the number of pollen grains is not different between ploidy levels (Fukuhara 2000).

9 Applications

A large number of rice varieties (Mishra and Rao 2016) using anther culture having superior grain quality characteristics, resistant to diseases like blast, bacterial blight, brown plant hopper and tolerance to abiotic stress have been released in different countries (Table 1). Rice anther culture has been used to produce DHs with multiple stress tolerances. Lee et al. (2003) developed a salt-tolerant DH rice variety through anther culture from F_1 hybrids of *indica* and *japonica* varieties. Similarly, Thomson et al. (2010) developed DH line AC-1 for the saline areas of Bangladesh through anther culture of crosses involving salt-tolerant rice lines from the International Rice Research Institute (IRRI). Dewi et al. (2009) developed varieties tolerant to aluminium toxicity using anther culture of *indica* genotypes, whereas Purwoko et al. (2010) used anther culture of upland rice and produced DH lines tolerant to aluminium stress, shade and blast resistance. Due to genetic drift, mutations, artificial and biological mixing and biotic and abiotic stress, the purity of the parental lines used for hybrid rice production is decreased. This may result in decline in the quantity and quality of hybrid rice. The conventional procedure is laborious, time-consuming and phenotype based, which does not assure its homozygosity and stability. DH lines developed through anther culture are homozygous (Zhu et al. 1998) and, therefore, can be used in purification effectively. Bai et al. (1991) and Wang et al. (1994) used anther culture for purification of restorer lines used in hybrid rice production. They reported improved purity, seed setting rate, yields and pest resistance. Haploid cells can be treated with physical (X-rays, UV rays or gamma rays) or chemical mutagens and can be used in inducing and fixing mutations by developing DH lines from mutated cells. Jiang et al. (2002) reported enrichment of rice germplasm by fixing and expressing recessive traits through anther culture by introducing these traits using mutagens or hybridization. For QTL studies segregating populations, F_2 or backcross populations have been used. But these populations are difficult to replicate thereby not useful in obtaining accurate phenotypic data for preparing a precise QTL map. The recombinant inbred lines (RILs) can be used for this purpose. But RILs require long duration to develop. Thus, DH populations can be successfully used to construct genetic maps and to locate QTLs. Because each DH line is homozygous, uniform and produce sufficient seeds for replications at several locations. Several QTLs have been mapped using DH populations as the mapping populations (Table 1). The IRRI conducted a project supported by the HarvestPlus Challenge Program to produce nutritious rice. In the project DH lines are being generated to increase the grain micronutrient content of existing and future high-yielding *indica* varieties using high-zinc, high-iron *japonica* donors,

Table 1 Applications of anther culture in rice

Varieties released			
Variety	**Characteristics**	**Country**	**References**
Huayu I, Huayu II, Xin Xiu, Late Keng 959, Tunghua 1, Tunghua 2, Tunghua 3, Zhonghua 8, Zhonghua9, Huahanzao, Huajian 7902, Tanghuo 2, Shanhua7706, Huahanzao 77,001, Nanhua 5, Noll and Hua 03	High-yielding varieties with superior grain quality, resistant to blast and bacterial blight diseases	China	Hu and Zeng (1984), Chen (1986), loo and Xu (1986), and Yang and Fu (1989)
Milyang 90	Good grain quality, resistant to brown plant hopper and stripe virus disease	China	Chung (1987)
Milyang 90, an anther culture-derived line	Superior grain quality and overall good performance	China	Chung (1987)
Hwacheongbyeo, Joryeongbyeo, Hwajinbyeo	Resistant to brown plant hopper, rice stripe Tenuivirus, blast and bacterial blight	South Korea	Lee et al. (1989)
Guan 18	Early maturity, good quality and disease resistance	China	Zhu and Pan (1990)
Guan 18, an *indica* rice hybrid	Superior grain quality	China	Zhu and Pan (1990)
Huayu 15	Resistant to lodging and diseases, good quality	China	Shouyi and Shouyin (1991)
Parag-401	Superior grain quality and resistant to iron chlorosis	India	Patil et al. (1997)
Bicol (IR51500AC11–1)	Salt tolerant	Philippines	Senadhira et al. (2002)
CR Dhan 10 (CRAC2221–43), Satyakrishna	Resistant to neck blast, sheath rot and yellow stem borer	India	CRRI annual report 2007–2008
Risabell	High milling and cooking quality, resistant to blast	India	Pauk et al. (2009)
Janka	Drought tolerance, good grain quality	India	Pauk et al. (2009)
Abel	Cold tolerance at early stage	India	Pauk et al. (2009)
CR Dhan 801 (CRAC2224–1041, IET18720), Phalguni	Resistant to leaf blast, gall midge; moderately resistant to sheath rot, rice stripe Tenuivirus, yellow stem borer and brown spot	India	CRRI annual report, 2009–2010
DH lines derived from Koshikari	Superior visual grain quality and excellent eating quality	China	Xa and Lang (2011)

(continued)

Table 1 (continued)

QTLs mapped		
DH population used	**QTLs mapped**	**References**
–	Resistance to rice blast, bacterial blight, and sheath blight disease	Wang et al. (2001)
–	QTLs for plant growth, yield and yield-related traits across three diverse locations	Hittalmani et al. (2002)
Cross between upland and lowland *japonica* rice in three environments	QTL mapping of root traits	Li et al. (2003)
Cross between IR64 and Azucena	Six QTLs associated with resistance to brown plant hopper	Soundararajan et al. (2004)
Cross between *japonica* rice Chunjiang 06 and *indica* rice TN1	Five QTLs for panicle-layer-uniformity, heading synchrony	Ma et al. (2009a, b)
DH populations of Maybelle, an American *japonica* variety, and Baiyeqiu, a Chinese *indica* landrace	QTLs linked to sheath blight resistance	Xu et al. (2011)
DH population of Cheongcheong (*indica*) and Nagdong (*japonica*)	Two QTLs affecting yield and yield components	Park et al. (2014)
DH population of Cheongcheong (*indica*) and Nagdong (*japonica*)	Four QTLs related to amylose content, two QTLs related to protein content and two QTLs associated with lipid content for rice quality analysis	Lee et al. (2014)

Source: Mishra and Rao (2016)

which are known to be highly responsive to anther culture. Grewal (2009) evaluated more than 1500 DH lines through anther culture for their agronomic potential and for high iron and zinc contents. Similarly, Hu et al. (2004) used rice doubled haploid lines and mapped QTLs for rice protein and fat content.

In conclusion the doubled haploid technology along with marker-assisted selection (MAS) and transgene technologies can effectively expedite the crop breeding programme. *Japonica* rice and many genotypes of *indica* rice respond very well to androgenesis, but many others are still recalcitrant, and the cellular, biochemical and molecular bases for the transformation of microspores into pollen embryoids are still poorly understood. Therefore, there is an urgent need to develop new genotype-independent methods through the study of QTLs responsible for microspore embryogenesis and further plant regeneration from the microspore embryoids. Anther culturability can also be improved by studying and improving existing protocols of anther/microspore culture. The haploidy technique has played an important role in practical plant breeding as can be seen in release of DH cultivars, use of DH in QTL mapping. This process of improvement in the androgenic response from nonresponsive genotypes can be expected to continue in the race to improve the efficiency and yield of rice at a time of increasing challenge to maintain food security.

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Accelerated Wheat Breeding: Doubled Haploids and Rapid Generation Advance

Puja Srivastava and Navtej Singh Bains

Abstract New breeding objectives, evolving disease organisms, changing climate patterns and the need for quick genetic ameliorations make accelerated breeding an important aspect of wheat improvement work. The emergence and deployment of wheat x maize system of DH production over the last few decades are discussed as an important option for accelerated wheat breeding. The lack of acute genotypic specificity favours the application of this method in wheat breeding. A low-cost, high-throughput system based on detached tiller culture and pre-regeneration chromosome doubling is presented. Recent studies in wheat have focused on development of alternative accelerated breeding systems based on modulation of growth environments and compression of crop cycle duration. Multiple crop generations obtained in this manner allow homozygosity to be approached in a single year. Directed assemblage of recurrent parent background and selection in nontarget environments have been enabled by use of molecular markers and complement accelerated breeding through these means. The future prospects of accelerated breeding are enriched by recent advances in deciphering molecular genetic basis e.g. *CEN H3* and *Mtl*-1 genes of haploid induction.

Keywords Wheat · Embryo culture · Detached tiller culture · Chromosome elimination · Pre-regeneration colchicine treatment · Rapid cycle breeding

1 Introduction

The wheat-breeding strategy underlying the green revolution relied on simple genetic changes, complemented by improved management practices and higher inputs. Future genetic improvements in crop productivity may need to be made without enhanced use of fertilizer, irrigation and other inputs, in deference to

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natural resource depletion and environmental concerns. The list of target traits for breeding, particularly in relation to nutritional and quality concerns, is growing rapidly. Climate perturbations also pose an unprecedented challenge to the wheat improvement strategy. Thus there is a need for diverse and multiple genetic ameliorations, often accompanied by demanding time frames. All these add up to a tall order for a crop with a narrow genetic base, notwithstanding the earlier breeding successes. Breeding programmes in the conventional mould may not be able to measure up to these challenges. The need for an accelerated system which integrates a wide spectrum of the available and emerging molecular-genetic technologies is inevitable. Hallmarks of such a breeding programme would be:

- Working with a multitude of traits and objectives by virtue of depth of germplasm and access to fast developing gene and genomic resources
- Molecular marker tools for precise and rapid mobilization of genes to genotypes suitable for various cultivation contexts
- An information management system for allelic, haplotypic and genealogical data of the breeding programme
- Accelerated progress to homozygosity through doubled haploids (DH) or rapid generation advance

Accelerated breeding generally aims at cutting out the protracted segregating phase either through single step homozygosity as delivered by the DH systems or shortening the time for generational turnover. Selecting the desired chromosomal constitution through molecular markers provides another avenue of acceleration, particularly in context of backcrosses. All modes of acceleration strengthen the 'breeding option' in face of sudden biotic and abiotic challenges. Overall efficiency of the breeding programme also improves on account of precision of evaluation (being based on fixed, homozygous material) and exclusively additive nature of genetic variation – resulting in a much higher genetic advance. Application of such a system, however, requires considerable groundwork for development of an efficient DH technology.

2 Development of Wheat x Maize System of Doubled Haploid Production

2.1 Early Haploid Induction Systems

The first reports of haploidy in plants came from anther culture in *Datura* spp. by Guha and Maheshwari (1964, 1966). Later, Ouyang et al. (1973) attempted anther culture and obtained haploid plants in wheat for the first time. But the major constraint in this system was the poor plant regeneration in majority of wheat genotypes and a high frequency of albino plants in the haploid population. Chromosome elimination in wide crosses opened up an alternate route to haploidy in cereals when Kasha and Kao (1970) developed barley haploids from *Hordeum vulgare* x *Hordeum*

bulbosum crosses. This system was extended to wheat by Barclay (1975) who developed haploids by crossing wheat with *Hordeum bulbosum*. However, the system was compatible only with wheat genotypes carrying the crossability alleles *kr1* and *kr2* (Falk and Kasha 1981). Most of the improved germplasm relevant to breeding programmes lacked these genes.

2.2 Emergence of the Wheat x Maize System

Arrival of wheat x maize system was marked by the observation of microscopic, early stage embryos in crosses between hexaploid wheat and maize (Zenkteler and Nitzsche 1984). In this study a set of exploratory crosses between different Gramineae species including wheat, maize, pearl millet, rye, barley, etc. had been analysed for early post-pollination events. Later, Laurie and Bennett (1986) at Plant Breeding Institute (PBI), Cambridge, studied the early post-pollination events in wheat x maize crosses and demonstrated that both the wheat and maize chromosomes were present in the zygote, but the maize chromosomes were eliminated during the initial cell divisions. Another significant observation from their study was that in such crosses the endosperm was absent and resulted in embryo abortion before the embryo could develop to a rescuable size. Surprisingly, despite of the wide genetic difference between the parents, the frequency of fertilization was high (about 25%). Further, Laurie and Bennett (1987) showed that the wheat x maize system in contrast to the wheat x *H. bulbosum* system was free from the effects of non-crossability conferring alleles *Kr1* and *Kr2*. Later in 1988, Laurie and Bennett devised a system of in vitro culture of pollinated wheat spikelets and recovered the first haploid plant from wheat and maize crosses.

2.3 Crossing Technique

The idea that conventional crossing techniques used in wheat may not be suitable in case of wheat x maize crosses, because of greater genetic distance involved, led to the efforts towards modifications of these techniques. Laurie and Bennett (1988) modified the crossing techniques in such a way that one of the primary florets was cross-pollinated and the other was allowed to self-pollinate. They recovered more embryos in case of self-pollinated/cross-pollinated spikelets. It was suggested that the self-pollinated spikelets gave a feeder effect, may it be via release of a growth substance. The importance of keeping glumes intact was also emphasized by Laurie (1989). Once the potential of wheat x maize crosses for haploid breeding became evident, modifications that saved time and effort became important. Pollinations without emasculation were found to be effective as wheat ovaries become receptive 2–3 days ahead of anthesis (Matzk and Mahn 1994; Suenaga et al. 1997), but the pollinations must be timed close to date of anthesis (e.g. 1 day ahead) to have

minimum reduction in fertilization frequency. Differences in embryo formation frequency with respect to the position of the spikelet were indicated as spikelets from the middle of the spike gave higher embryo formation frequency (Martin et al. 2001).

2.4 Use of Growth Regulators

Growth regulators play an important role in caryopsis and embryo development. Gibberellic acid was used in wheat wide crosses primarily to delay endosperm abortion, but it did not seem to be useful in wheat x maize crosses (Laurie 1989). Application of growth regulators was not known to be an essential requirement for the success of wheat wide crosses. Laurie and Bennett (1988) had relied on in vitro culture of spikelets, 2 days after pollination, to ensure caryopses growth and embryo development. Thus it was unexpected when Suenaga and Nakajima (1989) demonstrated that injection of 2,4-D solution (100 ppm) in the uppermost internode was sufficient to sustain caryopsis growth and development on the plant itself for 15 days, till embryos reached a rescuable stage. Use of a growth regulator (auxin) and that too in an unconventional target tissue (hollow of the stem) turned out to be the single most important requirement for caryopsis development in case of in vivo embryo formation in wheat x maize crosses. This intervention involved two consecutive injections (0.3–0.5 ml each of 100 ppm 2,4-D), 24 and 48 h after pollination. The potential of this technique to bypass the need for cumbersome spikelet culture was quickly confirmed by studies (Inagaki and Tahir 1995). Laurie and Reymondie (1991) proposed an alternative mode of 2,4-D application by showing floret drops at relatively low concentration, to be equally effective. A single treatment of 10 ppm to the florets, one day after pollination or continuous availability of lower doses of 2,4-D (0.5–2.0 ppm) was also found to be effective for embryo formation. The 2,4-D application eased out haploid embryo formation to such an extent that it came to be perceived as a potential system for haploid breeding. The timing of 2,4-D application was also seen to be critical. The use of 2,4-D before pollination drastically reduced the embryo formation frequency (Suenaga and Nakajima 1989; Matzk and Mahn 1994). 2,4-D treatments immediately after and again 1 day after pollination increased the frequency of embryo formation to 11.8% as compared to 1% in the pre-pollination treatments. Delaying the application to 24 h or more after pollination gave higher embryo formation frequency. Many other plant growth regulators like NAA, IAA, zeatin, kinetin and ABA with various concentrations were tried, and the result from 100 ppm 2,4-D was significantly better (Suenaga 1994). Gracia-Llamas et al. (2004) proposed the application of dicamba alone or in combination with 2,4-D as a means for further improving the yield of haploid plants though other studies had not indicated its superiority over 2,4-D (Matzk and Mahn 1994; Knox et al. 2000). Use of 2,4-D thus continues to be an integral component of wheat x maize system.

2.5 Comparison of Wheat x Maize System with Other Haploid-Inducing Systems

Response in wheat to anther or microspore culture tends to be genotype specific, making this technique less suitable for breeding work though it has resulted in development of varieties like Florin in France (De Buyser et al. 1987). A few comparative studies on haploid induction systems in wheat are available. Inagaki and Tahir (1990) reported that the average embryo formation frequency was 0.2% in crosses with *H. bulbosum* as compared to 9.5% in crosses with maize. Kisana et al. (1993) found wheat x maize system to be better than anther culture in terms of efficiency. Another comparison with anther culture was made by Sadasivaiah et al. (1999). They concluded that wheat x maize system was better in terms of lower genotype specificity, absence of albinism and ease of application.

2.6 Alternative Pollinator Species for Induction of Wheat Haploids

Apart from maize, pearl millet (Laurie and O' Donoughue 1989) and sorghum (Ohkawa et al. 1992) have been found to be effective pollinators for inducing haploidy in wheat. Teosinte was found to be a better pollinator than maize, in a study by Ushiyama et al. (1991), when they evaluated 39 maize and teosinte genotypes. Matzk and Mahn (1994) reported higher frequency of embryo formation using pearl millet (27%) as compared to maize (22%), while Inagaki and Mujeeb-Kazi (1995) had compared maize, pearl millet and sorghum as potential pollinators and found maize to be better than others. Dusautoir et al. (1995) crossed various durum wheat varieties with maize and teosinte and found that teosinte pollen was more effective for inducing haploids. Li et al. (1996), while using tetraploid *Tripsacum dactyloides* as pollinator, obtained high frequency of fertilization and embryo formation in crosses with hexaploid wheat as female. An alternative pollinator species *Coix lacryma-jobi* (Job's tears) was identified by Mochida and Tsujimoto (2001). A perennial wild grass *Imperata cylindrica* was shown by Chaudhary et al. (2005) to have a haploid induction efficiency comparable to that of wheat x maize crosses. Wheat x maize system proved to be a ready template for uncovering several similar cross combinations offering the potential of haploid induction through chromosome elimination in wheat. The complete elimination of one of the parental genomes may not be very uncommon; it has been documented in 74 hybrids involving monocotyledonous species and 35 involving dicotyledonous species (Ishii et al. 2016). The choice of haploid-inducing pollinator in wheat however continues to be maize in most cases, primarily due to ease of access and abundance of pollen production besides absence of a major or qualitative advantage, if any, offered by alternative pollinator species.

2.7 Quantitative Variation in Haploid Induction Response of Maize and Wheat Lines

Suenaga (1994) crossed maize with 47 wheat varieties and observed a wide range for caryopsis formation and embryo development (33.3–99.1% and 2.2–50.0%, respectively). Similar results were obtained by Amrani et al. (1993) for tetraploid wheat. On the other hand Matzk and Mahn (1994) found little difference for embryo formation between wheat lines, when they used crosses between eight wheat and nine maize lines. Similarly significant differences for embryo formation and plant regeneration were not observed by Zhang et al. (1996). Laurie and Reymondie (1991) found differences for embryo formation between spring and winter wheat lines but not within the two sets. The genotypic differences, by and large, are not acute enough to prevent use of the system for target wheat genotypes. Efficacy of maize genotypes as pollinators was also investigated (Suenaga and Nakajima 1989; Zhang et al. 1996), and significant differences for embryo formation were observed. Differences in haploid embryo formation and regeneration frequencies on account of maize genotype can be exploited to improve output of the system by identification of superior pollinators. Superior maize genotypes reported by some of the early studies included CM75 (Suenaga 1994), ZML (Matzk and Mahn 1994) and Pearl Popcorn (Verma et al. 1999). In absence of information on genotypic responses, collection of pollen from a set of maize lines or populations may be a practical strategy (Inagaki and Tahir 1995; Lefebvre and Devaux 1996; Mangat 2000).

2.8 Detached Tiller Culture

The wheat ear can be detached from the plant with a sharp, often underwater cut below first or second node and maintained for some days by placing the cut end in a solution carrying sucrose and basal salts. Grain development in ears cultured in this manner at around anthesis stage and under appropriate conditions is seen to progress well. The detached tiller culture system was initially developed to study nutrient translocation and seed development physiology in wheat spikes (Graham and Morton 1963; Donovan and Lee 1977; Singh and Jenner 1983). The culture solution in these studies was generally maintained at low temperature or under aseptic conditions through specially designed or improvised equipment to avoid microbial contamination of the nutrient-rich medium. Kato and Hiyashi (1985) attempted to forego sterilization and simplify the tiller culture system, but seed development was very poor due to contamination of the sucrose-containing medium. A remarkable modification involved the use of sulphurous acid to suppress contamination in the culture solution and culm decay as first introduced by Kato et al. (1990). Sulphurous acid (H_2SO_3) is a weak inorganic acid, which is considered an aqueous solution of sulfur dioxide in water. Kato et al. (1990) successfully cultured selfed wheat ears at room temperature on liquid medium containing 100 g/l sucrose and

0.075% sulphurous acid. The study aimed at conferring cold treatment to growing caryopsis on detached tillers, as a substitute to seedling vernalization. The relevance of detached tiller culture system for wheat x maize crosses was first indicated by Riera-Lizarazu and Mujeeb-Kazi (1990). Ushiyama et al. (1991) used detached spike culture system in wheat x teosinte and wheat x maize crosses and recommended MS-based medium containing 100 mg/l 2,4-D, 10 ml/l ethanol, 8 ml/l sulphurous acid and 40 g/l sucrose for tiller culture. Riera-Lizarazu et al. (1992) used detached tiller culture in triticale x maize crosses, while Inagaki (1997) applied it in studies on selection of efficient pollen donor for double haploid production and reported positive effect on embryo formation and plant regeneration frequency. Detached tiller culture system was also shown to be more efficient than the conventional on plant alternative by Cherkaoui et al. (2000). The tiller culture medium containing 100 mg/l 2,4-D, 40 g/l sucrose, 10 mg/l silver nitrate, 8 ml/l sulphurous acid and 3 g/l calcium phosphate was recommended by Jian et al. (2008), who obtained caryopsis formation in 95% of pollinated florets and an embryo formation frequency of about 30%. MS-based medium containing 40 g/L sucrose, 100 mg/L 2,4-D and 8 ml/L sulphurous acid was recommended by several studies (Inagaki and Hash 1998; Hussain et al. 2012). Detached tiller culture is primarily of interest to researchers working with field-grown wheat plants, whose tillers could be detached post-pollination and brought indoors to obtain embryo development under optimal environmental conditions.

2.9 Embryo Rescue Media and Culturing Techniques

The double fertilization norm is rarely fulfilled in fertilization of wheat with maize pollen, and embryo formation is mostly not accompanied by the endosperm development. The post-pollination caryopsis growth, unless supported by 2,4-D application or in vitro culture, is imperceptible, and the embryo degenerates. If the recommended growth regulator-based interventions are followed, a proportion (25–30%) of the well-developed caryopsis carry irregularly shaped embryos, floating in a watery fluid. About 15 days after pollination, these need to be rescued aseptically on artificial medium. Early removal of caryopses from spikes results in low number and small size of embryo. Delayed culture on the other hand adversely affects the regeneration potential of embryos. Ten- to eleven-day-old embryos gave higher regeneration percentage (78.3%, Suenaga 1994), while Kammholz et al. (1996) found 12–15-day-old embryos having better regeneration. Younger and older embryos gave a much lower percentage of regeneration. Fifteen days are optimum for developing a rescuable sized embryo without compromising its regeneration potential. Another important factor that is expected to impact regeneration is embryo rescue media. The common synthetic nutrient medium includes half-strength MS (Murashige and Skoog 1962) or B5 (Gamborg et al. 1968), and both have been extensively used for culture of wheat embryos from wheat x maize crosses (Suenaga and Nakajima 1989; Comeau et al. 1992; Kammholz et al. 1996; Cherkaoui et al.

2000; Dogramaci-Altuntepe and Jauhar 2001; Sourour et al. 2012; Niu et al. 2014). Medium-solidifying agents were studied by Morshedi and Darvey (1997) who identified Gelcarin GP812 as significantly better than other gelling agent like agar, agarose, wheat starch, etc. At present, agar or synthetic gelling agents like gelrite are employed without major implications for regeneration. Nurse culture, common with microspore culture protocols, was studied by Niu et al. (2014) where excised embryos were placed on a 20-day-old seed endosperm tissue and then cultured on the MS medium. This method helped small-sized embryos but is laborious and time-consuming compared with the regular embryo rescue method. Use of additional media components like hormones, vitamins, amino acids, etc. to enhance embryo regeneration frequency finds few mentions (Kammholz et al. 1996; Sourour et al. 2012; Niu et al. 2014). Various modifications have been studied by adding different organic supplements (Zenkteler and Nitzsche 1984; Zhang et al. 1996; Suenaga et al. 1997; Campbell et al. 2000; Singh et al. 2004; Ayed et al. 2011), but none seems to be a critical requirement. This is unlike wheat anther culture and isolated microspore culture protocols which require very specific medium composition besides pretreatment conditions. Such stringent conditions with respect to culture conditions have not been worked out for wheat x maize system. Embryo development conditions prior to rescue, age and size of embryo seem to be more important, and several basic media are known to support adequate regeneration.

2.10 Chromosome Doubling

Haploid plants derived from wheat x maize show almost no spontaneous chromosome doubling (Sadasivaiah et al. 1999). Chromosome doubling can be achieved through various compounds of which colchicine is the most widely used (Subrahmanyam and Kasha 1973; Hassawi and Liang 1991; Ouyang et al. 1994; Soriano et al. 2007). In wheat x maize crosses, colchicine treatment is normally given to haploid seedlings at 3–4 tiller stage for 5–8 h by submerging the whole root system in colchicine solution followed by washing with water and reestablishment of seedlings. Chromosome doubling efficacy depends upon colchicine dose and duration of treatment. Use of colchicine solution having concentration of 0.1% for 5 h (Inagaki 1997; Thiebaut et al. 1979), 0.2% for 5 h (Sadasivaiah et al. 2001; Maluszynski 2003) and 0.45% colchicine for 6–8 h (Niu et al. 2014) has been found effective for doubling with some differences. Higher doses are effective in chromosome doubling but may result in deformed plants, low survival rate and increased cost, while lower doses require prolonged exposure and may not result in doubling. The standard post-regeneration chromosome doubling treatment leads to formation of chimeric plants. These show partial seed production, and therefore, an additional growth cycle for seed multiplication before evaluation in the field is required (Chen et al. 1994; Islam 2010). A chromosome doubling technique integrated into the haploid induction procedures given prior to regeneration may be more effective. In this context, colchicine when applied to embryo rescue media reduced the rate of

embryo germination (Niu et al. 2014). In addition to colchicine, alternate doubling agents have also been studied for chromosome doubling in wheat like use of nitrous oxide (Hansen et al. 1998), caffeine (Thomas et al. 1997) and herbicides trifluralin, oryzalin, etc.(Dhooghe et al. 2011).

3 Application of Wheat x Maize System in Wheat-Breeding Programmes

The wheat x maize approach of doubled haploid production is finding use in several wheat-breeding programmes worldwide. The technique is an integral part of the wheat-breeding methodology at Australian Grain Technology Pty. Ltd., the largest wheat-breeding company in Australia (Kuchel et al. 2005). Longreach Plant Breeder, another wheat-breeding company in Australia, also employs doubled haploid breeding, wheat variety Longreach Reliant, being a DH product released in 2016. Public institutes in Australia, e.g. Plant Breeding Institute, Sydney, South Australian Research and Development Institute and Department of Agriculture and Food, Western Australia, have also employed this technique. In Japan, wheat cultivar Sanukioyume-2000 is one of the cultivars developed by using the wheat x maize system (Yuichi et al. 2002). In the USA, wheat variety Bond CL was developed by Colorado State University using wheat x maize crosses and released in 2004 (Haley et al. 2006). Oklahoma State University released Gallagher, a DH, in 2012 (Carver 2016). Elite crosses in the OSU wheat programme are processed with DH technology, often contributing about one fourth of the lines for advanced trials. Kentucky Small Grain Growers Association approved wheat DH lines BW 965 and BW 966 in 2015. CIMMYT, Mexico, worked towards refining the DH production protocol and used it to generate populations for gene mapping and genetic analysis but did not make it a part of the breeding programme as advantages were not perceived over the long-standing shuttle breeding system, involving two seasons of selection in large populations per year (Huihui et al. 2013). In Canada, the first wheat DH variety, McKenzie, was developed through anther culture and released in 1997. In the next about 12 years, 27 wheat DH cultivars were released in Canada mostly developed through wheat x maize crosses at wheat-breeding centres of Agriculture and Agri-food Canada and Universities of Saskatchewan and Manitoba. In 2009 DH cultivar Lillian, developed using marker-assisted selection to improve protein content with gene *Gpc-B1*/*Yr36*, occupied the largest area under any single variety in Canada (De Pauw et al. 2010); in 2010, DT801, first DH variety of durum wheat, was released in Canada. In India the wheat x maize system is being applied to selected crosses from wheat-breeding programmes at Punjab Agricultural University, Ludhiana (Singh et al. 2012), and at Himachal Pradesh Agricultural University, Palampur. India's first DH wheat cultivar, Him Pratham, released in 2013, has been developed at Palampur through wheat x *Imperata cylindrica* crosses (Chaudhary et al. 2014). Some of the wheat DH laboratories work in the service mode as well. Washington

State University, Pullman, produces wheat DH plants for $15 for on-campus and $25 for off-campus breeders (www.css.wsu.edu/facilities/dhlab/). One of the big service providers in wheat DH, Heartland Plant Innovations Inc. (HPI) in Kansas, USA, produces up to 50,000 DH plants per year and charges partner and commercial breeders $35 and $50 per plant, respectively (www.heartlandinnovations.com/our-programs/doubled-haploid-production).

4 Adapting the Wheat x Maize System to a Wheat-Breeding Programme

Having discussed above the studies which led to development of wheat x maize system as a potential wheat-breeding tool, we describe research aimed at adapting this system to needs of a wheat-breeding programme, located in a subtropical clime with extremes of winter and summer and modest controlled plant growth facilities. This programme at Punjab Agricultural University, Ludhiana, caters to the state of Punjab in particular and north-western plain zones in general, the largest and most productive wheat zone of India. The wheat breeding in the region is challenged by fast-evolving stripe rust races, abiotic stresses imposed by rising temperature and other climatic changes, urgent need for improving nutrient and water use efficiencies and taking up of nutritional and processing quality as hard core breeding objectives. The region is the mainstay of national food security, and acceleration in breeding outcomes can be highly rewarding. First demonstrations of wheat x maize system in India came from work at PAU, Ludhiana (Bains et al. 1995; Dhaliwal et al. 1995). The crosses were effective between field-grown wheat and maize plants raised in the polyhouse for a short (2–3 weeks), optimal environmental span in the main season. The window for crossing work needed to be expanded for DH work. The haploid plants generated in the main season had to be shifted to off-season location where chromosome doubling was not efficient because of long days and other conditions not conducive to tillering. Shifting wheat x maize crossing work to off-season location, solved the chromosome doubling issue, as plants were brought to main location and doubled during short day, winter conditions. The primary success of the wheat x maize crosses in terms of embryo formation frequency, however, was low (~10%) as night temperature at off-season location tended to fall below the required threshold of 15 °C. These major bottlenecks and several other issues were resolved within the framework of infrastructural capacity and environmental constraints. In fact, application and modification of the system went hand in hand leading to the current protocol development as discussed below.

Unlike conventional wheat crosses, wheat x maize crosses involve keeping the glumes intact (Laurie and Bennet 1988) to support better caryopsis growth and embryo development. This emasculation technique is skill and labour intensive. The pollination of spikes with intact glumes involves opening of individual florets for dispensing the pollen, generally requiring a team of two workers. Possibility of

employing a chemical hybridizing agent (CHAs) being used in hybrid wheat research for facilitating wheat x maize crosses was explored (Sandhu et al. 2002) and found to drastically lower haploid formation frequency, probably due to phytotoxic effects of CHA. Under an alternate strategy aimed at striking a favourable trade-off between caryopsis development and labour saving, unemasculated- clipped and emasculated-clipped florets were seen to be at par with standard (glumes intact) method in terms of haploid formation and plant regeneration (Sandhu et al. 2003). Application of this methodology on larger set in subsequent work, however, revealed that results may vary widely as ear health and other conditions have to be optimal for this strategy to work. We reverted back to use of standard method. Unemasculated florets with glumes intact could be useful to hasten up crossing work in the field, but pollinations have to be timed very carefully to 1 or 2 days prior to anthesis. Selfed caryopsis, which is expected in some proportion in unemasculated ears pollinated with maize, proved to be detrimental by competing for nutrition and starving out the crossed caryopsis. This observation is in contrast to the benefits reported for maintaining selfed florets in one part of the ear (Laurie and Bennet 1988). Later, when our DH protocol shifted to detached tiller culture system, it excluded the use of unemasculated spikes altogether, as the very process of detachment triggers anthesis resulting in high proportion of selfing. It was also observed that clipped florets were less suitable for detached tiller culture-based system as caryopsis health tended to fall below desirable level for regeneration ability. Thus different components of the protocol have to be compatible, and more instances of this requirement will come up in subsequent paragraphs. One emasculation shortcut which may be compatible with detached tiller culture approach is hot water dip as spikes with intact glumes are used and detached spikes can be very conveniently administered the hot water dip. Dipping of detached ears packed loosely in water-tight polythene bags in a water bath held at 43 °C for 3 min (Singh 2016) is partly successful and is being refined further to greatly enhance the throughput of the system. With respect to growth regulators, use of auxins like picloram and dicamba were also studied (Puja 2007, Kansal 2011), but use of 2,4-D was found to be optimal and cost-effective for embryo frequency and plant regeneration. Initially Pearl Popcorn, an open-pollinated maize cultivar released by PAU, Ludhiana, was picked up by us as pollinator (Verma et al. 1999) as it was found to confer comparatively high embryo formation and also high regeneration frequency, an unexpected outcome for a pollinator. Practical considerations that emerged over time made us to look for pollinators which flowered early and were thermo and photoperiod insensitive with respect to flowering time, so as to achieve synchronizations more predictably for maize plants grown in polyhouse or open field. A tassel which is resilient to environmental stress (cold in our case) in terms of tassel and floret size as well as pollen production is the second important requirement in this regard. Over the last few years, a local maize population adapted to Lahaul valley in the Himalayas (Himachal Pradesh, India) has met these requirements well for wheat x maize crosses at main as well as off-season location (also in Lahaul valley). It maintains a time to flowering of 35–40 days in various environmental conditions. Resource partitioning in the plant favours good tassel development even under stress.

In wheat x maize crosses, caryopsis development results from 2,4-D application, irrespective of fertilization by maize pollen. Thus while caryopsis development is found in majority of the florets treated with 2,4-D, embryo formation can vary greatly (0–40%), particularly for crosses performed under variable or suboptimal conditions. The caryopses which carry an embryo are apparently indistinguishable from others. At low embryo formation frequencies, embryo rescue thus becomes highly labour intensive. A method to identify and sort out embryo carrying caryopsis can be useful in this regard; Lefebvre and Devaux (1996) attempted to identify embryo carrying caryopsis prior to dissection using X-ray radiography, but they were not successful. Bains et al. (1998) devised a simple method of screening a 15-day-old, harvested caryopsis in a glass petri dish against light incident from above, which makes the floating embryos settled at bottom of the caryopsis visible as dark spots. Using this technique, 98% of caryopsis with embryo could be detected prior to dissection. For chromosome doubling, the standard colchicine treatment is targeted to crown region of well-tillered, 3–5-week-old wheat plants, after uprooting them from soil. The treated seedlings need to be planted back in soil under tiller promoting conditions. The off-season conditions, to which haploids produced in main season are shifted, are essentially long day and favour flowering rather than tillering. For haploids produced in off-season location, better conditions prevail, but it is a relatively small period in the main season which is conducive. Further, the standard colchicine treatment is low throughput and was originally devised for wide hybrids in wheat, which are typically few in number. Responding to the local and general need for alternate chromosome doubling technique, two strategies aimed at doubling the chromosome number of haploid embryos prior to plant regeneration were pursued (Sood et al. 2003). In the first approach the haploid embryos were rescued on medium containing colchicine (at concentrations of 0.2%, 0.3%, 0.4% and 0.5%) and moved to a colchicine-free regeneration medium 48 h later. Embryos exposed to 0.5% colchicine had 91.67% of their regenerated plants showing chromosome doubling. In the second approach based on tiller injections, different concentrations (0.5%, 0.75% and 1.0%) of colchicine solution, which also contained 2,4-D (100 ppm), were injected into the uppermost internode of crossed tillers 48 and 72 h after pollination. The chromosome doubling efficiency was high for 1% treatment which became the most attractive alternative. The treatment targeted early zygotic divisions. Chimeras of doubled/haploid sectors were generally not observed in the case of the tiller injection treatment, and most of the florets showed seed set in the doubled plants. In absence of chimeras, stomatal guard cell length provided rapid, early-stage analysis of ploidy level. This approach found favour with our team for field-based crosses, though use of high colchicine concentration (1%) involved greater expense. The caryopsis were rounded (less plump) and opaque, which served as an indicator of an effective treatment but prevented the application of pre-dissection identification of embryo carrying caryopsis. The shift to detached tiller culture-based system for reasons discussed below, however, excluded the tiller injection of colchicine, as unlike whole plants, cultured tillers did not tolerate the 1% injections well and lower doses were less effective. The colchicine treatment compatible with detached tiller culture consists of two applications of 0.2%

colchicine (prepared in 100 ppm 2,4-D), administered as drops inside the pollinated florets, 24 and 48 h after pollination (unpublished results). While exploring APM and trifluralin as replacements of colchicine, we found that APM at low concentration (50 μM) enhanced the embryo formation, whereas trifluralin promoted plant regeneration. In context of chromosome doubling, initial results have shown that 150 μM of APM and 350 μM of trifluralin gave doubling in about 75% of the plants when applied as drops to florets as explained for colchicine above (Singh 2014).

Staggered planting and low-cost interventions like polyhouses make wheat spikes and maize tassels available for several months during both main and off-season, virtually covering the whole year. However, fertilization-promoting conditions for wheat x maize crosses are prevalent for a small part of the year. The detached tiller culture system thus widens the crossing window to several months by easily providing optimal conditions in relatively small controlled environment facility. We extended the tiller culture system to include indoor pollinations as well at a raised temperature (25–27 °C). Besides consistent and improved embryo formation frequency, the most laborious step, i.e. pollinations, could be conducted around the clock. The dehiscing maize tassels are also detached and maintained in culture to provide fresh pollen, which is critical for high embryo formation. The system augers well for the breeding objectives as it allow a wide and flexible choice of plant material from the field for inducing the doubled haploids. The administration of colchicine to the florets can be done more safely, precisely and effectively under detached tiller culture system. The system makes very efficient use of space as 10–20 times more wheat spikes being used for haploid induction can be accommodated as compared to potted plants. Batch handling for various steps in the protocol is facilitated and promotes high throughput. In our early work, however, issues of spike bleaching, inadequate embryo size and regeneration potential were faced in the tiller culture system. Bleaching was typically noticed in small reach-in growth chambers, obviously due to accumulation of sulphurous fumes. Larger chambers with ventilation and improved internal air movement were found to restore ear health. The problem of embryo size/regeneration was sought to be solved by extending the culture phase. This tended to improve embryo size, but regeneration could not be improved as ageing sets in. A solution was found in the form of cold treatment to ears completing the tiller culture phase. For this purpose, compact bunches of detached tillers with culm ends dipped in water are kept at 4 °C in refrigerator for 3–5 days. The regeneration frequency improves to a tune of 60–70%, and unexpectedly the embryo recovery shows an improvement of about 40–50%. The cold treatment can be extended in interest of phasing the embryo rescue work. Several embryo rescue media compositions were investigated over the years. These include use of casein (Kaur 2004), activated charcoal in addition to casein (Puja 2007), amino acids (Bains et al. 2009) and polyamines (Goyal 2016). A major impact of tissue culture constituents and conditions was not observed on haploid production parameters. Thus, the embryo size and developmental stage are primary determinants of regeneration ability and basic media composition suffices.

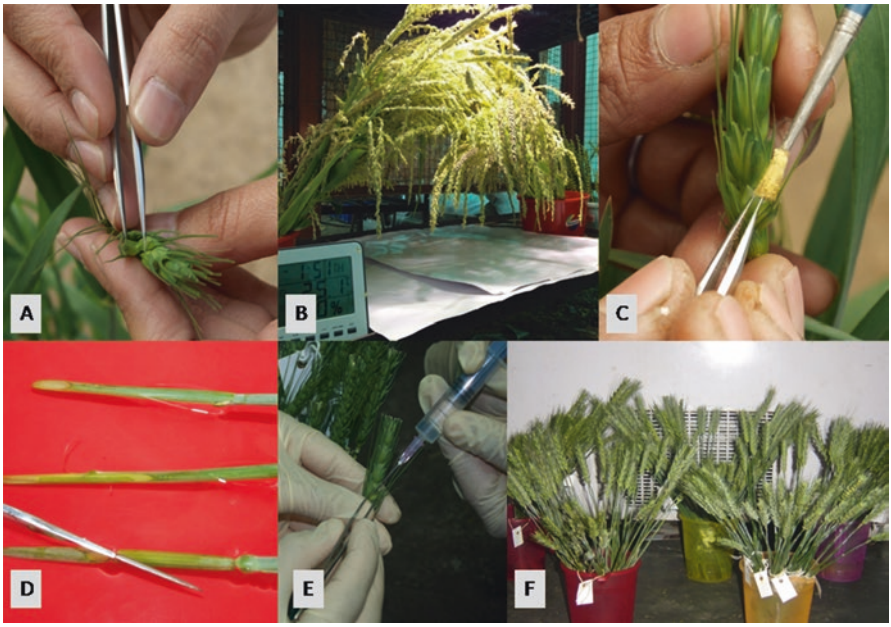

Fig. 1 Steps in wheat x maize system. (**a**) Emasculation of wheat ears without removing the glumes. (**b**) Collection of maize pollen after giving artificial light to maize tassels. (**c**) Pollination of emasculated ears with maize with the help of paint brush. (**d**) Underwater cut given to tillers prior to detached tiller culture. (**e**) 2,4-D drops being given to florets 24 h after pollination. Pre-regeneration colchicine, if given, is added to 2,4-D solution. (**f**) Tillers being maintained under detached tiller culture

Aspects of experimentation and observation above were aimed at adapting the DH production to local requirements. It has resulted in a low-cost, high-throughput protocol as given below, which can be useful in several situations:

1. Emasculation of wheat ears on field-grown plants with removal of central florets and keeping glumes of retained florets intact (Fig. 1).
2. Detachment of wheat tillers 2–3 days after emasculation, followed immediately by underwater cut with sharp scalpel on the stems, retaining uppermost node. The cut tillers are kept with ends dipped in water in small open-top buckets (each containing 100–200 ears) for about the next 24 h.
3. Pollination of detached ears maintained in water with freshly collected maize pollen from detached tassels in the same chamber under controlled conditions (25 °C, 70% relative humidity).
4. Colchicine (0.2%) + 2,4-D (100 ppm) + DMSO (2%) solution administered as drops to inside of florets 24 h after pollination.
5. Shifting of detached tillers to culture medium (1/2 MS + 40gm sucrose/L + 0.8% H_2SO_3).
6. Second dose of colchicine (0.2%) + 2,4-D (100 ppm) + DMSO (2%) to florets, 48 h after pollination.

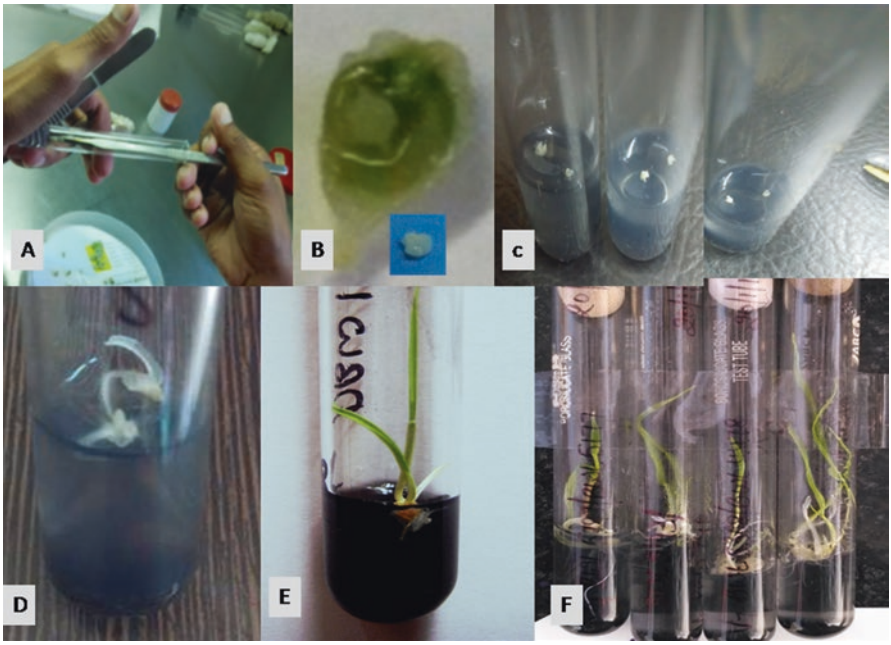

Fig. 2 Steps in wheat x maize system continued. (**a**) Embryo rescue being carried out. (**b**) Dissected caryopses showing free floating embryo without endosperm. (**c**) Embryos being kept on artificial medium for regeneration to plants. (**d**) Initiation of regeneration; plants are shifted to 16 h light and 8 h dark after this stage. (**e**) Regenerated green haploid plant. (**f**) Regenerated haploids ready to be transferred to soil for hardening

7. Detached tillers maintained at approximately 18–22 °C, 16 h light/day, 70–75% relative humidity and good air circulation in a spacious chamber (e.g. walk-in rather than reach-in) for 15 days after pollination.
8. Shifting of detached tillers in compact bunches with ends dipped in water to 4 °C for 3–5 days in refrigerator.
9. Embryo rescue under aseptic conditions on solid media (½ MS+ 20gm sucrose+1 ppm kinetin+100 mg myoinositol+2.6 gm Gelrite+2gm activated charcoal powder) (Fig. 2).
10. Cultures kept in dark till shoot emergence and shifted to 8 h light/16 h dark.
11. Regenerated plants transferred to vermiculite for hardening (15 days) and shifted to soil till maturity and seed set (Figs. 3 and 4).

The optimal doubled haploid efficacy parameters based on the above protocol, viz. caryopses formation frequency (>80%), embryo formation frequency (~40%), plant regeneration frequency (> 60%) and chromosome doubling frequency (~80%), jointly confer doubled haploid production of about 15 DH plants per 100 florets.

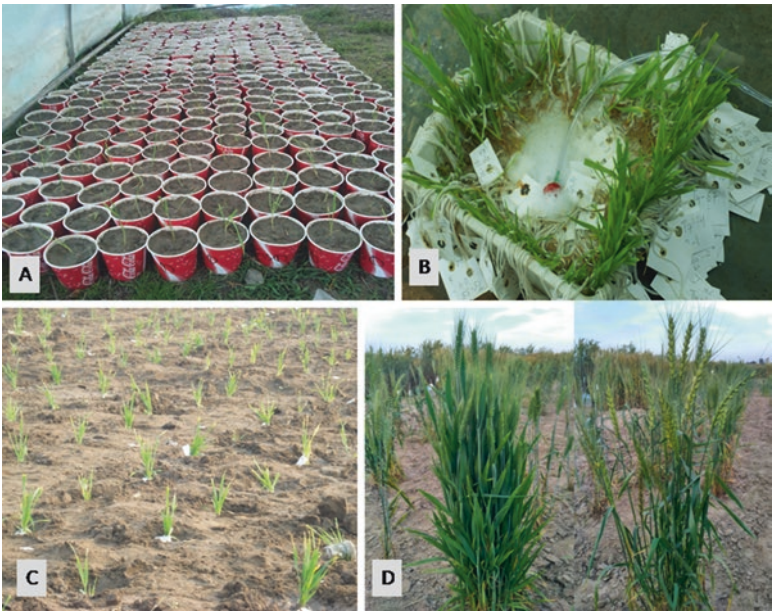

Fig. 3 Steps in wheat x maize system continued. (**a**) Regenerated haploids being grown in medium of soil+ vermicompost for hardening. (**b**) Colchicine treatment being given to haploid seedlings (in case where pre-regeneration colchicine was not given). The colchicine solution is kept aerated with the use of air bubblers. (**c**) Post colchicine treatment or post-hardening (pre-regeneration colchicine was applied), the seedlings are shifted to field under tiller promoting conditions. (**d**) Mature plant having seed set

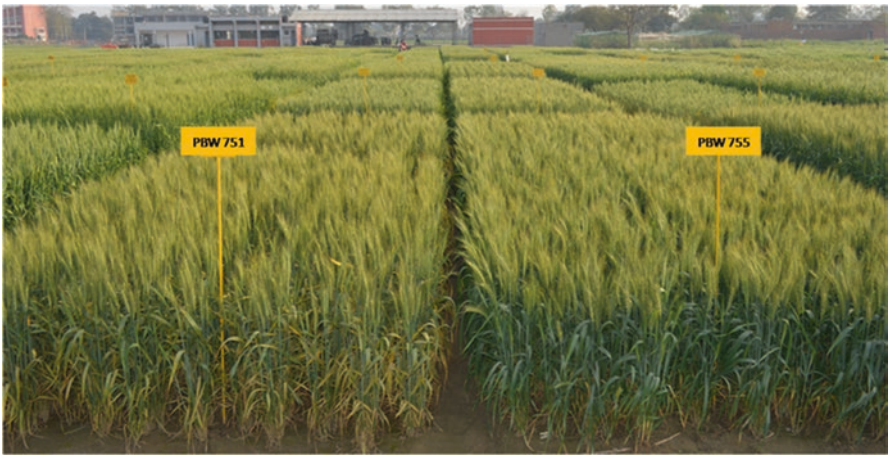

Fig. 4 Evaluation of DH lines in trials at Punjab Agricultural University, Ludhiana, India

5 Prospects of Developing Same Species Haploid Inducers

Chromosome elimination in interspecific crosses is seen as an established route to haploidy for crop improvement (Kasha and Kao 1970; Laurie et al. 1990; Forster et al. 2007; Wezdzony et al. 2009). Uniparental chromosome elimination can occur due to difference in the timing of mitosis, asynchrony in nucleoprotein synthesis, formation of multipolar spindles, and a spatial separation between genomes at interphase and/or at metaphase. The complete elimination of one of the parental genomes is not uncommon. It has been documented in 74 wide hybrids involving monocotyledonous species and 35 involving dicotyledonous species (Ishii et al. 2016). Haploid production by intraspecific hybridization is less frequent (Chang and Coe 2009; Geiger 2009), and one of the first reports came in 1959 with the discovery of maize haploid inducer Stock 6, producing 2–3% maternal haploids when outcrossed as a male. Over the years, improved inducer lines such as WS14, RWS, UH400, BHI306 and CAU5 with haploid induction rates (HIR) of 8–10% were developed. Combined with a haploid identification system based on a dominant scutellum and aleurone pigmentation gene *R1-nj*, the current levels of haploid induction have been successfully adapted for commercial DH production. In recent years this has fuelled dramatic increase in inbred line development in hybrid maize programmes. The advantage of intraspecific inducers is that the haploid production is an in vivo system with no requirement for the throughput lowering embryo rescue step. The important question for future developments in case of a naturally available intraspecies haploid inducer is the genetic basis and transferability of the haploid induction trait to another target species. Molecular genetic advances have narrowed down to underlying basis of chromosome elimination in interspecies crosses (most likely centromere-related genes) as well as intraspecies crosses (e.g. *Mtl1* gene in maize haploid inducers) as described briefly below.

The path-breaking report for development of haploid inducers (through CENH3 manipulations in *Arabidopsis*) was published by Ravi and Chen (2010). Soon after, CENH3 was also implicated in chromosome elimination in bulbosum system (Sanie et al. 2011). Distant CENH3s (e.g. *Zea mays*) were seen to complement the *Arabidopsis* null mutants but cause haploid induction (HI) in crosses (Maheshwari et al. 2015) on account of a divergent CENH3 N-terminal tail. Kuppu et al. (2015) showed that point mutations including preexisting ones in 'histone-folding domain' could confer HI in *Arabidopsis*. In crop species, CENH3 modifications were seen to generate HI trait in maize (Kelliher et al. 2016). Britt and Kuppu (2016) called CENH3 an emerging player in haploid induction technology and strongly recommended non-transgenic approaches involving alien gene introgression and mutagenesis for creating haploid inducers.

The genetic basis of haploid induction behaviour in the commercially successful Stock 6-based maize system has been recently reported by three independent research teams within the space of 1 month (Gilles et al. 2017; Kelliher et al. 2017; Liu et al. 2017). It was shown that a 4 bp frameshift mutation in a pollen-specific phospholipase gene is responsible for haploid induction. RNAi, TALENS, and

CRISPR-Cas suggest of a loss of function in this gene (named *MATRILINEAL1* or *NOT LIKE DAD*) as the trigger for haploid induction behaviour. Wheat sequences showing partial homology to this gene have been identified in the wheat genome (Kelliher et al. 2017). These studies represent major recent breakthroughs that chart a feasible path to haploid induction, and newer gene targets are expected to emerge shortly.

6 Rapid Generation Advancement

CIMMYT's long-standing shuttle breeding system between Obregon and Toluca sites within Mexico is well known, whereas wheat breeders in England and Canada often move plant material to New Zealand for generation advancement. Wheat-breeding programmes in India rely on high-altitude locations such as Lahaul-Spiti (Himachal Pradesh) and Wellington (Tamil Nadu) for obtaining acceleration of breeding cycle by taking two generations per year. Enhancing the number of seed to seed cycles beyond two requires special techniques and controlled conditions. Preliminary experiments showed that 6–8 crop cycles can be achieved within a calendar year. Recently such methods have been referred to as 'faster generation cycling system' (FGCS, Yan et al. 2017) or rapid cycle breeding (RCB) or simply rapid generation advancement (RGA) by different workers (Wang et al. 1999, 2003; Ochatt et al. 2002; Zheng et al. 2013; Forster et al. 2014; Liu et al. 2016; Yao et al. 2016, 2017). Seed to seed crop duration is shortened by culturing young embryos and managing plants (through induced stress) to greatly reduce the time for flowering and seed maturity. FGCS involves two steps: firstly, plants are grown in a controlled environment where specific irrigation and nutrient management practices accelerate the vegetative growth and flower differentiation. Secondly, culturing of young or immature embryos is taken up for reducing the time required for seed maturation (Wang et al. 1999, 2003) or even to growing caryopsis through detached tiller culture, prior to embryo rescue. Immature embryo culture is advantageous, as when seed matures inhibitors to germination or promoters of dormancy set in (Chawla 2002) and seed dormancy in wheat develops before the hard dough stage (Lan et al. 2005). For rapid generation advancement in winter wheats, vernalization treatment may be given to germinated embryos before transplanting them into soil (Wang et al. 1999, 2003). Rapid generation advance system application revealed that only older embryos (more than 20 days post-anthesis) required vernalization to initiate flowering, whereas plants generated from younger embryos (15 dpa) flowered without vernalization (Qin and Wang 2002). In addition, in vitro protocol for faster generation cycle has also been reported for wheat (Yao et al. 2016).

Successful development of rapid generation advance systems have been reported in several major crops, which significantly shortened the generation time and enabled 6–9 generations per year, viz. barley and wheat (Zheng et al. 2013; Yao et al. 2017); maize (Pioneer 2008); oat and triticale (Liu et al. 2016); *Brassica* spp. (Yao et al. 2016) and legumes (Ochatt and Sangwan 2010; Ribalta et al. 2014). The

key advantage of rapid generation advance over DH lies in the greater opportunity for genetic recombination through multiple segregating generations. Also, selection can be incorporated in any generation, and NILs or RILs can be developed (Yan et al. 2017). Zheng et al. (2013) demonstrated a rapid generation advance system based on immature embryo culture and raising of miniaturized plants under controlled conditions. Plates containing the newly cultured embryos were kept at 20–22 °C constant temperature without any extra lighting. When the cultured embryos started to germinate (which took between 24 and 72 h), the plates were transferred into an incubator with 16 h lighting (fluorescent lamps) and 25 °C day/22 °C night temperatures. When the coleoptiles of the young seedlings reached about 1.5–2.0 cm in length, the young seedlings were transferred into trays having potting mix. Seed to seed cycle was shortened by providing water stress during plant growth. Seedlings were watered only when wilt symptoms appeared. This protocol offers the potential of producing up to eight generations of wheat and nine generations of barley per annum. A speed breeding protocol which does not involve embryo culture has been established as a result of an international collaborative study (Watson et al. 2018) and uses extended photoperiod with specific light spectrum and harvesting of immature caryopses. The protocol gives six generation of wheat in a year as demonstrated for spring bread wheat, durum wheat, barley and the model grass *Brachypodium distachyon*. These species were grown under controlled environment room with extended photoperiod (22 h light/2 h dark). Plants grown under speed breeding progressed to anthesis (flowering) in approximately half the time of those from glasshouse conditions. Depending on the cultivar or accession, anthesis was reached in 37–39 days (wheat – with the exception of Chinese Spring) and 37–38 days (barley), while it took 26 days to reach heading in *B. distachyon*. While in the same duration, same species grown in glasshouses with no supplementary light or heating reached the early stem-elongation growth stage or three-leaf stage, respectively. In wheat, grains per spike decreased to some extent in the speed breeding chamber as compared to the glasshouse with no supplementary light, but both wheat and barley plants produced a healthy number of spikes per plant, despite the rapid growth. Viability of mature seeds was unaffected by speed breeding with similar seed germination rates observed for all species. The method can be used for carrying out wheat x wheat crossing and backcrossing and phenotyping for different disease as well. In Australia, DS Faraday is the first variety to be developed using speed breeding method and is likely to be released during 2018. Completely controlled state-of-the-art facility may not be available everywhere; also these processes limit the number of crosses that can be handled at time. At PAU, a field-scale, inexpensive method for raising an extra crop generation between the off-season (May to mid-September) and the main wheat season (November–May) is being followed. A protocol based on raising of seedlings in growth chamber (in propagation trays at 25 °C during mid-September) and then transplanting to field in the first week of October. This accelerated caryopsis development by post-anthesis culture of tillers under high temperature and long photoperiod, harvest of 13-day-old caryopsis followed by drying at 38 °C (4–5 days) and cold treatment at 4 °C (2 days) is followed (Gill 2017). An ideal rapid generation advance sequence

using this method involving fresh crosses in off-season as a starting point would offer selection opportunities in main season during F_2 and F_5 generation. It would take two to two and a half years from a fresh cross to reach bulking stage. Though not as rapid as the other studies (Yan et al. 2017; Watson et al. 2018), the proposed protocol has higher applicability and scale of operation with fewer resources.

In conclusion, both wheat x maize crosses and rapid generation advance systems offer excellent opportunities for accelerated breeding in wheat, which is likely to become the norm rather than a rarely used option in wheat improvement.

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In Vitro-Assisted Compression of Breeding Cycles

Janine Croser, Federico Ribalta, Maria Pazos Navarro, Christine Munday, Richard Bennett, Parwinder Kaur, and Sergio Ochatt

Abstract The compression of breeding cycles to quickly progress segregating material to homozygosity has attracted substantial international research interest for some decades. Modified pedigree breeding methods such as single seed descent (SSD) have enabled faster generation turnover and commercialization of new crop cultivars. Since the latter part of the last century, doubled haploid technology has revolutionized the progression to genome fixation in responsive species. In unresponsive but economically important families, biotechnological tools are being developed to accelerate traditional SSD – either by completing the full plant life cycle in vitro or by coupling controlled environmental conditions in the soil to elicit rapid floral onset with germination of immature seed in vitro to truncate seed filling. Both techniques have resulted in step-change efficiencies in generation turnover with up to fourfold improvements in species such as grain legumes. Such enhanced SSD systems are also valuable for breeding complex traits across a range of species. In this chapter, we explore the recent advances in in vitro-assisted breeding cycle compression in crops, opportunities to combine rapid phenotyping for key traits and the benefits of in vitro life cycle completion when researching under restrictive regulatory frameworks and working with enfeebled or rare material.

Keywords In vitro flowering · In vitro seed set · Single seed descent · Embryo culture · Phenotyping · Multiparental populations · Precocious germination

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1 Introduction

From the start of reasoned agriculture through to the exploitation of plant breeding approaches (Roumet and Morin 1997), the main goal of most breeders has been to introduce important agronomic traits or those with the highest economic impact into a stable genome within the shortest time span (Bean et al. 1997; Ochatt et al. 2002, 2004). In traditional breeding, the development of plant varieties with resistance to abiotic or biotic stress is a costly and time-consuming process. Selection of individuals during the early generations of a segregating population can lead to phenotypic bias created by the rejection of non-competitive individuals. To overcome this problem, breeders may delay intense selection until individual genetic lineages approach homozygosity (Caligari et al. 1987). Given the fundamental role of homozygosity in plant breeding, it is not surprising that in recent decades a large international effort has targeted the development of biotechnology-based tools designed to achieve gene fixation as efficiently as possible.

The production of monoploid or dihaploid plants and the subsequent chromosome doubling, termed 'doubled haploid' (DH) technology (detailed in Chap. 1), has become the most widely applied and beneficial biotechnological tool for the rapid development of homozygosity. The use of DH technology in the breeding programs of responsive species such as cereals and oilseeds has led to impressive gains in productivity (Jain et al. 1996/1997; Maluszynski et al. 2003). Unfortunately, there remain whole families of important crop species, such as the *Fabaceae*, which are recalcitrant to the widespread adoption of this tool (Ochatt et al. 2009; Germanà 2011; Lulsdorf et al. 2011; Ribalta et al. 2012). There are also situations across a range of crop species where the linkage of genes or the need for further recombination requires an alternative approach. In these cases, conventional single seed descent (SSD), pioneered by Goulden (1939), has often been adopted for the relatively rapid attainment of homozygosity. The SSD method compresses the breeding cycle by the advancement of one randomly selected seed per plant through the early segregating stages. As only one seed is needed to produce the next generation, plants can be grown under conditions that accelerate flowering and seed set, but do not encourage high yield. This leads to a higher rate of generation turnover by reducing the length of the generation cycle. The SSD strategy is beneficial in situations where simultaneous selection is required for several characteristics with different heritability, and it is efficient compared to conventional in-season generation turnover. It is our intention to review here research progress towards the use of in vitro tools to accelerate generation turnover within the SSD framework.

2 Strategies for In Vitro-Assisted Breeding Cycle Compression

In this chapter, we focus on in vitro modifications to the conventional SSD approach. It is always pertinent to reduce the duration of generation cycles, as genotypes cannot be registered as new varieties until they reach a stable, near-homozygous or

homozygous state (Kasha and Maluszynski 2003).The need for a fast-track breeding method becomes clear when, as is the case for many species, turnover of only one to two generations per year are feasible in the field (Roumet and Morin 1997; Castello et al. 2015; Croser et al. 2016).

Since the late 1990s, various systems exploiting SSD in vitro have been proposed, all aimed at shortening the generation time in crop species starting from immature seeds or very young seedlings forced to flower precociously. The in vitro modifications can, in general, be divided into two strategies:

1. In vitro SSD (IVSSD), which is the precocious completion of the full life cycle in vitro
2. In vitro-assisted SSD (IVASSD), which grows the plant under in-soil conditions designed for rapid floral onset and embryo maturity coupled with the truncation of the seed filling by in vitro germination of the immature seed

Both approaches have potential to improve SSD generation turnover up to fourfold and provide platforms for rapid gains in productivity across a range of crop species.

In vitro methods to accelerate the turnover of generation cycles have been applied to a range of species from monocots to dicots, even woody species (Tisserat and De Mason 1985; Ochatt et al. 2002; Teixeira da Silva et al. 2014a). There is a particularly compelling requirement for technologies such as IVSSD that enable completion of the full life cycle of the plant in vitro, in cases where the studied species are rare or difficult to root (Bean et al. 1997; Ochatt 2015; Gatti et al. 2016) or where there is limited seed supply (Dickens and van Staden 1988; Tisserat and Galletta 1988; Al-Wareh et al. 1989).

Both IVSSD and IVASSD require the following: (a) identification of physical and nutritional parameters to elicit rapid floral onset and successful fruiting, (b) miniaturization of plants to efficiently turnover high numbers without high resource inputs and (c) development of a robust protocol for in vitro germination from immature seed. Publications relating to the use of these strategies for breeding cycle compression in economically important species are summarized for monocotyledonary species (Table 1), leguminous and oilseed species (Table 2) and horticultural species (Table 3).

3 In Vitro Flowering and Fruit Set

In vitro flowering is one of the most elusive and fascinating in vitro developmental processes (Teixeira da Silva et al. 2014a, b). It plays a central role in selective hybridization, particularly when pollen from rare stocks is used, and is the likely first bottleneck to be sidestepped in the recombination of genetic material through in vitro fertilization of noncompatible lines (Murthy et al. 2012; Haque et al. 2016). There are some advantages to completing the full plant life cycle in vitro. In vitro flowering and fruit set can assist breeding programmes in the intra- or intercrossing of varieties via synchronization of flowering. Additionally, in vitro flowering and

Table 1 Publications relating to breeding cycle compression in economically important monocot species

Technique	Reference	Species	Immature seed culture	Media	Growth conditions			Generations/ year
					Temperature (day/night)	Photoperiod	Type of light	
In vivo/in vitro	Liu et al. (2016)	Oat	12 days after the first spikelet of inflorescence visible	In vitro: Modified MS In vivo: Sand-peat (50:50)	25/22 °C	16 h	In vitro: White fluorescent in vivo: Artificial	Up to 7
		Triticale	12 days after flowering					
	Rizal et al. (2014)	Sorghum	8–12 DAP	In vitro: Seeds on MS medium, then seedlings in Yoshida culture solution In vivo: Sterilized soil	In vitro: 30 °C In vivo: Not provided	In vitro: 24 h In vivo: Not provided	In vitro: Artificial In vivo: Not provided	>4
	Adamski et al. (2014)	Wheat	15–20 days after flowering	In vitro: Gamborg B5 medium	Not provided	In vitro: 16 h In vivo: Not provided	In vitro: White fluorescent In vivo: Natural	Up to 2
	Zheng et al. (2013)	Barley	39–50 DAP depending on genotype	In vitro: Modified MS In vivo: Sand-peat (50:50)	In vitro and in vivo: 25/22 °C	In vitro and in vivo: 16 h	In vitro: White fluorescent In vivo: Artificial	Up to 9
		Wheat	42–55 DAP depending on genotype					Up to 8
In vitro only	Yao et al. (2016)	Wheat	8 DAP	Seed germination on modified MS. seedlings on half strength modified MS	22 °C for 20 days and then 28 °C	16 h for 20 days and then 20 h	Not provided	6–8

Table 2 Publications relating to breeding cycle compression in economically important legume and oilseed crop species

Technique	Reference	Species	Immature seed culture	Media	Growth conditions			Generations/ year
					Temperature (day/night)	Photoperiod	Type of light	
In vivo/in vitro	Ochatt et al. (2002) Ochatt and Sangwan (2010)	Pea Bambara groundnut	Yellow pods	In vitro: Modified MS In vivo: Vermiculite	24/20 °C for pea, 27 °C for bambara	16 h pea 10 h bambara	Sodium lamps and incandescent	4.5–5.5 (pea) 4–4.6 (Bambara)
	Surma et al. (2013)	Pea Yellow lupin Narrow-leaf lupin	21 DAP (pea) 28 DAP (lupins)	In vitro: Modified MS In vivo: Soil	22/20 °C (pea) 16/12 °C for 2 weeks, then 22/20 °C (lupins)	16 h	Not indicated	Up to 3(low survival rate)
	Croser et al. (2016)	Narrow-leaf lupin	26 DAP	In vitro: Modified B5 In vivo: Potting mix	In vitro: 25 °C for 24 h,24/20 °C for 6 days In vivo: 24/20 °C	In vitro: Dark for 24 h, 20 h for 6 days In vivo: 20 h	Far-red enriched LED	>5
	Bermejo et al. (2016)	Lentil	18 DAP	In vitro: Modified MS + IAA In vivo: Peat + perlite	24 °C	16 h	White fluorescent	4 (low survival)
	Yao et al. (2016)	Canola	10 DAP	In vitro: Modified MS In vivo: Potting mix	In vitro: Germination at 20–22 °C, then 25/22 °C In vivo: 25/22 °C	In vitro: Germination in dark, then 16 h In vivo: 20 h	Not indicated	Up to 7
	Ribalta et al. (2017)	Pea	18 DAP	In vitro: Modified MS In vivo: Potting mix	In vitro: 25 °C In vivo: 25 °C for 24 h, then 24/20 °C 6 days	In vitro: Dark for 24 h, 16 h for 72 h In vivo: 20 h	In vitro: White fluorescent In vivo: Far-red enriched LED	6–7 (early, mid and late types)

(continued)

Table 2 (continued)

Technique	Reference	Species	Immature seed culture	Media	Growth conditions			Generations/ year
					Temperature (day/night)	Photoperiod	Type of light	
In vitro only	Narasimhulu and Reddy (1984)	Peanut	No	Blaydes + kn or BAP + auxins	25 °C	24 h	White fluorescent	–
	Dickens and van Staden (1988)	Soybean	No	Modified MS	25/20 °C	8 h	Not indicated	–
	Franklin et al. (2000)	Pea	No	Shoot development: MSB5 + BAP Flowering induction: Modified MS + IBA or NAA + GA_3	25 °C	16 h	White fluorescent	–
	Ochatt et al. (2002) Ochatt and Sangwan (2010)	Pea Grass pea	No	Modified B5 and modified MS	24/20 °C	16 h	White fluorescent	5–7 (pea) 3.2–3.5 (grass pea)
	Asawaphan et al. (2005)	Peanut	No	Modified MS + kn	25 °C	16 h	White fluorescent	–
	Sarker et al. (2012)	Lentil	No	Shoot development: Modified MS + BAP, Kn, GA_3 and tyrosine Flowering induction: Modified MS + IBA + NAA	25 °C	16 h	Not indicated	–

	Ribalta et al. (2014)	Pea		Modified MS + antigibberellin	25 °C	16 h	White fluorescent	>6 (early types) >5 (mid/late types)
	Mobini et al. (2015)	Lentil Faba bean	14 DAP (lentil) 18 DAP (faba)	Modified MS + IAA and zeatin (faba) or 4Cl-IAA (lentil) + antigibberellin	22/18 °C 3 days, then 20/18 °C	18 h for 3 days, then 20 h	White fluorescent	6.7 (faba)[a] 8.1 (lentil)[a]
	Ochatt and Sangwan (2008, 2010)	*Arabidopsis*	No	Modified MS + picloram and BAP	24 °C	16 h	White fluorescent	>10
	Lentini et al. (1988)	*Brassica campestris*	No	White	25 °C	16 h	White fluorescent	–

[a]Turnover predicted based on time to turnover one generation

Table 3 Publications relating to breeding cycle compression in economically important horticultural species

Technique	Reference	Species	Immature seed culture	Media	Growth conditions			Generations /year
					Temperature (day/night)	Photoperiod	Type of light	
In vivo/in vitro	Bhattarai et al. (2009)	Tomato	Yes	In vitro: Germination on modified MS solid until germination; liquid media until differentiated; rock wool cubes with liquid media for 3 weeks In vivo: Vermiculite and potting mix	In vitro: 25 °C	In vitro: 36 h darkness followed by 16 h light In vivo: Low light, moderate temperature and high RH	In vitro: F36 W/GRO fluorescent lamps	5 possible
	Ameha et al. (1998)	Cucumber	No	In vitro: Modified MS				
In vivo/in vitro	Metwally et al. (2012)	Garlic	No. root segments containing apical meristems	In vitro/in vivo: First generation, followed by three generations in the soil				
	Asawaphan et al. (2005)	Peanut	Yes	In vitro: Modified MS	25 °C	16 h light. Low light intensity induced flowering	Cool white fluorescent tubes	Unknown

In vitro only	Al-Wareh et al. (1989) Abstract only seen (see below)	Potato	No. apical meristems					
	Ameha et al. (1998)	Cucumber	No	In vitro: Modified MS	20–22 °C	16 h light	Cool white fluorescent tubes	Rapid flowering but no further plant development
	Bodhipadma and Leung (2003)	Capsicum (*Capsicum annum*)	Yes	In vitro: Initially solid modified MS, later liquid modified MS on solid modified MS	22 °C	Constant illumination	Philips cool white fluorescent tubes	Generation 2 seeds did not develop
	Haque et al. (2016)	Capsicum (*C. annuum*, *C. chinensis*, *C. frutescens*)	No	Modified MS	22 °C	16 h	Philips cool white fluorescent tubes	Only some cultivars produced viable seed in vitro. Average generation length 144 days (2 per year)

seed harvest avoid potential glasshouse losses at transfer and the production of sterile plants or plants with reduced fertility (Bean et al. 1997; Ochatt and Sangwan 2008, 2010). It can also be used as an agent to initiate flowering in material which takes many years to flower in its natural environment, such as bamboo (Gielis et al. 2002; Dutta Mudoi and Borthakur 2012).

Within this context, it is well recognized that the process of flowering is complex; it is regulated by scores of genes and is tightly linked to the floral biology and physiological status of the mother plant. In vitro floral onset and successful fruit set depend on a combination of factors, including plant growth regulators, nutrients and carbohydrates but also light (intensity, quality and duration in the case of photoperiodic regimes), temperature and pH of the culture medium (Murthy et al. 2012; Croser et al. 2016; Ribalta et al. 2017). Growth in vitro offers the possibility for precise control of environmental and nutritional factors and hence will assist in understanding the mechanism(s) underlying their role(s) in both flowering and fruiting (Ochatt 2015, 2017). With increased understanding of the factors implicated in floral onset and embryo maturation, these processes can be modulated and shortened, thereby rendering it possible to manipulate the transition from the vegetative to the reproductive phase.

To successfully develop an in vitro flowering protocol, we must consider the role of endogenous plant growth regulators and their effect on the physiological status of the cultured plant. While in vitro flowering has been achieved in the absence of plant growth regulators in a handful of species (Dickens and van Staden 1988; Lentini et al. 1988; Al-Wareh et al. 1989; Ochatt et al. 2002; Ribalta et al. 2014; Yao et al. 2016), it is recognized that auxins are usually needed to induce in vitro flowering, either alone (Sarker et al. 2012; Mobini et al. 2015; Bermejo et al. 2016) or in combination with gibberellins (Franklin et al. 2000) or cytokinins (Narasimhulu and Reddy 1984; Ochatt and Sangwan 2008; Mobini et al. 2015). Less commonly, cytokinins have been found to promote in vitro flowering. Examples include *Arachis*, *Trifolium*, *Momordica*, *Kniphofia*, *Perilla*, *Ipomoea*, *Bacopa* and *Withania* (Wang et al. 2001; Asawaphan et al. 2005; Taylor et al. 2005; Zhang 2007; Haque and Ghosh 2013a, b; Baghel and Bansal 2014; Castello et al. 2015; Sivanesan and Park 2015; Haque et al. 2016). This promotive effect of cytokinins may be linked to the induction of in vitro flowers in rootless shoots (e.g. as in bamboo species, see Haque et al. 2016), while in others, shoots without roots would never flower, as in *Perilla* (Joshi and Nadgauda 1997). Interestingly, *Capsicum* species, although recalcitrant to in vitro approaches, were able to flower in vitro regardless of the presence or absence of roots (Zhang 2007), similar to grain legumes (Ochatt et al. 2002).

Unsurprisingly, it is often the synergistic effect of plant growth regulators with other medium additions that promotes flowering. A good example is the combination of cytokinins with silver ions, such as those provided by silver nitrate, a known inhibitor of ethylene action, which can promote or enhance successful in vitro flower induction (Bais et al. 2000; Sharma et al. 2008; Haque et al. 2016). The role of gas exchange requires careful consideration, and evidence suggests it to be a key component in many in vitro flowering protocols. For example, culturing *Capsicum*

shoots in airtight containers resulted in leaf and immature flower drop (Bodhipadma and Leung 2003), an effect also observed in legumes (Ribalta et al. 2014).

Given the obvious synergistic effects between medium additives, it is imperative that researchers wishing to develop successful in vitro flowering protocols consider not only the role of plant growth regulators but also nutrient provision and sucrose concentration. Recently, a novel strategy enabling maturation of in vitro-grown embryos and seeds in a similar way to that observed *in planta* was developed (Gallardo et al. 2006; Ochatt 2015, 2017). This strategy, after modification to avoid the blockage of precocious germination, can be used directly to understand the mechanisms underlying the maturation process and thus contribute to the design of efficient and reproducible methodologies to accelerate cycles in vitro (Ochatt et al. 2004; Gatti et al. 2016; Ribalta et al. 2014, 2017; Croser et al. 2016). Indeed, this strategy has elucidated the role of nitrogen (Gallardo et al. 2006); sulphur (Ochatt 2015; Ochatt and Revilla 2016) and sugar (Ochatt 2011, 2015), as well as auxins (Atif et al. 2013b), gibberellic acid (Ochatt 2011), abscisic acid (Ochatt 2017) and, quite recently, phytosulfokine-alpha (Ochatt et al. unpublished).

While flowering is evidenced in a broad range of species, even in situations where this is not the intent of the experiment, successful fruit set can be harder to achieve. In terms of economically important crop and horticulture species, seed set without a need for hand pollination in vitro has been observed in *Lens* (Sarker et al. 2012; Bermejo et al. 2016); *Pisum* (Ochatt et al. 2002, 2004; Ribalta et al. 2014); *Triticum* (Yao et al. 2016) and various members of the *Solanaceae*, including *Lycopersicon* (Sheeja and Mandal 2003) and *Capsicum fruitescens* (Tisserat and Galletta 1995). Conversely, in vitro fruiting was only possible after hand pollination in *C. annuum* (Bodhipadma and Leung 2003). The relative responsiveness of different species to tissue culture manipulation is thus a key consideration in the development of IVSSD technology (Ochatt 2015).

A further consideration in the development of a successful in vitro flowering protocol is the ability to control plant size. Ribalta et al. (2014) reported the use of antigibberellin flurprimidol to reduce plant height within an in vitro flowering protocol. More recently, flurprimidol has been adopted in the development of in vitro flowering protocols for faba bean and lentil (Mobini et al. 2015). The addition of flurprimidol to the culture medium has also enabled the production of smaller plants required for in vitro growth, permitting in vitro flowering across a range of pea cultivars. This is of particular relevance for the in vitro culture of tall genotypes. The authors also observed a differential response to flurprimidol on plant height reduction across genotypes, with taller genotypes showing a stronger response to the antigibberellin treatment. These differences were attributed to the variable endogenous content of active gibberellins between genotypes.

In recent times, researchers have also paid attention to the genetic determinism of flower induction and seed set and have included studies where the expression of genes involved in these phenomena was modified through genetic manipulation (GM). In this chapter, we focus on non-GM material, especially since global legislation on the use and cultivation of GM plants is not presently available (Davison and Ammann 2017) and, therefore, their subsequent use in breeding programs not yet

assured. However, it is worth noting that the in vitro flowering technique offers opportunities to overcome legislative impediments for in vivo cultivation of genetically modified genotypes when the research is aimed at gene function validation through the use of transformants with a modified gene(s) expression level (Ochatt 2015; Davison and Ammann 2017).

In summary, protocols for completion of the plant life cycle solely in vitro offer undeniable benefits in cases of enfeebled hybrids, synchronization of flowering for crossing efforts and overcoming legislative barriers and for use as models for unravelling the modulation of flowering. However, protocols for the induction of flowering in vitro are usually complex and genotype-specific (Table 1), and many species and genotypes remain unresponsive to the triggering of early flowering in vitro, limiting its general use within crop improvement programs (Ribalta et al. 2017). For this purpose, the SSD protocols that alternate between in vivo growth and in vitro germination circumvent the issues related to in vitro flowering, allowing a wider applicability of the system.

4 In Vitro-Assisted Single Seed Descent Strategy

The combination of in-soil growth conditions designed to trigger early flowering with technology to enable precocious in vitro germination of the seed has facilitated the development and exploitation of techniques to accelerate breeding across a range of species. Changes in environmental conditions have a significant effect on plant photomorphogenic responses including flowering (Zhou and Singh 2002; Spalding and Folta 2005). The influence of temperature and light (quality, intensity and duration) on the rate of progress towards flowering is well recognized (Friend et al. 1963; Murfet and Reid 1974; Moe and Heins 1990; Searle and Coupland 2004; Nelson et al. 2010; Croser et al. 2016; Ribalta et al. 2017). Under field conditions, a model has been developed linking the rate of progression towards flowering to temperature and day length. A relationship, $1/f = a + bT + cP$, was established where f is the time in days from sowing to first flowering, T and P are the respective mean pre-flowering values of temperature and photoperiod and a, b and c are genotypic constants (Summerfield et al. 1983). This model was successfully applied to a range of legume species (Summerfield et al. 1983; Roberts et al. 1985; Summerfield and Lawn 1987). In all species, genetic variation was identified for the constants of the model. Vernalization notwithstanding temperatures greater than a critical minimum base to an upper maximum is known to speed plant development (Iannucci et al. 2008; Vadez et al. 2012). In long-day plants, extended photoperiods promote flowering by reducing the synthesis or transport of an inhibitor of flowering. On the other hand, in short-day species like soybean and bambara groundnut, long nights are most inductive (Cregan and Hartwig 1984). Growth under controlled environments and the development of more affordable lighting systems for photoperiod extension have opened the door to large-scale efforts to manipulate plant morphogenesis for accelerated breeding objectives. These efforts include classical SSD,

undertaken under conditions optimized for flowering and harvest of a single seed to germinate to the next generation and, more recently, the in vivo acceleration of flowering time combined with in vitro precocious germination.

The acceleration of flowering initiation with photoperiod manipulation can be further improved when light quality parameters are optimized. Understanding the role light quality plays on floral initiation is key to a range of pre-breeding tools (Croser et al. 2016). Research in the model long-day species pea suggests that extended exposures to light with a low red to far-red ratio (R: FR) are most effective for early flower induction (Runkle and Heins 2001; Weller et al. 2001; Cummings et al. 2007). An R: FR ratio close to the natural daylight level of 'around 1' can accelerate floral induction in long-day plants (Vince-Prue 1981; Croser et al. 2016; Ribalta et al. 2017). In addition to light relative values, the importance of considering absolute light values (in particular, the number of photons in the FR region) for flower induction has been demonstrated in a range of legume species and models developed for prediction of flowering behaviour in response to light (Croser et al. 2016). To effectively manipulate plant morphology, it is important to understand the amount of plant-usable light that different light sources emit. Traditional lighting technologies, such as metal halide, fluorescent or high-pressure sodium, provide fixed emission spectra composed of many bands in the wavelength range from 320 to 800 nm (Naznin and Lefsrud 2014). These types of light sources are the most commonly used in in vitro-based systems (Table 1). The recent rise of affordable light-emitting diode (LED) lighting systems has facilitated more accurate manipulation of light quality. LED optics deliver a uniform and precise beam of usable light and allow the selection of specific wavelengths to elicit specific photomorphogenic responses (Stutte 2009; Cope and Bugbee 2013; Naznin and Lefsrud 2014). The use of LED arrays to provide photoperiod, combined with photoperiod duration and temperature manipulation at the upper limits of the plant's physiological tolerance, has provided the capacity to trigger rapid flowering across a broad range of legume crops, pasture species and flowering type genotypes (Croser et al. 2016; PazosNavarro et al. 2017).

Plant miniaturization is also required to facilitate efficient resource use under controlled environment conditions. The use of the antigibberellin agent flurprimidol to control plant size under glasshouse conditions has been reported in some species (Hamid and Williams 1997; Ochatt et al. 2002; Pobudkiewicz and Treder 2006; Burton et al. 2007). Ochatt et al. (2002) proposed the use of flurprimidol as a soil drench in an in vivo-assisted SSD system. The antigibberellin acts to suppress gibberellic acid (GA) biosynthesis, thereby reducing internodal spacing (Rademacher 2000). Treated plants can flower and set seed at a much-reduced height, enabling the adoption of multi-tier shelving units in controlled environment growth facilities. When antigibberellins are applied in vivo within a regime of physical containment of root size and highly regulated water and nutrient supply, a density of up to 100 grain legume plants m^{-2} can be grown to successful seed set (Croser unpublished results). It is important to note that the exact concentration and timing of application of the antigibberellin needs to be optimized for each growth environment, new species and even across genotypes within the same species (Ribalta et al. 2014; Mobini and Warkentin 2016).

The induction of flowering under controlled environment conditions designed to accelerate flowering is the first of two key components in an IVASSD protocol. The second component is the precocious germination of immature seed. This phase is common to both IVSSD and IVASSD protocols.

5 Embryo Development Leading to Precocious Germination

To further speed generation turnover, IVASSD strategies exploit precocious in vitro germination of immature seed in the selfed material (Table 1). To achieve precocious germination, embryo developmental processes and timing need to be well understood. Seed development can be divided into a sequence of four stages from ovule fertilization by pollen through to germination of the mature seed. The first phase is cell division, which results in embryogenic axis development and cell number determination. The second phase is embryo physiological maturation from globular to heart then torpedo and finally cotyledonary embryos. The third phase is seed filling which results in storage product accumulation and acquisition of germination competence and tolerance to desiccation. The fourth and final phase is the dehydration that precedes embryo quiescence and dormancy until seed germination (Kucera et al. 2005; Weber et al. 2005). The duration of these phases, measured in terms of days after pollination (DAP), is typically species-specific, and the transition between them is transcriptionally regulated (Ochatt 2017). The importance of seed physiological maturity for successful precocious in vitro seed germination and subsequent robustness of germinated plants is well recognized (Gallardo et al. 2006; Ochatt 2015; Croser et al. 2016; Ribalta et al. 2017). In species such as field pea (*Pisum sativum* L.), the acquisition of germination competence is associated with an increase in seed dry matter during phase three when all embryo structures have been formed (Gallardo et al. 2003). Embryo physiological maturity occurs at the end of seed filling when the seed reaches its maximum dry weight (Ellis et al. 1987).

During embryogenesis, the seed attains its final size through cell division coupled with increased cell size and is accompanied in dicots by a transient endosperm that is progressively replaced by the developing embryo. During morphogenesis, the supply of nutrients to the embryo, modulated by the maternal seed coat, determines final seed size and weight acting on the size of cotyledonary cells particularly during seed filling. If any of these phases is impaired, so will be seed germination. The transition between embryogenesis and seed filling is thus paramount for seed development. In this short period, generally 3–4 days and between 11 and 14 DAP in legumes (Gallardo et al. 2008; Ochatt 2015, 2017), cotyledonary cells divide actively (Atif et al. 2013a or b?), start to differentiate and transform the embryo into a complex organ with capacity to store a wide array of products while simultaneously becoming photosynthetically active. It is at this time that expression of genes involved in storage product accumulation peaks and endoreduplication (the cytogenetic imprint of this transition period) starts (Ochatt 2015, 2017; Ochatt and Revilla 2016).

During seed filling, the quantity and quality of storage products within the seed are determined, and competence for germination and desiccation tolerance (some 4–5 days later) is acquired. This stage is characterized by an intense endoreduplication, resulting in significant cell enlargement, a concomitant increase in dry weight and a corresponding decrease in relative water content. Some factors have been implicated in the modulation of embryo physiological maturity including hormone regulation, sugar metabolism and moisture content (Obendorf and Wettlaufer 1984; Le Deunff and Rachidian 1988; Gallardo et al. 2006; Ochatt 2011; Slater et al. 2013; Ribalta et al. 2017). Although hormonal regulation of legume seed development and germination has been extensively studied (Weber et al. 2005; Ochatt 2015), the role of hormones on immature seed in vitro germination requires further elucidation. Sucrose and moisture contents have been proposed as reliable indicators for the identification of the exact stage for immature seed culture (embryo physiological maturity) (Le Deunff and Rachidian 1988;Ribalta et al. 2017). For example, in pea, embryo physiological maturity is attained at 18 DAP, when seed moisture content is below 60% and the sucrose level is below 100 mg g^{-1} DW. The in vitro culture of seeds at 18 DAP not only resulted in more robust in vitro germination but also faster seedling development and accelerated floral onset in the resulting plant compared with the in vitro culture of immature seeds at 14 and 16 DAP (Ribalta et al. 2017). The success of any IVASSD strategy mediated by a shortening of each generation cycle will thus depend on modulation and exploitation of this key transition between embryogenesis and seed filling.

Predicting the optimal developmental stage of the seed for robust precocious in vitro germination is of particular relevance in seeds exhibiting dormancy (PazosNavarro et al. 2017). There are two types of dormancy: the first is caused by a water impermeable ('hard') seed coat that needs to be softened by either chemical or mechanical means before imbibition can occur (Taylor et al. 2005), and the second is regulated by hormonal processes following imbibition (Kucera et al. 2005). For example, in the field, *Trifolium subterraneum* (subterranean clover) can overcome dormancy mechanisms with a combination of high and diurnal temperature fluctuations over the summer-autumn period (Nichols et al. 2013). Under in vitro conditions, seed coat dormancy can be overcome by scarification or removal of the seed coat. Embryo dormancy has been successfully overcome in species such as *Lupinus* through the addition of plant growth regulators to the culture medium (Kasten et al. 1991).

Optimization of in vivo and in vitro growth conditions for early flower induction combined with an improved understanding of the physiology of seed development has enabled the rapid development and exploitation of IVASSD protocols across a range of species, including cereals, oilseeds, legumes and horticultural crops (Tables 1, 2 and 3). The combination of growth under optimized environmental conditions with plant miniaturization and in vitro precocious germination has provided protocols that turn over 5–8 generations per year in grain legumes (Ochatt et al. 2002, 2010; Ribalta et al. 2017) and up to 19 generations per year in *Arabidopsis* (Ochatt and Sangwan 2008, 2010). It is expected that this technology will be adopted in a

range of research and industrial applications as the full benefits of accelerated generation turnover systems become evident within genetic improvement programs.

6 Integration of Abiotic/Biotic Stress Screening within Protocols for Breeding Cycle Compression

Just as each recombinant inbred line (RIL) generation in a SSD breeding program offers an opportunity to observe the development and performance of lines, so too does each generation in an IVASSD program. There is an additional driver to phenotype segregating RILs in an IVASSD program: the size of populations is a major contributor to the cost base of IVASSD (expense of space in controlled facilities, intensive management, etc.), so selecting the most promising lines early in the RIL generations can offer major cost savings to end users. There are, however, constraints imposed on phenotyping due to the IVASSD conditions. The nature of IVASSD growth conditions, where normal plant development is modified or even sacrificed to ensure timely production of seed for the following generation, makes it impossible to phenotype for certain traits such as yield and plant morphology, including branching characteristics. However, other traits (like abiotic stress tolerance) may be screened effectively, albeit under modified growth conditions (Bennett pers. comm.). Naturally, stress response metrics that can be measured in a nondestructive manner are best suited to phenotyping in an in vivo-based accelerated SSD system, as minimal disturbance to the plant should ensure rapid production of the next generation. Many abiotic stresses are particularly amenable to screening during an accelerated SSD generation, as the stress can be imposed, plant response measured, and then stress removed to enable the generation to be completed under the usual IVASSD conditions. For instance, mineral deficiencies or toxicities, such as boron, aluminium or salt toxicity, can be imposed on hydroponically grown plants, after which the solution can be replaced with a balanced solution, or plants transplanted to soil, quickly releasing them from the stress to allow normal seed development.

In vitro-assisted SSD systems are also likely to be amenable to phenotyping in the in vivo phase for tolerance to biotic stresses (particularly those scored from leaf symptoms) and environmental stresses such as chilling or frost tolerance, as plants are grown under tightly controlled environmental conditions that are open to manipulation. Plants can easily be subjected to altered temperature or light conditions for the desired duration and then returned to normal conditions to monitor plant response. To minimize delays in the phenotyped generation or to permit differentiation using destructive testing, this type of treatment can be undertaken on cloned material.

As noted above, there is a clear advantage to selecting the most promising lines early in the SSD progression from a cross to the fixed lines; however, there are risks to doing so. In early generations of a segregating population, the presence or absence of a trait in an individual does not guarantee that subsequent generations derived from that individual will share its genetic makeup. There is a risk of unwittingly

excluding promising material, and progressing material, that is not useful. Ultimately, a compromise must be achieved between cost savings and these risks, as is the case in any breeding program.

7 Genomic Applications of Breeding Cycle Compression

The IVASSD strategies rely on the understanding and manipulation of key physiological milestones such as flowering response and the competence for embryo germination. Recent advances in next-generation sequencing and high-throughput technologies with low-cost genotyping platforms are rapidly advancing the understanding of these parameters. These technologies provide novel insights into the molecular basis of floral initiation and plant development at the transcriptional network level that contribute to the floral initiation process and the transition between developmental stages (Atif et al. 2013a; Gupta and Jatothu 2013; Ochatt 2015, 2017). This knowledge is helping to identify major changes in the plant leading to the activation of flowering genes while also providing a better understanding of floral initiation, as well as the molecular networks underlying the developmental transition. Such studies at whole genome transcriptome levels have the potential to enhance the development of tools for accelerated breeding. Understanding the triggers for flowering and the effect of stress on time to flowering will assist in breeding cultivars adapted to specific production areas. Elucidation of the genes involved in these fundamental mechanisms will directly drive the advances in the development of platform technologies such as IVASSD for rapid genetic gain.

A key application of IVASSD breeding is in the development of mapping populations, particularly the more rapid development of multiparental populations. In conventional quantitative trait loci (QTL) mapping, a biparental population is used to identify the genomic location and magnitude of the effect of a locus that affects a phenotypic trait (Yu et al. 2013; Islam et al. 2014; Cao et al. 2015). QTL mapping using such biparental populations is usually low in resolution since only two alleles per locus are analysed and genetic recombination is limited (Cavanagh et al. 2008). A multiparent advanced generation intercross (MAGIC) strategy is anticipated to have higher genetic diversity, smaller haplotype blocks, higher recombination and better mapping resolution (Cavanagh et al. 2008). Thus, a MAGIC population has a better chance to break the negative linkage between complex traits. Recently, use of a MAGIC population to identify QTL has become a new approach (Islam et al. 2016), where platform technologies such as accelerated single seed descent and advancement of next-generation sequencing techniques coupled with novel statistical and bioinformatics tools have a key role to play. This will help in developing better and faster products for both farmers and markets while developing novel genomic resources for more precise trait mapping and molecular breeding (Fig. 1).

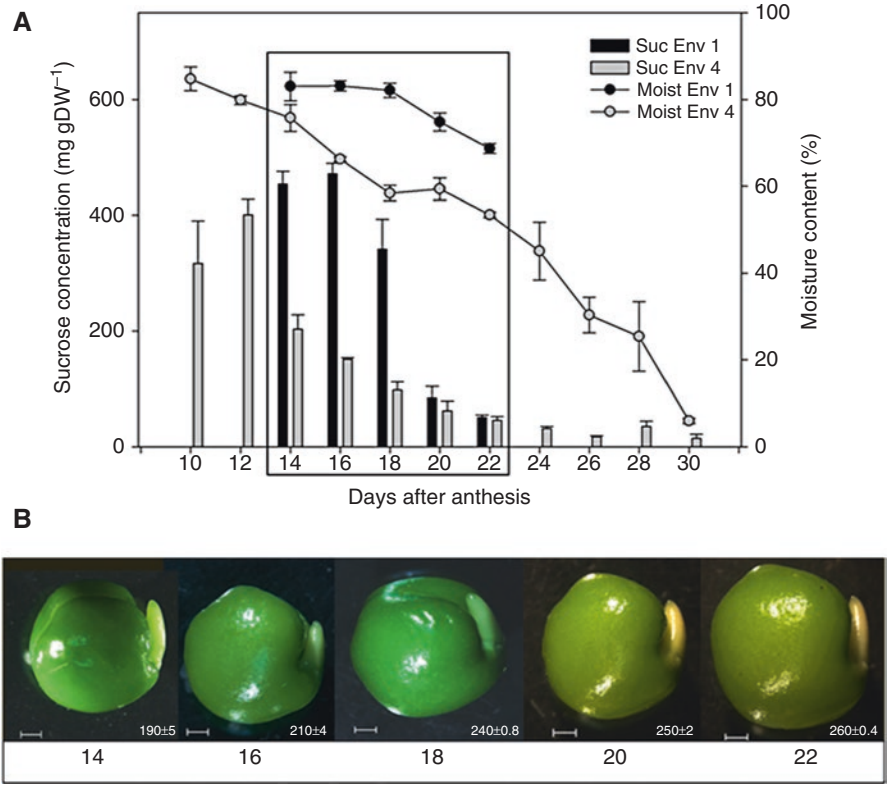

Fig. 1 A Sucrose concentration (Suc, mg g^{-1} DW) and moisture content (Moist, %) during pea seed development in natural light (Env 1) and artificial light (Env 4). The box represents the more narrow range of seed development used to compare between environments. Data represent mean values ± SE; n = 6–9. B Kaspa seed development and weight (mg; values ± SE; n = 5; scale bar 1 mm) from 14 to 22 days after pollination in Environment 4. (Adapted from Ribalta et al. 2017)

8 Conclusion

The development of IVASSD technology has paved the way for rapid genetic gains, particularly in those species recalcitrant to doubled haploid techniques. While IVASSD technology is only just beginning to be used in an organized manner within plant breeding and pre-breeding programs, it has excellent potential as a platform for a rapid return to homozygosity, the development of biparental and multiparental populations for a range of genomic applications and the exploitation of further levels of recombination. The integration of screening tools, including marker-assisted selection or phenotyping for biotic and abiotic stress tolerance/resistance will further enhance the value of this technique within commercial breeding programs as it will speed the differentiation of segregating material and capture specific traits of interest. For its part, in vitro completion of the plant life cycle has a key role to play in the isolation and unravelling of factors affecting progression to flowering and

embryo developmental processes. A better understanding of these processes will enable further refinement of both IVSSD and IVASSD protocols. We expect that IVASSD technologies will play a significant and expanding role in plant improvement across the coming decade.

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Tissue Culture Approaches in Relation to Medicinal Plant Improvement

Frank Marthe

Abstract The medicinal and aromatic plant species owe their importance to the production of secondary metabolites. These species with worldwide highest volume of business for phytopharmaceuticals, herbal supplements and functional food ordered by size are ginseng (*Panax ginseng* C.A. Mey.), ginkgo or maidenhair tree (*Ginkgo biloba* L.), noni or Indian mulberry (*Morinda citrifolia* L.), saw palmetto (*Serenoa repens* [W. Bartram] Small), coneflower (*Echinacea* spp. Moench), valerian or all-heal (*Valeriana officinalis* L.), green tea (*Camellia sinensis* [L.] Kuntze), garlic (*Allium sativum* L.), Saint John's wort (*Hypericum perforatum* L.), black cohosh, black snakeroot (*Actaea racemosa* L., syn. *Cimicifuga racemosa* [L.] Nutt.), great nettle (*Urtica dioica* L.), horse chestnut (*Aesculus hippocastanum* L.), hawthorn (*Crataegus* spp.) and apricot vine or maypop (*Passiflora incarnata* L.) (Hoppe (2013) Handbuch des Arznei- und Gewürzpflanzenbaus (book in German), vol 1. Saluplanta, Bernburg, pp 509–513). Plant tissue and protoplast culture/somatic hybridization methods offer a huge scope for creation and conservation of genetic variability for the improvement of a wide variety of medicinal plants. In addition to this, tissue culture methods are now being used for the production of secondary metabolites in vitro. The micropropagation is the best example of the commercial application of tissue culture technology. Meristem-tip culture helps in developing disease-free plants. Development of efficient methods for somatic embryogenesis and embryo desiccation and encapsulation technology may lead to the production of 'synthetic seeds' for mass cloning of plants. Somatic embryogenesis in plants further helps in cloning and genetic transformation. Production of haploids anther/pollen culture from wide hybrids has been exploited for the early release of varieties. Embryo culture is very useful technique to obtain interspecific and intergeneric hybrids among otherwise difficult to cross parents. Protoplast culture and somatic cell hybridization help in combining characteristics even from otherwise sexually incompatible plant species and to obtain cytoplasmic hybrids (cybrids). In vitro freeze-storage and cryopreservation in liquid nitrogen at ultra-low temperature of –196 °C (−320 °F) are very important methods for germplasm conservation especially of the vegetatively propagated crops. Since the possibility

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of producing useful secondary products in plant cell cultures was first recognized in the 1970s, significant progress has been made, and a number of plant species have been found to produce secondary products such as capsaicin, shikonin, diosgenin, caffeine, glutathione and anthraquinone.

Keywords Medicinal plants · Micropropagation · Tissue culture · Anther culture · Haploids · Secondary metabolites · Cryopreservation

1 Introduction

The medicinal and aromatic plants species are known to produce wide variety of secondary metabolites. Whereas, mostly other crop species are being used for primary metabolites, except dyeing plants and fibre plants. More than hundred species are cultivated worldwide for medicinal use, in traditional medicine ore as spice. Species of the families Gramineae and Brassicaceae are overwhelming basis of food, feed and fuel production but has almost no importance in medicinal and aromatic use. A huge genetic variability among medicinal plant families/species and even within species holds significant promise for the further improvement of the adopted varieties. On the other hand for most of the species, no methods for cell, tissue and organ culture exist (Gosal et al. 2010). Therefore, a project has been undertaken to improve the neglected but important medicinal and aromatic plants using in vitro techniques developed for other species and mostly from other families. For the production of medicinal and aromatic plants, a general tendency exists from collection of the plants to cultivation under controlled conditions. Reasons for this trend are growing demand of high and stable quality drugs and the availability of plant material. The area of cultivation per species of medicinal and aromatic plants is relatively small. But the production of this specialized crops realizes a high agricultural value added. Between species of these specialized crops, remarkable differences exist in scale of production, quality requirements and price on the international market. The species with worldwide highest volume of business for phytopharmaceuticals, herbal supplements and functional food ordered by size are ginseng (*Panax ginseng* C.A. Mey.), ginkgo or maidenhair tree (*Gingko biloba* L.), noni or Indian mulberry (*Morinda citrifolia* L.), saw palmetto (*Serenoa repens* [W. Bartram] Small), coneflower (*Echinacea* spp. Moench), valerian or all-heal (*Valeriana officinalis* L.), green tea (*Camellia sinensis* [L.] Kuntze), garlic (*Allium sativum* L.), Saint John's wort (*Hypericum perforatum* L.), black cohosh, black snakeroot (*Actaea racemosa* L., syn. *Cimicifuga racemosa* [L.] Nutt.), great nettle (*Urtica dioica* L.), horse chestnut (*Aesculus hippocastanum* L.), hawthorn (*Crataegus* spp.) and apricot vine or maypop (*Passiflora incarnata* L.) (Hoppe 2013). All this 14 species come from different plant families (Araliaceae, Ginkgoaceae, Rubiaceae, Arecaceae, Compositae, Valerianaceae, Theaceae, Alliaceae, Guttiferae, Ranunculaceae, Urticaceae, Hippocastanaceae, Rosaceae, Passifloraceae). This illustrates the great challenges of cell, tissue and organ culture for medicinal and aromatic plants composed of many different species and small

areas of cultivation per species. But these techniques are powerful especially in growing special genotypes or productive varieties for production of high-quality drugs under particular conditions. Plant cell, tissue, organ and protoplast culture methods offer a huge scope for creation, conservation and utilization of genetic/epigenetic variability for the improvement of medicinal and aromatic plants. Besides, tissue culture techniques are now increasingly being used for the production of bioactive compounds in vitro.

2 Micropropagation

It involves the production of plants from very small plant parts through tissue culture (in vitro cloning of plants) and is one of the most successful examples of commercial exploitation of tissue culture. Micro means "very small"; therefore, as the name implies, micropropagation is done by using very small plant tissue in vitro. This involves the production of plants from very small plant parts (0.2–0.5 mm), usually shoot apices, under in vitro conditions in the laboratory and subsequent growth and multiplication of in vitro obtained plants in the glasshouse/field. In the absence of seasonal constraints, 10–20 cycles, depending upon the plant species, can be completed in 1 year, ensuring 5–30 times multiplication per cycle. Micropropagation possesses special significance in the rapid spread of new varieties of vegetatively propagated species, rejuvenation of old varieties/clones of vegetatively propagated crops for improving their yield and quality, production of disease free plants, high-quality superelite planting material for commercial seed production, mass cloning of rootstocks, mass production of plants which are otherwise difficult to multiply, multiplication of rare species, multiplication of inbred lines and male-sterile lines for hybrid seed production, multiplication of selected hybrids and interstate/international exchange of germplasm. Gantait and Kundu (2017) present optimization of micropropagation, in vitro cultures, multiple shoot regeneration, rooting and acclimatization of *Vanilla planifolia* in a review. For basil (*Ocimum basilicum* L.) Manan et al. (2016) present an optimised protocol for micropropagation and in vitro flowering. The micropropagation technology can certainly help in improving yield and quality especially of the vegetatively propagated species. We have taken up a project for in vitro culture, maintenance and multiplication of some important medicinal plants.

3 Meristem Culture

In vegetatively propagated plants, the pathogens keep accumulating generation after generation, resulting in the varietal decline. There are no effective chemical methods to control viral diseases. In this regard, meristem culture (Morel and Martin 1952) is a practical approach for producing disease-free plants. Shoot meristems (0.2–0.4 mm) excised from plants are aseptically cultured in vitro on suitable

culture medium under controlled conditions. Meristem-derived plants are indexed by using electron microscopy/immunological methods for selecting pathogen-free plants (Mori and Hosokawa 1977). Disease-free plants are then micropropagated for production of superelite planting material.

4 Somatic Embryogenesis and Artificial Seeds

Induction of somatic embryos and their encapsulation can be tried for mass cloning of plants (Helal 2011). For the production of artificial seeds, the regeneration process of callus aggregates to seedlings is stopped. The callus with beginning of differentiation has to be covered to be stable for transport and processes similar to germination in soil. Most of projects for development of artificial seeds were stopped because of the relatively high costs and the instable results for establishment of equal crop covering. It is likely that automation of multiplication systems (Paek et al. 2005) will be commercially feasible within the next few years.

5 In Vitro Conservation of Germplasm

In vitro freeze-storage and cryopreservation are very important techniques for germplasm conservation especially of the vegetatively propagated species. Plants have been successfully regenerated from tissues cryopreserved as −196 °C (−320 °F) in liquid nitrogen for several months to years in several crops (Panis et al. 2001). In vitro conservation of plant parts has several advantages over in vivo conservation, e.g. in vitro techniques allow conservation of plant species that are in danger of becoming extinct. In vitro storage of vegetatively propagated plants can result in great savings in storage space and time (Prasad et al. 2015), and seed sterile plants that cannot be reproduced sexually can be maintained in vitro. This method is now being practically used at several national and international gene banks. Successful cryopreservation of plant shoot tips is dependent upon effective desiccation through osmotic or physical processes. The technique of long-term storage opens also the chance to use incomplete systems for production of F_1 hybrids. In each case, a source for male sterility must be found. For species producing leaf and root drugs, the absence of restorer lines can be gaped by in vitro propagation of the male-sterile component. The male-sterile line for hybrid cultivar ‘Varico 3’ of thyme (*Thymus vulgaris* L.) is propagated vegetatively. The seed setting of the used hybrid is very low (Carlen et al. 2010). Low or no seed set can induce higher number of flowers and prolongation of flowering period. For production of drugs from flowers, this can be useful. Multiplication by in vitro propagation is also valuable for preservation of endangered plants. The plant material from in vitro propagation can be basis for restoration programs and for cultivation. Examples for many successful protection projects by in vitro propagation can be *Nilgirianthus ciliatus* a globally

endangered aromatic slender shrub of Western Ghats, India, with extensive applications in Ayurveda (Rameshkumar et al. 2017), *Salvia brachyodon*, a Balkan endemic plant (Misic et al. 2006) and *Clerodendrum* spp. of family Lamiaceae which are sources of many unique bioactive secondary metabolites (Nataraj et al. 2016).

6 In Vitro Production of Haploids

Using conventional methods, in self-pollinated plants, the true-breeding lines are obtained after 7–8 generations through self-pollination, whereas in cross-pollinated crops, because of inbreeding depression, it becomes difficult to develop vigorous inbred lines for hybrid seed production programmes. But the production of haploids and subsequent doubled haploids through anther/pollen culture from F_1 plants occurs in less than 1 year. But in many instances, poor androgenesis and occurrence of albino plants and mixoploids have been the recurring problems. Success in development of homozygous plants via haploid induction and redoubling of haploid genome, instead of classical inbred line production by self-pollination steps, can accelerate the breeding process also for species of medicinal and aromatic plants. For this, the generation of haploids by anther culture, isolated microspore culture and ovule or ovary culture is required (Fig. 1). Besides the adaptation of in vitro techniques, extensive characterization of species and/or genera is prerequisite for

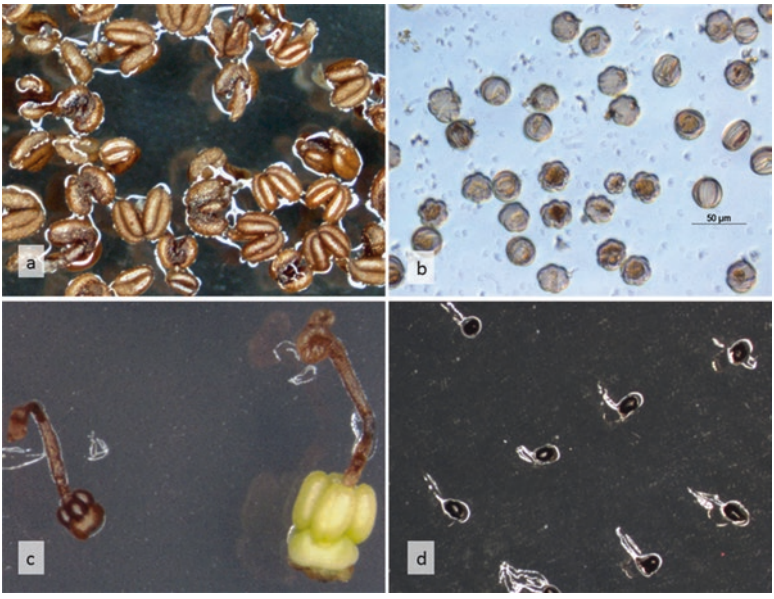

Fig. 1 Different explants for haploid induction in lemon balm (*Melissa officinalis* L.) of family Labiatae. (**a**) Anther culture. (**b**) Isolated microspore culture. (**c**) Ovary culture. (**d**) Ovule culture

selection of the most promising parent material to reach the aim of crop improvement. For this, black nightshade (*Solanum nigrum* L. complex), of Solanaceae family, can act as a good example. Because African type of *S. nigrum* which is used in medicine and folk medicine is phylogenetic different from poison European sibs of the same species (Klocke et al. 2016). Another example is the common purslane (*Portulaca oleracea*), an annual succulent herb in the family Portulacaceae which has been recognized as the richest source of essential omega-3 and omega-6 fatty acids (Amirul Alam et al. 2014).

For many species of different families, the induction of haploid and double haploid lines has been done. Because of extensiveness of information, the situation is focused on the two prominent families Labiatae (syn. Lamiaceae) and Umbelliferae (syn. Apiaceae). The family Labiatae includes high number of major medicinal and aromatic plants such as mint (*Mentha* spp.), sage (*Salvia officinalis* L.), clary sage (*Salvia sclarea* L.), lavender (*Lavandula angustifolia* Mill.), basil (*Ocimum basilicum* L.), marjoram (*Origanum majorana* L.), oregano (*Origanum vulgare* L.), rosemary (*Rosmarinus officinalis* L.), summer savory (*Satureja hortensis* L.) and thyme (*Thymus* spp.). But only for mint and clary sage formation of callus is documented (Ferrie 2007). In *Satureja khuzistanica* Jamzad and *Satureja rechingeri* Jamzad, both from Iran, Hadian et al. (2012) referred about embryoids in microspore culture. Kastner et al. (2016) report microcalli in lemon balm (*Melissa officinalis* L.) (Fig. 2). No haploid or doubled haploid plant is reported for family Labiatae.

In family Umbelliferae with the important medicinal and aromatic plants dill (*Anethum graveolens* L.), celery (*Apium graveolens* L.), caraway (*Carum carvi* L.), coriander (*Coriandrum sativum* L.), cumin (*Cuminum cyminum* L.), domesticated carrot (*Daucus carota* L. subsp. *sativus*), fennel (*Foeniculum vulgare* Mill.) and parsley (*Petroselinum crispum* (Mill.) Nym.) for many species haploid or doubled haploid plants were created. These are laceflower (*Ammi majus* L.), toothpick-plant

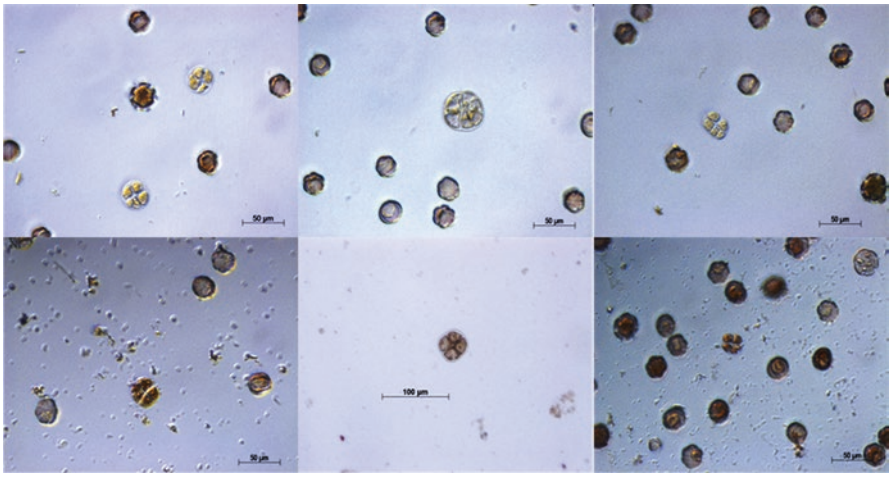

Fig. 2 Embryoids in microspore culture of lemon balm (*Melissa officinalis* L.)

(*Ammi visnaga* L.), dill, garden angelica (*Angelica archangelica* L.), caraway, carrot, fennel, lovage (*Levisticum officinale* W.D.J. Koch), parsnip (*Pastinaca sativa* L.) and anise (*Pimpinella anisum* L.) (Ferrie et al. 2011a, b). ChengMin et al. (2011) reported this for Chai Hu (chin.) (*Bupleurum chinense* DC). The results of these two exemplary families illustrate the differences and challenges for production of doubled haploids for each species.

7 Embryo/Ovule Culture

Embryo culture is a simple and cost-effective approach to obtain interspecific and intergeneric hybrids among otherwise hard to cross parents. It has been successfully used to transfer desirable genes from wild relatives into cultivated varieties of several field, fruit and vegetable crops. For interspecific and intergeneric hybridizations, genetically widely distant genetic systems have to be combined. The resulting embryos may be aborted because of parental mutual incompatibility. The technique of embryo rescue offers the facility to keep such embryos. They have to excised aseptic from surrounding pericarp and endosperm during early stage of seed development. The in vitro cultivation mostly on hormone-free media leads to a growing process similar to germination of seeds. (Sharma et al. 1996). This technique is now routinely used for several plant species. Common purslane (*Portulaca oleracea*) may be a good example (Amirul Alam et al. 2014).

8 Protoplast Culture and Somatic Hybridization

Somatic cell hybridization involving fusion of protoplasts from different species is considered an important approach to combine characteristics even from otherwise sexually incompatible species and to obtain cybrids and organelle recombination not possible through conventional hybridization. It is well known that alloplasmic association leads to male sterility as a consequence of interactions among nucleus and mitochondria. The fusion of protoplasts, somatic cells of different species, for example, from hypocotyl or mesophyll, is a promising relatively new tool in plant breeding because of its potential to create so-called cybrids. This technique enables to combine genotypes interspecific and intergeneric which cannot sexually crossed successfully. But also for interspecific sexual crosses which are more or less successful, protoplast fusion can be of interest. Besides the combination of genes or alleles for new resistances and new metabolite profile, for example, by nuclear genetic information (DNA) in cybrids, additionally, the organelles of cytoplasm are also combined. Cytoplasmic encoded traits are usually inherited through the maternal parent. In cases of missing variability for cytoplasmic male sterility (CMS) within a species, this is of importance to overcome this restriction. For protoplast fusion, some important prerequisites exist. It starts with the preparation of single cells of the desired explant in a shaking liquid media in vitro culture. Also small

aggregates in the cultures are unusable. Protoplasts are cells without cell wall which contains cellulose as principle component. To reach cell wall free Protoplasts a permanent digest by enzymes is necessary. For protoplasts from different species placed together in liquid medium, the fusion has to be induced. Fusogenic agents such as polyethylene glycol or physical means like electric shock can start the fusion process. The regeneration of somatic hybrid plants starts with tacking off the cell wall digesting enzymes. Dividing cells build aggregates from which transferred to solid media green sprouts start growing. After growing on different media with or without hormones, the transfer to soil can occur. For practical use of cybrids from protoplast fusion as well as hybrids from sexual crossings, restrictions from different pharmacopoeia have to be considered. For medicinal plants, monographs usually determine species for use.

The genus *Brassica* is almost a model regarding somatic hybridization. Beside resynthesized allotetraploids of genus *Brassica* also Chinese woad or tein-cheing (*Isatis indigotica* Fort.) was hybridized by protoplast fusion. The root of *I. indigotica* is a commonly used traditional Chinese medicine. Successful protoplast fusions exist with radish (*Raphanus sativus* L.) and bok choy (*Brassica rapa* subsp. *chinensis*) (Tu et al. 2008; Liu et al. 2007). *Duboisia* spp. contain scopolamine. The synthesized derivative butylscopolamine is used as antispasmodic. For *Duboisia leichhardtii* F. Muell and *D. myoporoides* R. BR., cybrids were produced and characterized by molecular marker (Mizukami et al. 1993). For protoplast, fusion chances are discussed. For *Leptadenia reticulata* (Jeewanti), a member of Asclepiadaceae, an efficient in vitro regeneration protocol via callus was induced (Patel et al. 2014). Lambert and Geelen (2010) produced protoplasts in *Maesa lanceolata*, a species of the family *Maesaceae* traditionally used as a cure for Leishmania in Africa.

9 In Vitro Production of Secondary Metabolites

In general, the medicinal plant species are known to produce bioactive compounds (secondary metabolites) in vivo or in vitro. These metabolites act as a valuable source of medicine and chemical compounds, including fragrances, flavours and natural sweeteners. Cultured cells/organs (more particularly roots) produce a wide range of secondary metabolites in vitro. For in vitro production of secondary metabolites, three approaches have been followed, viz. raising cell cultures, immobilization of cultured cells and growing untransformed/transformed hairy root cultures in vitro. There are several advantages of in vitro culture systems such as the following: independence from various environmental factors, any plant cell can be multiplied to yield specific metabolite and culture of cells may prove suitable where plants are difficult or expensive to grow in the field because of their long life cycles. A number of plant species have been found to produce secondary products, such as shikonin, diosgenin, caffeine, glutathione, capsaicin and anthraquinone (Havkin-Frenkel et al. 1997; Varindra et al. 1997; Fischer et al. 1999; Varindra et al. 2000; Sandhu et al. 2003; Pandhair et al. 2006; Amoo et al. 2012; Gayathri and Archana

2012; Wang et al. 2012; Amoo et al. 2013; Gonçalves and Romano 2013; Jassim and Ameen 2014; Sangwan et al. 2014). Transformed hairy root cultures, which are considered genetically more stable and keep growing fast, are now increasingly being used for the production of secondary metabolites in vitro (Srivastava and Srivastava 2007).

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