Jameel M. Al-Khayri
S. Mohan Jain
Dennis V. Johnson *Editors*

Advances in Plant Breeding Strategies: Vegetable Crops

Volume 10: Leaves, Flowerheads, Green Pods, Mushrooms and Truffles

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Preface

Contemporary plant breeders no longer need to rely solely on traditional methodologies in their work of assuring a sustainable and elastic level of world food production. However, human population is increasing at an alarming rate in developing countries and food availability could gradually become a serious problem. Agriculture production is severely affected because of environmental pollution, rapid industrialization, water scarcity and quality, erosion of fertile topsoil, limited remaining arable land to expand production area, lack of improvement of local plant types, erosion of genetic diversity, and dependence on only few crop species for food supply worldwide. According to FAO, 70% more food must be produced over the next four decades to feed a projected population of 9 billion people by the year 2050. Currently, only 30 plant species are used to meet 95% of the world's food requirements, which are considered as the *major crops.* The breeding programs of these crops have been very much dependent on the ready availability of genetic variation, either spontaneous or induced. Plant breeders and geneticists are under constant pressure to sustain and increase food production by using innovative breeding strategies and introducing minor crops that are well adapted to marginal lands and can provide source of nutrition through tolerance of abiotic and biotic stresses. In traditional breeding, introgression of one or a few genes into a cultivar is carried out via backcrossing over several plant life cycles.

With the development of new molecular tools, molecular marker-assisted backcrossing has facilitated rapid introgression of a transgene into a plant and reduced linkage drag. Continued development and adaptation of plant biotechnology, molecular markers, and genomics have established ingenious new tools for the creation, analysis, and manipulation of genetic variation for the development of improved cultivars. For example, molecular breeding has great potential to become standard practice in the improvement of several fruit crops. Adopting a multidisciplinary approach comprised of traditional plant breeding, mutation breeding, plant biotechnology, and molecular biology would be strategically ideal for developing new improved crop varieties. This book highlights the recent progress in the

development of plant biotechnology, associated molecular tools, and their usage in plant breeding.

The basic concept of this book is to examine the best use of both innovative and traditional methods of plant breeding to develop new crop varieties suited to different environmental conditions to achieve sustainable food production and enhanced food security in a changing global climate, in addition to the development of crops for enhanced production of pharmaceuticals and innovative industrial uses. Three volumes of this book series were published in 2015, 2016, and 2018, respectively: Volume 1. *Breeding, Biotechnology and Molecular Tools;* Volume 2. *Agronomic, Abiotic and Biotic Stress Traits;* and Volume 3. *Fruits*. In 2019, the following four volumes were published: Volume 4. *Nut and Beverage Crops;* Volume 5. *Cereals*; Volume 6. *Industrial and Food Crops;* and Volume 7. *Legumes*. In 2021, three volumes are being concurrently published: Volume 8. *Vegetable Crops: Bulbs, Roots and Tuber;* Volume 9. *Vegetable Crops: Fruits and Young Shoots;* and Volume 10. *Vegetable Crops: Leaves, Flowerheads, Green Pods, Mushrooms and Truffles*.

This Volume 10, entitled *Vegetable Crops: Leaves, Flowerheads, Green Pods, Mushrooms and Truffles,* consists of 14 chapters focusing on advances in breeding strategies using both traditional and modern approaches for the improvement of individual vegetable crops. Chapters are arranged in 4 parts according to the edible vegetable parts. Part I: Leaves – Chicory (*Cichorium intybus* L.), Chinese cabbage (*Brassica rapa* L. var. *pekinensis*), Rocket salad (*Eruca vesicaria* ssp. *sativa* Mill.), Spring onion (*Allium fistulosum* L.),Water spinach (*Ipomoea aquatica* Forsk.), and Watercress (*Nasturtium officinale* R. Br.); Pat II: Flowerheads and Green Pods – Cauliflower (*Brassica oleracea* var. *botrytis* L.), Globe artichoke (*Cynara cardunculus* var. *scolymus* L.), Garden pea (*Pisum sativum* L.), and Yardlong bean (*Vigna unguiculata* (L.) Walp. ssp. *sesquipedalis* (L.) Verdc.); Part III: Mushrooms – Enoki mushroom (*Flammulina velutipes* (Curtis) Singer) and Shiitake mushroom (*Lentinula edodes* (Berk.) Sing.); Part IV: Truffles – Desert truffles *(Terfezia* spp.) and White truffle (*Tuber magnatum* Picco and *T. borchii* Vittad.).

Chapters are written by internationally reputable scientists and subjected to a review process to assure quality presentation and scientific accuracy. Each chapter begins with an introduction covering related backgrounds and provides in-depth discussion of the subject supported with high-quality color photos, illustrations, and relevant data. This volume contains a total of 91 figures and 73 tables to illustrate presented concepts. Each chapter concludes with an overview of the current status of breeding and recommendations for future research directions as well as appendixes listing research institutes and genetic resources relevant to the topic crop. A comprehensive list of pertinent references is provided to facilitate further reading.

The book is an excellent reference source for plant breeders and geneticists engaged in breeding programs involving biotechnology and molecular tools together with traditional breeding. It is useful for both advanced undergraduate and postgraduate students specializing in agriculture, biotechnology, and molecular breeding as well as for seed companies and policy makers.

We are greatly appreciative of all chapter authors for their contributions towards the success and quality of this book. We are proud of this diverse collaborative undertaking, especially since this volume represents the efforts of 67 scientists from 16 countries. We are also grateful to Springer for giving us an opportunity to compile this book.

Al-Ahsa, Saudi Arabia — Jameel M. Al-Khayri

Helsinki, Finland — Shri Mohan Jain

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About the Editors and Contributors

Editors

Jameel M. Al-Khayri is a professor of plant biotechnology affiliated with the Department of Agricultural Biotechnology, King Faisal University, Saudi Arabia. He received his B.S. in biology in 1984 from the University of Toledo, M.S. in agronomy in 1988, and Ph.D. in plant science in 1991 from the University of Arkansas. He is a member the International Society for Horticultural Science and Society for In Vitro Biology as well as the national correspondent of the International Association of Plant Tissue Culture and Biotechnology. For the last three decades, he dedicated his research efforts to date palm biotechnology. Dr. Al-Khayri has authored over 70 research articles in refereed international journals, 30 chapters, and edited several journal special issues. In addition, he has edited 18 reference books on date palm biotechnology, genetic resources, and advances in plant breeding strategies. He has been involved in organizing international scientific conferences and contributed numerous research presentations. In addition to teaching, students advising, and research, he held administrative responsibilities as the assistant director of Date Palm Research Center, head of the Department of Plant Biotechnology, and vice dean for Development and Quality Assurance. Dr. Al-Khayri served as a member of

Majlis Ash Shura (Saudi Legislative Council) for the 2009–2012 term. Currently he is maintaining an active research program on date palm focusing in vitro culture, secondary metabolites production, genetic engineering, and mutagenesis to enhance tolerance to abiotic and biotic stress.

Shri Mohan Jain is a consultant and plant biotechnologist, Department of Agricultural Sciences, University of Helsinki, Helsinki, Finland. He received his M.Phil., 1973, and Ph.D., 1978, from Jawaharlal Nehru University, New Delhi, India. He was a postdoctoral fellow in Israel and the USA, and was a visiting scientist/professor in Japan, Malaysia, Germany, and Italy. He was a technical officer, Plant Breeding and Genetics, at the International Atomic Energy Agency (IAEA), Vienna, Austria, from 1999 to 2005. Dr. Jain is a member of the International Association of Plant Tissue Culture and Biotechnology, and editorial board member of *Euphytica*, *In Vitro*, *Propagation of Ornamental Plants*, *Emirates J. Food and Agriculture*, and a series on forest biotechnology. He has published more than 160 book chapters and conference proceedings in peer-reviewed journals, and edited 55 books; been invited speaker; and acted as a chairperson in several international conferences worldwide. He was awarded Nobel Peace Prize in commemoration the awarding to IAEA of the Nobel Peace Prize for 2005; also former consultant to IAEA, the European Union, The Government of Grenada, Iranian Private Company and the Egyptian Government. Currently, his research interests are somatic embryogenesis, organogenesis, haploidy, somatic cell hybridization, somaclonal variation and mutagenesis mainly in medicinal plants, date palm, and banana genetic improvement, genetic diversity, erosion, conservation, and utilization in the context of climate change and food and nutritional security.

Dennis V. Johnson is a consultant and former university professor. He is a graduate of the University of California Los Angeles where he completed his B.A. (1966), M.A. (1970), and Ph.D. (1972) degrees in geography, with specialization in agriculture and biogeography. He has taught at several colleges and universities, including the University of Houston, and was a visiting professor for 2 years at the University of Ceará, Fortaleza, Brazil. Dr. Johnson also has worked extensively with international development agencies providing technical assistance to agriculture and forestry on projects and programs in Africa, Asia, Europe, and Latin America. He has published numerous articles on palm utilization and conservation and has edited or written books for FAO, IUCN, and UNEP. He has also translated into English plant science books from Portuguese and Spanish. A decade ago, Dr. Johnson began to focus his research on date palm, in particular its introduction to non-traditional areas such as Spain, North and South America, and Australia. He co-authored a book on date growing in the USA and has made presentations at five international date palm conferences, and co-edited books on date palm, sago palm, and plant breeding.

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Part I
Leaves

Chapter 1
Advances in Chicory (*Cichorium intybus* L.) Breeding Strategies

Laila Aldahak, Khaled F. M. Salem, Salih H. F. Al-Salim, and Jameel M. Al-Khayri

Abstract Chicory (*Cichorium intybus* L.) is a perennial plant of the Asteraceae (Compositae) family, collected, domesticated and cultivated in Europe, India and Egypt, like endive (*C. endivia* L.), its closest related species. It grows as a weed in temperate climatic regions and is widely cultivated in northern Europe. Chicory is beneficial to both humans and animals due to its high protein content, carbohydrates, minerals, vitamins and phytoactive compounds. It is consumed as a vegetable, edible flowers, coffee substitute and for medicinal and cosmetic metabolites. It is also used in hepatoprotective compounds and as a flavoring in beer. Its extract is an inhibitor of salmonella. It is consumed as an animal feed, always with great care to avoid toxicity. Industrial chicory is developed mainly for its inulin content. There is recent interest in genetically engineering chicory to obtain higher yields and create new cultivars, but chicory potential still awaits development, especially in Asian

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countries. This chapter presents an overview of the origin, distribution, taxonomy and conservation of genetic resources, as well as crop cultivation practices and advances in modern biotechnology and molecular biology and their application for crop improvement concurrently with traditional chicory breeding.

Keywords Biodiversity · Biotechnology · Breeding · Chicory · Coffee substitute · Inulin · Self-incompatibility · Vegetables

1.1 Introduction

The genus *Cichorium* (Asteraceae), with major geographic presence in Europe and Asia, is a perennial or annual herbaceous plant, usually with bright blue flowers, occasionally white or pink (FAO 2013). Although cultural practices differ widely, chicory is part of the diet in almost every country of most Western and Eastern populations (Barcaccia et al. 2016). The Asteraceae family contains 23,000 species in 1535 genera belonging to 1 of 3 subfamilies: Barnadesioideae, Cichorioideae and Asteroideae. *Cichorium* plants belong to the subfamily Cichorioideae (Kiers et al. 1999), which consists of 4 classes within *Cichorium intybus* – (a) Root cultivars, (b) Witloof cultivars, (c) Sugarloaf cultivars and (d) the Radicchio cultivars. Usually, endive (*C. endivia*) is divided into 3 groups (a) the broad-leaved group of escarole, (b) the crispy and curly narrow-leaved group of Frisé endives and (c) the narrow, incised leaves of ancient endive cultivars (Kiers et al. 1999; Ryder 1999) (Fig. 1.1).

The structuring among groups of wild and cultivated chicory species is weak, which is unusual considering that crops and wild recipients are genetically close.

Genes are often shared between those two forms. Emphasis should be on reducing crop-wild gene flow until transgenic varieties are openly cultivated. Wild and cultivated chicory varieties of *Cichorium intybus* can be intentionally crossed (Kiers 2000). But little is known about the accidental introgression between crop and wild chicory. Thus, one of the possible factors for chicory evolution is largely unstudied (Kiær et al. 2008). Wild *C. intybus* covers a large part of the entire European continent. As an important ingredient in traditional local dishes, it has traditionally been part of the local diet. It may be both products of the great difference between various types and the cause of that. Gradually, chicory and endive *C. endivia* became two typical European crops. Over time, these two species have grown into several cultivated varieties (Deryckere 2013). Both cultivated species account for various cultivars of raw or cooked leafy vegetables (Raulier et al. 2016).

Fig. 1.1 Chicory exhibits different growth forms depending on cultural and training practices. (**a**) Chicory field, (**b**) Chicory flowers, (**c**) Chicory roasted roots, (**d**) Chicory roots, **(e)** Chicory seeds, (**f**) Chicory leaves. (Source: Ackley 2018), (**g**) Chicory rosette type chicory with narrow, spatulate leaves, (**h**) Chicory Witloof chicons forced in the dark. (Source: Photos **a–e**, Nwafor et al. 2017; Photos **g**–**h**, Ryder 1999)

1.1.1 Botanical Classification and Distribution

Chicory is commonly grown in a range of temperate regions around the world, like South Africa, but it originated in Germany, Central Russia, Western Asia, as well as in Egypt and North America (Koch et al. 1999). In New Zealand, chicory was reported in 1867. In Pennsylvania until 1993, chicory was known as a harmful weed (Jung et al. 1996). *Cichorium intybus* (regular chicory) in eastern Anatolia, Turkey, is known as *tahlisk*, *kanej* or *hindiba*; there the topography is rugged and intensely divided providing perfect conditions for various types of plant growth (Tabata et al. 1994). In Italy, wild chicory plants can be found in beachfront areas and in the mountains (Conti et al. 2005).

The genus *Cichorium* comprises six species, listed in Table 1.1, of which only four are wild, including *C. bottae*, *C. spinosum*, *C. calvum* and *C. glandulosum*. Only two species are known as cultivated and wild, endive (*C. endivia*) and chicory (*C. intybus*) (Raulier et al. 2016). The taxonomic classification of the latter is summarized below:

Domain: Eukaryota
Kingdom: Plantae (Plants)
Subkingdom: Tracheobionta (Vascular plants)
Phylum: Spermatophyta (Seed plants)
Division: Magnoliophyta (Flowering plants)
Order: Asterales
Suborder: Asterids
Family: Asteraceae (Compositae)
Subfamily: Asteradeae
Tribe: Cichorieae
Genus: *Cichorium*
Species: *C. intybus*

There are five cultivated varieties of *Cichorium intybus* known by different common names depending on the region, as summarized in Table 1.1 (Barcaccia et al. 2003, 2016; Das et al. 2016; Hammer and Gladis 2014; Lucchin et al. 2008; Závada et al. 2017). Inference of population structure in the Eurasian collection of 11 cultivars and nine wild accessions of chicory based on 12 SSR markers are shown in Fig. 1.2 (Závada et al.2017).

1.1.2 Chicory Importance

1.1.2.1 Economic Importance

Chicory is a valuable and economically-important vegetable crop, produced commercially in many countries worldwide (Fig. 1.3). It is especially important as a commercial crop in Europe (96.3%), Africa (2.3%), Asia (0.9%) and Americas

Table 1.1 List of most important *Cichorium* species and varieties including common names, utilization and geographic distribution

No.	Scientific name	Common names	Domestication	Utilization	Geographic distribution
1	*C. intybus* L.				Northern and Central Europe, Siberia, Turkey, Afghanistan, North and Central China, South America, South Africa, Ethiopia, Madagascar, India, Australia, New Zealand
	C. intybus L. ssp. *intybus*	Chicory	Wild	Cooked, salads	
	C. intybus L. ssp. *intybus* var. *foliosum* Hegi.	Witloof chicory, Belgian endive	Cultivated	Forced and blanched shoots (chicons), cooked, salads	
	C. intybus L. ssp. *intybus* var. *porphyreum* Alefeld Landw.	Sugar chicory, sugarloaf	Cultivated	Cooked, salads	
	C. intybus L. ssp. *intybus* var. *latifolium* K. Hammer Gladis	Leaf chicory, Radicchio, Italian chicory, red chicory	Cultivated	Salads	
	C. intybus L. ssp. *intybus* var. *sylvestre* Bisch.	Catalogne	Cultivated	Cooked	
	C. intybus L. ssp. *intybus* var. *sativum* Lam. DC.	Root chicory, roasted chicory, industrial chicory	Cultivated	Coffee substitute, inulin extracts, cooked	
	C. intybus L. ssp. *glabratum* (C. Presl) Arcang.	Glabratum group	Wild	Cooked, salads	
2	C. *endivia* L.				Southern Europe, Turkey, Egypt, Tunisia
	C. *endivia* ssp. *endivia*	Endive group	Wild	Salads	
	C. *endivia* L. ssp. *endivia* var. *latifolium* Lam.	Endive group	Cultivated	Salads	
	C. *endivia* L. ssp. *endivia* var. *crispum* Mill.	Crispum group	Cultivated	Salads	
	C. *endivia* L. ssp. *pumilium* Jacq.	Pumilium group	Wild	–	
3	*C. spinosum* L.	Spinosum group	Wild, perennial	–	Eastern Mediterranean, Italy

(continued)

Table 1.1 (continued)

No.	Scientific name	Common names	Domestication	Utilization	Geographic distribution
4	*C. bottae* Deflers.	Bottae group	Wild, perennial	–	Saudi Arabia, Yemen
5	*C. calvum* Sch. Bip. ex Asch.	Calvum group	Wild, annual	–	Egypt, Ethiopia, Palestine, Jordan, Afghanistan, Pakistan
6	*C. glandulosum* Boiss	Glandulosum group	Wild	–	Syria, Turkey, Armenia, Iran, Iraq

Source: This table is based on Barcaccia et al. (2003, 2016), Das et al. (2016), Hammer and Gladis (2014), Lucchin et al. (2008), and Závada et al. (2017)

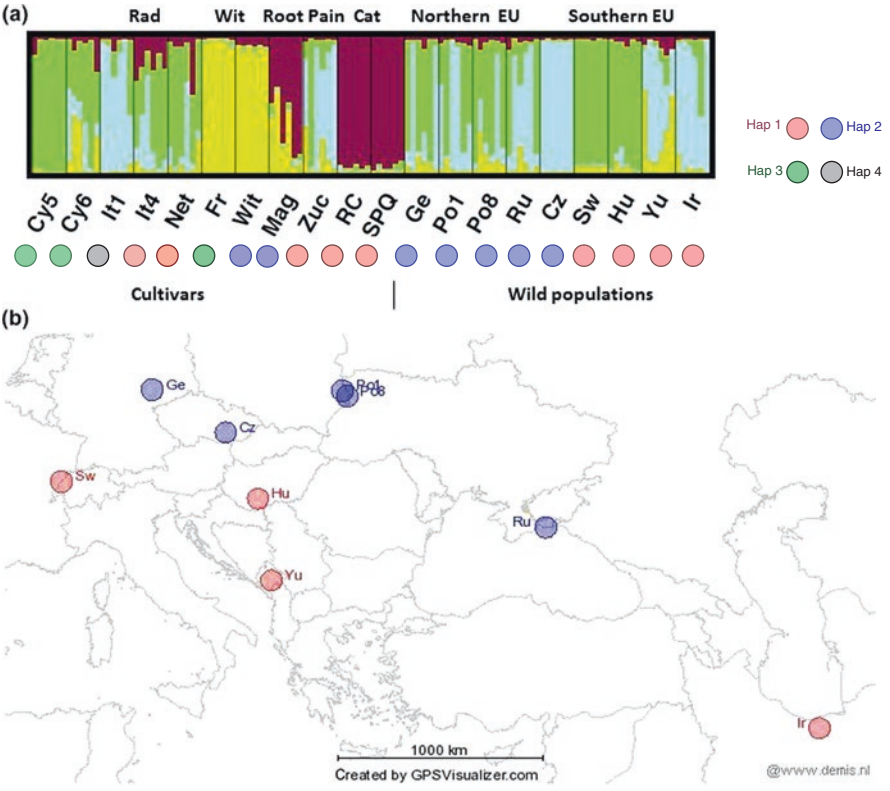

Fig. 1.2 Inference of population structure in the Eurasian collection of 11 cultivars (left side populations Cyprus 5 (Cy5), Cyprus 6 (Cy6), Italy 1 (It1), Italy 4 (It4), Netherland (Net), France (Fr), Witloof (Wit), Magdeburgh (Mag), Zuckerhu (Zuc), Radichetta (RC) and San Pasqual (SPQ)) and 9 wild accessions (right side, populations Germany (Ge), Poland 1 (Po1), Poland 8 (Po8), Russia (Ru), Czech Republic (Cz), Switzerland (Sw), Hungary (Hu), Yuman (Yu) and Iran (Ir)) of chicory. (**a**) Structure analysis of the 20 accessions each separated by a black bar and based on 12 SSR markers with $K = 4$ and the cpDNA haplotypes (Haplotype 1 (Hap 1): red, Haplotype 2 (Hap 2): blue, Haplotype 3 (Hap 3): green, Haplotype 4 (Hap 4): black) defined by a color-coded circle below each accession. (**b**) Geographic distribution of cpDNA haplotypes in the nine wild Eurasian populations. (Source: Závada et al. 2017)

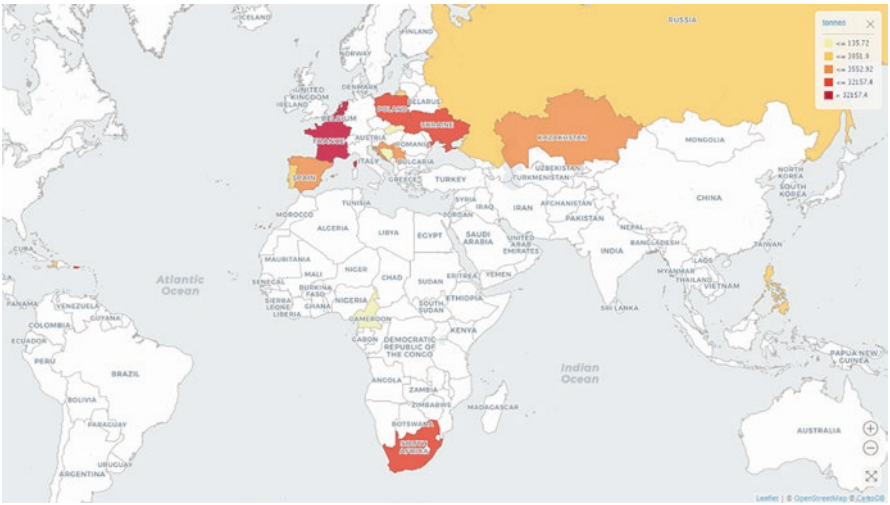

Fig. 1.3 Map of world chicory roots production in 2018. (Source: FAOSTAT (2018), http://www.fao.org/faostat/en/#data/QC/visualize)

Table 1.2 Top twenty producers of chicory roots worldwide in 2018

Country/territory	Production (mt)
Belgium	459,220
Belgium-Luxembourg	448,451
France	116,842
Netherlands	58,990
Poland	32,157
South Africa	15,671
Ukraine	4609
Puerto Rico	3989
Croatia	3553
Serbia	3488
Spain	3426
Kazakhstan	3287
Philippines	3051
Russian Federation	2628
Portugal	2598
Haiti	200
Cameroon	136
Bosnia and Herzegovina	95
Qatar	93
Slovakia	61

Source: FAOSTAT 2018, http://www.fao.org/faostat/en/#data/QC/visualize

(0.5%) (FAOSTAT 2018). In 2018, the top twenty producers of chicory roots and amounts of their production worldwide are listed in Table 1.2. Besides, it may be worth mentioning that chicory and endive are not only important to local economies, but they may also have significance for international trade. In 2002, the US chicory sales amounted to 5996 mt for a value of USD 8,193,000. Around half of these quantities are expressed by witloof chicory ("white leaf" chicory), both in quantity and volume, which accounts for over 90% of the total imports from Belgium and the Netherlands (Lucchin et al. 2008). Global market scope of chicory was projected to reach USD 220.6 million in 2018. By the completion of 2025, the chicory industry is expected to reach USD 294.2 million according to a research report on global chicory market analysis available at: https://www.millioninsights.com/industry-reports/chicory-market).

1.1.2.2 Nutritional and Pharmaceutical Properties

Globally, the cultivation and processing of chicory plants is for various uses, such as the root biomass to prepare a coffee substitute. Because of the economic value of its flower the plants are a source of basic phytochemicals (Bais and Ravishankar 2001).

Some chicory varieties are used as food (Table 1.1). Root chicory (*Cichorium intybus* var. *sativum*) is a horticultural crop grown for direct consumption as cooked food, where its green leaf types are eaten fresh or as a stewed vegetable in northern Italy, including catalog, blond and red forms of the Radicchio cultivar group (Fig. 1.4) (Barcaccia et al. 2016; Lucchin et al. 2008).

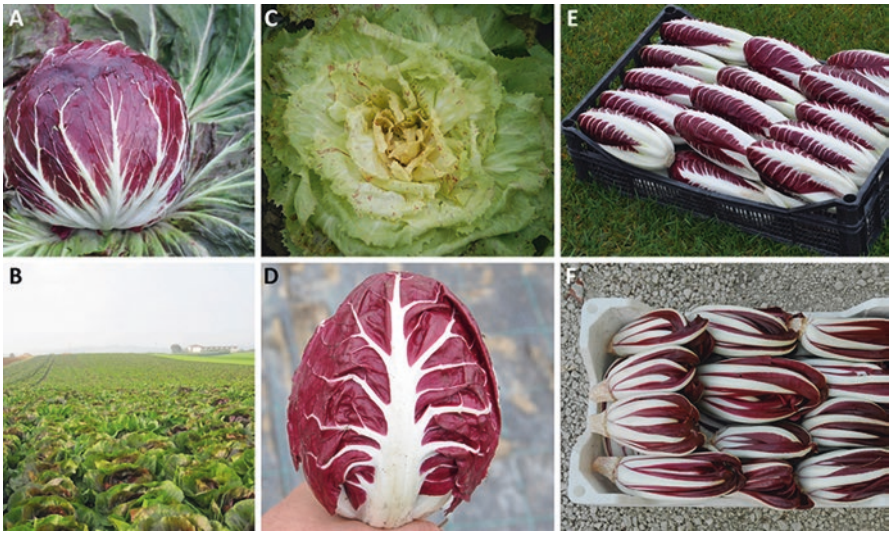

Fig. 1.4 An overview on the main cultivated biotypes of Italian Radicchio. (**a**, **b**) Rosso di Chioggia (Red of Chioggia), (**c**) Variegato di Castelfranco (Variegated of Castelfranco), (**d**) Rosso di Verona (Red of Verona), (**e**) Rosso di Treviso Precoce (Early Red of Treviso), (**f**) Rosso di Treviso Tardivo (Late Red of Treviso). (Source: Barcaccia et al. 2016)

Chicory leaves are especially rich in carbohydrates, Ca, Mn, Fe and vitamins B1, B2, and B6 (Tables 1.3 and 1.4) (Nwafor et al. 2017). Because of their low energy content, they can also be used in reduced-calorie diets. A better understanding of the nutritional value of conventional foods serves as the basis for greater incorporation into modern dietary trends (Jančić et al. 2016). The leaf extract is reportedly used to treat jaundice, hepatic enlargement, gout and rheumatism (Pushparaj et al. 2007; Saeed et al. 2017) (Table 1.5).

Chicory roots may be used after processing to make a supplement, especially in India, and serves as a chewing gum (Arya and Saini 1984; NISCAIR 1992; Taylor 1981). Chicory roots allegedly reduce minor digestive disorders such as gastrointestinal flatulence and poor digestion and acute loss of appetite. In addition, the aqueous root extract is said to be a light-sensitive malaria plant remedy (Street et al. 2013).

Table 1.3 Mineral content of chicory plant (leaves and roots) along with the recommended daily allowance (RDA)

	Content mg/100 g		
Element	Leaves	Roots	RDA
Ca	292.61 ± 13.35	181.26 ± 4.40	1000–1300
K	166.57 ± 3.43	103.7 ± 4.62	–
Mg	6.944 ± 5.86	20.14 ± 1.69	240–420
Na	88.84 ± 2.58	67.42 ± 2.45	1600
Fe	9.178 ± 0.85	1.77 ± 0.21	8–11
Cu	0.60 ± 0.06	0.36 ± 0.02	0.8–1.2
Mn	0.90 ± 0.01	0.31 ± 0.10	1.6–2.3
Zn	0.91 ± 0.03	0.39 ± 0.03	12–15
Pb	0.03 ± 0.01	0.04 ± 0.003	–

Source: Nwafor et al. (2017)

Table 1.4 Composition of chicory plant macronutrient[a]

	Content %	
Nutritional components	Leaves	Roots
Ash	10.91 ± 1.86	04.25 ± 0.11
Crude fiber	16.78 ± 2.20	05.12 ± 1.55
Crude protein	14.70 ± 1.03	04.65 ± 0.25
Total soluble sugar	07.80 ± 1.45	11.06 ± 1.00
Crude ether extra	03.68 ± 0.19	01.69 ± 0.71
Inulin	10.95 ± 2.56	44.69 ± 0.88
Moisture content	83.06 ± 1.55	75.63 ± 0.39
Total carbohydrate	70.71 ± 3.08	89.41 ± 1.07
Soluble dietary fiber	ND[b]	00.42 ± 0.07
Insoluble dietary fiber	ND	30.73 ± 0.33
Total dietary fiber	ND	31.15 ± 0.40

Source: Nwafor et al. (2017)

[a]Based on dry weight; mean ± 5.0 (each value is the sum of three determinations ± standard deviation)

[b]*ND* not determined

Table 1.5 Reported beneficial health effects of *Cichorium intybus* plant parts

Plant parts	Purported health benefits	References
Aerial decoction	Liver disorders, spasmolytic, cholesterol, antiseptic	Hanlidou et al. (2004)
Chicory seeds	Liver disorders	Ahmed et al. (2003)
Root	Jaundice, liver enlargement, gout and rheumatism cough	Pushparaj et al. (2007)
Whole plant	Eupeptic, stomachic, depurative, choleretic, laxative, hypotension, tonic and antipyretic	Miraldi et al. (2001)
Leaves	Blood cleansing	Pieroni (2000)
Leaves	High blood pressure and blood purification	Guarrera et al. (2005)
Leaves/roots	Arteriosclerosis, anti-arthritis, antispasmodic, digestive	Loi et al. (2005)
Whorls	Depurative	Pieroni et al. (2002)
Leaves	Choleretic, hepatoprotective against jaundice, mild laxative, hypoglycemic	Leporatti and Ivancheva (2003)
Aerial/roots	Renal disease	Jouad et al. (2001)
Whole plant	Kidney disorders and diabetes	El-Hilaly et al. (2003)
Roots	Diabetes	Ahmad et al. (2009)
Flower	Diarrhea	Savikin et al. (2013)
Aerial part/root	Cholagogue, digestive and hypoglycemic	Kokoska et al. (2002)
Leaves, stems, roots	Jaundice and tonic	VanWyk et al. (1997)
Leaf	Wound healing	Sezik et al. (2001)
Aerial	Hemorrhoids, urinary disorders	Tetik et al. (2013)

Source: Saeed et al. (2017)

Plant parts are used in folk medicine for hepatic disorders, namely aerial components in Bosnia and Herzegovina, and seeds and roots in Serbia and India (Hanlidou et al. 2004; Jaric et al. 2007). Chicory flowers allegedly are a medicinal remedy and have an anthocyanin pigment manifested in the blue color of the perianth and have cholagogic activity (Norbak et al. 2002).

Chicory has been a part of natural grasslands for thousands of years in many areas of the world, but it relatively new as a forage crop. Also, it is used for the production of accessible forage with high nutritional value for grazing ruminants in summer (Barry 1998). Under optimal conditions, forage chicory provides a large amount of high-quality feed. During the warm season, it is comparable to legumes and superior to grass-based pastures for good livestock performance. Therefore, chicory minimizes certain internal parasites in livestock and has the potential to reduce anthelmintic consumption (Guangdi and Kemp 2005). However, when used to feed livestock, Pune chicory does not induce flatulence (Barry 1998; Jung et al. 1996). Forage chicory appears to reduce symptoms of internal parasites in small ruminants such as sheep affected by gastrointestinal parasites as opposed to pasture (Marley et al. 2003; Scales et al. 1995). Puna (Grasslands Puna), Forage Feast, Choice, Oasis, Puna II, Grouse, Six Point are the most common forage chicory varieties.

Decontamination of heavy metal contaminated in soil is one of the most difficult problems facing remediation technologies. Phytoremediation is a modern and inexpensive technique that utilizes water, groundwater or plants to eliminate, convert or capture contaminants. *Cichorium intybus* exhibits more roots than many other species. Several research studies have shown that *C. intybus* absorbs high concentrations of lead (Pb). As a result, this species is considered a shoot hyperaccumulator for the disinfection of soil contaminated with Pb (Baker and Brooks 1989; Baker et al. 1994). Also, as a deep-rooted, perennial plant, chicory can minimize nitrate leaching, deep drainage and thus improve soil acidification and dryland salinity (Guangdi and Kemp 2005).

Numerous studies have confirmed the presence of phenolics and sesquiterpene lactones in chicory tissue. The main phenolics are derivatives of caffeic acid, coumarins and flavonoids (Kisiel and Michalska 2003; Malarz et al. 2002). Extracts from the rough roots and chicory leaves are most involved in radical scavenging. The main antioxidant present in the rough roots is 3,5-dicaffeoylquinic acid. Its root biomass content is 5.5%, determined by dry weight. 8-deoxylactucin glucoside (crepidiaside A) was the main hairy-rooted sesquiterpene lactone. The value was 1.4%, measured on a dry weight basis and almost two orders of magnitude greater than that in the wild chicory plant roots. The 8-deoxylactucin glucosidic derivative made up over 85% of the overall lactone content of sesquiterpene in long-term cultivated hairy chicory roots. The aglycone of this compound has shown anti-inflammatory activity (Malarz et al. 2013).

As for the purported therapeutic advantages of chicory, the various biological results originate from a variety of essential medicinal compounds, such as sesquiterpene lactone, alkaloids, unsaturated sterols, inulin, pigment chlorophyll, antioxidants, saponins, polyphenols, organic acids and tannins, which are found distributed in similar quantities in all parts of the plant (Abbas et al. 2015; Ferrazzano et al. 2011; Sampaio et al. 2009; Yoo et al. 2011). Therapeutic bioactive phytochemicals reported include hydroxycinnamic acids (e.g. chicoric, cholorogenic and caffeic acid derivatives), flavonoids (e.g. quercetin and kaempferol derivatives), anthocyanins and coumarins are listed in Table 1.6 along with their bioactivities (Peña-Espinoza et al. 2018). Moreover, chicory (*Cichorium intybus*) roots produce glucose and fructose syrup for the processing of inulin, either commercially or after partial hydrolysis (Kaur and Gupta 2002; Pool-Zobel 2005).

1.1.3 Domestication, Selection and Early Improvements

Chicory (*Cichorium intybus*) most likely originates from the Mediterranean Region while endive (*C. endiva*) comes from Central Asia (Vavilov 1992; Zeven and De Wet 1982). Pliny and Dioscorides suggested the origins of chicory and endive domestication were in Egypt but no supporting archeological evidence has been found (De Vartavan and Amoros 1997; Rivera Núñez and Obón de Castro 1996).

Table 1.6 Some bioactive phytochemicals of *Cichorium intybus* and their bioactivity

Bioactive group	Bioactive materials	Bioactivity	References
Guaianolide sesquiterpene lactones	Lactucin	Antiprotozoal	Bischoff et al. (2004)
	8-deoxylactucin	Anti-inflammatory; insecticidal	Cavin et al. (2005) and Rees and Harborne (1985)
Coumarins	Cichoriin	Insecticidal	Rees and Harborne (1985)
Anthocyanins	Cyanidin 3-O-(6-malonyl)-glucoside	Anti-inflammatory	Mulabagal et al. (2009)
Hydroxycinnamic acids	Chlorogenic acid	Antibacterial	Lou et al. (2011)
	Caffeic acid	Anti-cancer	Prasad et al. (2011)
	p-Coumaric acid	Anti-inflammatory	Pragasam et al. (2013)
Flavonoids	Kaempferol glucuronide	Anti-cancer and Anti-inflammatory	Chen and Chen (2013)
	Quercetin 3-O-glucuronide + Luteolin 7-O-glucuronide	Acaricidal	Ravindran et al. (2017)

Source: Peña-Espinoza et al. (2018)

In southern and eastern Asia, the two distinct species were distributed in the Mediterranean Basin before they diverged as horticultural crops. While the two species have similar origins, *Cichorium intybus* is primarily found in the peninsular area of the southern Balkans and the northern Middle East. *Cichorium intybus* was selected for a biennial cycle; during the first year, plants grow rosette leaves and a floral stem grows after a cold period. Capitula with flowers cluster within the mouth. Chicory is an allogamous and endogenous plant in which self-incompatibility is not strict, 10–20% of the seeds produced come from self-fertilization, which in some witloof chicory seed samples may also be as high as 33% (Bellamy et al. 1996).

1.2 Current Cultivation Practices and Challenges

1.2.1 Current Cultivation Practices

Chicory is an erect perennial plant, which requires well-distributed rainfall but can be cultivated under careful irrigation. When raised for roots, chicory requires a hot and humid climate and is mostly cultivated in southern regions. However, when raised for seed, the crop is grown in regions with a dry temperate climate. It is a hardy plant and can tolerate extreme temperatures during its vegetative and reproductive stages. For successful seed germination, chicory needs a minimum temperature of 21 °C; while, for good plant growth it requires a moderate and uniform temperature, with the optimum at 18–24 °C (Council of scientific and industrial

research 1992). First, the preparation of land is plowed deep, as the taproot penetrates to a depth of 38 cm or more. The field is formed into ridges and furrows, with a spacing of 45 cm between ridges. A row spacing of 20 cm and a plant spacing of 15 cm is maintained to yield a heavy tonnage of roots with a seeding rate (3–5 kg/ha) (Bais and Ravishankar 2001). Chicory needs high nutrient input to maintain high production, especially in soils with low fertility (Belesky et al. 2001). The best conditions for growing chicory are well-drained soils with medium to high fertility, which is supplied with nutrients such as phosphorus (P), potassium (K), sulfur (S) and nitrogen (N) fertilizer, especially during seedling development (Moloney and Milne 1993).

High root yield, inulin content and longer inulin chains are affected generally by sowing date, harvest date and genotype. Degrading fructan enzymes are active in the autumn, which reduces the average length of the inulin chain. So, early harvest is necessary for the longer-chain inulin. The best quality inulin is produced in September (Northern Hemisphere) while the highest root biomass is reached a few weeks later. Seed productivity of common chicory depends on the number of productive shoots per individual, the number of calathidia per shoot and the number of achenes per calathidium (Roustakhiz and Majnabadi 2017).

1.2.2 Current Agricultural Problems and Challenges

Due to the impact of climate change on agricultural development, it is important to identify the problems of the agricultural sector which are likely to occur in the coming years. In addition to greenhouse gas emissions, broad variations are predicted in the impacts of climate change on grassland production around the world because the manure of ruminants is the key source of methane from agriculture. In the coming years, farms will not only need to manage changes in order to maintain current performance, but will also improve their environmental footprint by reducing and strengthening factors such as grassland carbon sequestration and use of mathematical models, allowing policymakers and farmers to explore the possibilities and implications of their choices in a climate-change environment. Cross-disciplinary contact and information sharing must be developed among growers, scientists and modelers for these advances to take place (Wonfor 2016).

1.2.3 Genetic Improvement Objectives

Genomic development has played a vital role in improving vegetable crop yield. There are various vegetable crops growing worldwide and genomics, breeding and biotechnology research on them have been carried out to varying degrees (Kalloo and Bergh 1993). Inulin (soluble fiber) is present in large amounts in a variety of fruits and vegetables, but root chicory (*Cichorium intybus* var. *sativum*) is the main

source of industrial inulin output (De Leenheer 1996; Silva 1996). Although several root chicory cultivars have been developed, new root chicory cultivars are ideal for high inulin content and resistance to herbicides, insects and pathogens.

Classical methods of breeding are both time consuming and laborious. *Agrobacterium*-mediated plant transformation has become one of the favored methods for genetic engineering and rapid improvement of crops; somaclonal variation is often caused by the conditions of tissue culture in the differentiating vegetative cells (Larkin and Scowcroft 1981; Muller et al. 1990). However, older experiments were limited to similar varieties such as the witloof chicory and a few studies were carried out on the regeneration and genetic transformation of various root-type chicory cultivars such as *Cichorium intybus* var. *sativum* (Maroufi et al. 2012).

Transgenic chicory plants (*Cichorium intybus* var. *sativum*) cv. Melci was developed for overexpressing sucrose – sucrose 1-fructosyltransferase (1-SST) under the guidance of the cauliflower mosaic virus (CaMV 35S) promoter, in addition to the overexpression of 1-SST, the primary gene in chicory in inulin biosynthesis, may serve as a novel approach to plant production with long-chain inulin material (Maroufi et al. 2018).

1.3 Germplasm Biodiversity and Conservation

Germplasm is the raw material of the plant breeder, used to create new varieties and consists of different specimens such as traditional cultivars, natural hybrids, feral and weedy ancestors, wild species and obsolete varieties, elite lines, mutants and polyploids, and interspecific and intergeneric hybrids that are frequently developed (Haussmann et al. 2004).

1.3.1 Germplasm Diversity

Recent studies have addressed phylogenetic relationships within *Cichorium* (Gemeinholzer and Bachmann 2005; Kiers et al. 1999, Kiers 2000) (Fig. 1.5). Gemeinholzer and Bachmann (2005) could not discriminate between *C. intybus*, *C. spinosum* genetically with three different marker sets such as internal transcribed nuclear ribosomal spacer (ITS), amplified fragment length polymorphism (AFLPs) and simple sequence repeats (SSRs). There is no wild relative reported of *C. endivia*. There are three historically distinct cultivar classes within this genus (Scarole, Frisé, Endivia), but AFLP analyses do not support this definition (Kiers 2000). *Cichorium intybus* is classified as having both wild and cultivated forms. Analyses carried out by AFLP demonstrate that they are genetically distinct (Sørensen et al. 2007; Van Cutsem et al. 2003). However, the morphological features that distinguish between the two species are constant and heritable, meaning the morphological conditions for species delimitation are met (Raulier et al. 2016).

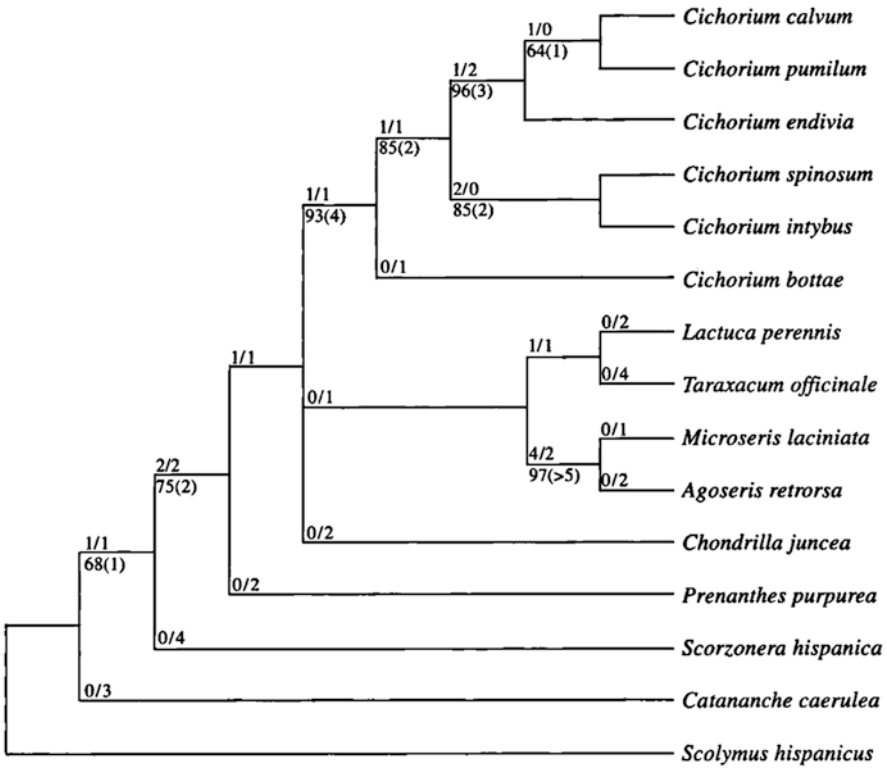

Fig. 1.5 The most parsimonious single tree based on restriction fragment length polymorphism (RFLP) data from chloroplast DNA. The length is 51 moves, and the index of consistency 0.65. Bootstrap values above 50% of 1000 replicates are given under the branches along with the bracket decay values. Changes in character condition are seen over the divisions, first the number of apomorphic shifts followed by the cumulative number of parallels and reversals. (Source: Kiers 2000)

1.3.2 Cultivars Characterization and Phylogeny

A significant diagnostic character at the chicory generic and species levels is unusual pappus configuration, which causes difficulties in the relationship of *Cichorium* with other genera of Lactuceae due to its unclear homology with other pappus structures. The group is quite separate from a morphological perspective. In addition to the two cultivated species, *Cichorium endivia* and *C. intybus*, there are four wild species known, *C. pumilum*, *C. calvum, C. bottae* and *C. spinosum*. The latter two species can be easily distinguished by their cushions, such as growth form and spiny terminal branches. The most important results are (1) *C. bottae* belongs to the remaining genus *Cichorium*, (2) *C. spinosum* is the closest relative to *C. intybus* and (3) *C. endivia* has the strongest relation to *C. pumilum* (*C. pumilum* × *C. intybus*). *Cichorium calvum* in chloroplast-based phylogeny but in nuclear ribosomal DNA linked phylogeny, the relationships between these three species remains unclear,

given the strong morphological overlap with *C. endivia*. *Cichorium intybus* does not support a close relationship between the three DNA-based analytical methods (chloroplast and nuclear DNA-based phylogenetic analysis and AFLP-based multivariate analysis). The wild ancestor *C. endivia* appears unclear (Kiers 2000). However, the information gathered in crop species of Asteraceae up to now can also be used for flowering time management in vegetable crop breeding (Leijten et al. 2018).

1.3.3 Genetic Resources Conservation Approaches

Cultivars, landraces and other genetic stocks (breeder lines, experimental lines), as well as wild relatives of cultivated plants, reflect the genetic diversity of a crop. All of these tools form a gene pool to improve important crop features, extend the cultivar genetic base and also serve as a source of a new varieties for agriculture (alternative crop use, use of unused crops, expansion of crop spectrum). At present, genetic variation is under critical threat in both agriculture and nature. Natural biodiversity has been decreasing due to industrialization, climate change and agricultural practices (Dotlačil et al. 2002). In most chicory combinations, germplasm is conserved as seed at either room temperature or refrigerated at 4 °C, in most chicory collections. Also, seeds can be stored in freezers at −18 °C. The survival of these specimens is very laborious as accessions have to be regenerated every 4–10 years because chicory is a cross-fertilizing and insect-pollinated herb (Vandenbussche et al. 2002).

1.3.3.1 In Situ Conservation

Cichorium, as a leafy vegetable, has minimal presence in gene banks and this form of germplasm suggests that they have attracted less attention than other crops. In the case of *Cichorium*, which integrates data tracked in Europe, 12 collections from 7 countries contain a total of about 1800 accessions (Lebeda and Boukema 2001; Van Hintum and Boukema 1999). The major accessions, 788 and 505, are in France and Germany, respectively (Maggioni 2004). The key solution to conserving crop wild relatives (CWR) species is to preserve them in situ within their existing wild ecosystems. There species can adapt to shifting climatic environments and insure their long-term survival chances (Palmé et al. 2019). CWR plants are best maintained in genetic reserves, conservation areas explicitly intended to conserve the long-term genetic variability of natural communities (Maxted et al. 1997). They can be established within conservation areas or outside existing conservation areas if required (Palmé et al. 2019).

Table 1.7 List of important Gene banks for *Cichorium* genetic resources

Gene bank	Country	Website
Institute for Plant Genetic Resources	Bulgaria	https://www.genesys-pgr.org/ar/wiews/BGR001
Institute of Crop Science, Chinese Academy of Agricultural Sciences	China	http://www.cgris.net/query/croplist.php#
Czech Republic Gene Bank	Czech Republic	https://grinczech.vurv.cz/gringlobal/search.aspx
Leibniz Institut für Pflanzengenetik und Kulturpflanzen forschung (IPK)	Germany	https://www.ipk-gatersleben.de/en/gbisipk-gaterslebendegbis-i/
USA Plant Germplasm Introduction and Testing Research Station, Pullman, WA	USA	https://npgsweb.ars-grin.gov/gringlobal/search.aspx

1.3.3.2 Cryopreservation

Cryopreservation is the conservation of living propagules at very low temperatures (−150 °C). Different methods have been developed to minimize the harm caused by desiccating and freezing while ensuring fast recovery of propagules. For certain instances, the apice shoot from in vitro growth is the plant material used to store vegetatively-propagated plants. Cryopreservation strategies rely either on freeze-induced dehydration (classical method) or internal solution vitrification (new method) (González-Benito et al. 2004). Several techniques have been developed for chicory in vitro shoot tip cryopreservation.

Cryopreservation experiments were conducted using controlled rate freezing (Demeulemeester et al. 1992) and encapsulation dehydration (Vandenbussche et al. 1993, 2002) for *C. intybus* ssp. *intybus* var. *foliosum*, cvs. Light, Rumba, Carolus. Controlled rate freezing is a conventional plant cryopreservation method. Shoot tips from the chicory line (Rosso di Chioggia) in vitro stock plants were cryopreserved by single-step vitrification. The genetic reliability of the cryopreserved line was maintained. A simple effective protocol for the cryopreservation of red chicory shoot tips was successfully developed by Benelli et al. (2011). A list of important world gene banks for the conservation *Cichorium* genetic resources is given in Table 1.7.

1.3.3.3 In Vitro Conservation

Progress in biotechnology has culminated in the creation of a whole range of germplasm, namely clones derived from elite genotypes, cell lines with unique characteristics and genetically modified content. This modern germplasm also has a high added value and is very hard to obtain (Engelmann 1994). Therefore, it is of utmost importance to establish appropriate strategies to ensure its healthy survival (Engelmann 1997, 2000). In vitro storage is routinely applied as medium-term storage to a range of crops, based on slow-growing techniques (Ashmore 1997).

Nonetheless, questions about the long-term genetic stability of crops with somaclonal variations can be raised (Larkin and Scowcroft 1981). Contamination avoidance techniques for in vitro cultivation have been widely developed and extended to more than 1000 different species (Bigot 1987). Tissue cultivation strategies are of great interest in plant germplasm selection, replication and preservation (Engelmann 1991). Tissue culture systems allow propagation in an aseptic environment of plant material with high multiplication rates (Engelmann 1997).

In vitro culture can be used for the clonal propagation of starting plant material and the development of virus-free plants. The distribution of plant germplasm in the form of in vitro cultures is less voluminous and improves the state of health. Indeed, somaclonal heterogeneity is less likely to occur when plant recovery is carried out directly from apices compared to other approaches (González-Benito et al. 2004). Sinkovič et al. (2020) reached the conclusion through their study, that in vitro and in vivo antioxidant assays of chicory *Cichorium intybus* plants, as influenced by organic and conventional fertilizers, demonstrated that organic and mineral fertilizer mix received the lowest in vitro antioxidant potential (AOP) for red chicory cultivars and the smallest in vitro AOPs for red and purple chicory cultivars. Besides, the fertilizer treatments had different impacts in terms of total flavonoid content (TFC) and total phenolic content (TPC) on the red and red-spotted chicory cultivars, resulting in separate in vitro and in vivo AOPs (Table 1.8).

Table 1.8 Phenolic compounds found in chicory (*Cichorium intybus*) extracts as identified by high-performance liquid chromatography (HPLC)

Chicory	Methanolic extracts (%)	Total phenolic content (mg GAE* g^{-1} dry extract)	Phenolic compound	Content (%)
Leaves	23.16	26.4 ± 1.05	Caffeic acid	35.22
			Chlorogenic acid	17.84
			p-Coumaric acid	9.65
			Gallic acid	1.96
			p-Hydroxybenzoic acid	11.04
			Isovanillic acid	1.97
			Protocatechuic acid	2.50
			Unknown compound	19.46
Roots	10.75	20.0 ± 0.9	Caffeic acid	24.36
			Chlorogenic acid	10.85
			m-Coumaric acid	27.90
			p-Coumaric acid	25.03
			Protocatechuic acid	1.77
			Unknown compound	10.09

Source: Nwafor et al. (2017)
GAE Gallic acid equivalent

1.3.3.4 Gene Banks

Gene banks play a crucial role in the survival, development and utilization of a broad range of plant genetic variations for food and nutrient-quality crop enhancement. They ensure the continued availability of genetic tools for science, breeding and enhanced seed production for a productive and robust agricultural system. They help connect the past and the future; sustainable maintenance of plant genetic resources relies on the efficient management of gene banks by implementing standards and procedures to ensure the continued survival and availability of plant genetic resources (FAO 2014).

Acknowledgement that domesticated plants soon lose much of their agrobiodiversity has prompted a worldwide campaign to gather and preserve germplasm. Knowledge of advanced agriculture and the small genetic base of crops and possible vulnerability to crop failure have encouraged research activities (National Research Council 1972). A network of national and international gene banks gradually accrued resources of 6.1 million specimens worldwide in 1300 gene banks (FAO 1996). Germplasm is preserved as seed for certain plants. So, under sufficient conditions of low temperature and humidity, seeds remain viable for 20 years or longer, although some organisms have seeds that quickly lose viability and often need to be vegetatively regenerated. Moreover, when a sample is regenerated from seed it has the potential to lose some genetic diversity, especially if it is regrown under greenhouse or laboratory conditions, removed from the natural evolutionary forces (Bretting and Duvick 1997).

1.3.4 Cytogenetics

Chromosome numbers of all 6 species of *Cichorium* were determined by the squash technique, using young root tips of 3 plants per genus (Dyer 1979). In the diploid process, 18 chromosomes were counted for all species (2n = 2x = 18), which was accepted in the case of *C. intybus*, *C. endivia* and *C. spinosum* (Sell 1976). However, chromosome numbers have not been ascertained for the other species (Kiers 2000).

To distinguish chromosomal variations between species, fluorescent in situ hybridization (FISH) 5S and 45S rDNA samples and chromomycin A_3 (CMA)/4′,6-diamidino-2-phenylindole (DAPI) staining were used to examine ten *Cichorium* accessions from *C. endivia* and five from *C. intybus*. The results revealed interesting chromosome polymorphisms, especially in the number of 45S rDNA sites between the two organisms, even revealing differences of chromosomes within *C. intybus* accessions (Bernardes et al. 2013) (Fig. 1.6).

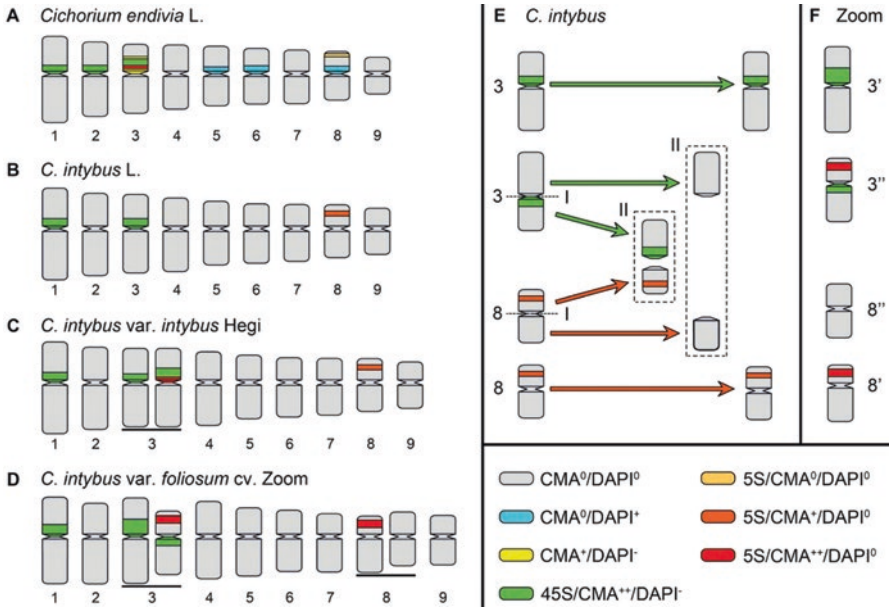

Fig. 1.6 Schematic description of *Cichorium endivia* and *C. intybus* chromosomal. (**a**) Idiomedia of *C. endivia*, (**b**) *C. intybus*, (**c**) C. *intybus* var. *intybus*, (**d**) C. *intybus* var. *foliosum* cv. Zoom, (**c**, **d**) Heteromorphic pairs in *C. intybus* var. *intybus* and *C. intybus* var. *foliosum* cv. Accessions to the Zoom are emphasized, (**e**) Representation of potential mutual translocation with breaks in the centromeres (centric fission-I and fusion-II) including a pair 3 and 8 homologue of *C. intybus*, (**f**) In the genesis of the heteromorphic chromosomes pairs of *C. intybus* var. *foliosum* cv. Zoom cytotype. (Source: Bernardes et al. 2013)

1.4 Traditional Breeding

1.4.1 Improvement of Strategies

Chicory is consumed for its leaves and roots. Effective methods to improve chicory have been particularly essential for inulin extraction and hydrolysis products such as oligofructose and fructose. Cultivation is assessed, outlining common phytotechnical problems (crop rotation, fertilization, sowing, variety choice, weed control, plant safety, harvesting) and carbohydrate content of the roots (Baert and Van Bockstaele 1992). The key goals of Radicchio chicory breeding are to improve both economic and qualitative characteristics. The basic and traditional objectives of the breeding of new varieties are (a) single plant production, weight and yield, (b) biotic stress resistance (fungal diseases and insects) and abiotic stress tolerance, (c) exposure to specific climatic or agronomic environments, (d) uniformity in crop maturity, size and development and (e) strong consumer acceptance as regards to extrinsic (color, style, consistency) and intrinsic (taste and texture) characteristics (Barcaccia et al. 2016) (Fig. 1.7). Recently-initiated breeding projects by local breeders and

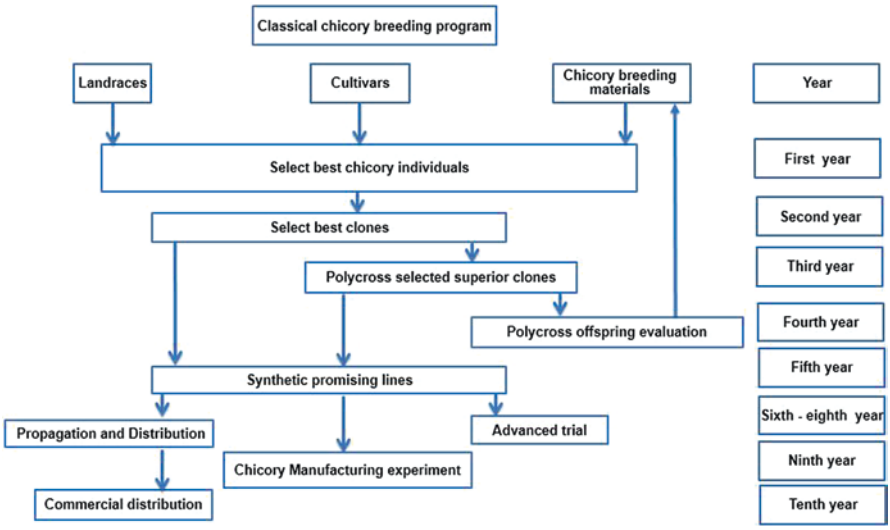

Fig. 1.7 Classical breeding program for the production of industrial chicory varieties. (Diagram prepared by L. Aldahak and K. F. M. Salem)

national seed institutions seek to (a) isolate individuals suitable for use as parents for the creation of synthetic varieties and while not readily feasible, within the best local selections and (b) pick inbred lines suitable for commercial F_1 hybrid growth (Barcaccia et al. 2016). On the other hand, Grasslands Puna (*Cichorium intybus*) was the first chicory cultivar explicitly bred for forage (Rumball 1986). A breeding experiment was conducted to enhance durability in chicory by selecting (*Sclerotinia* spp.) for greater tolerance. This can be achieved by both artificial inoculation and plant processing explicitly and indirectly by selecting lactones, lactucin and lactucopicrin for higher levels of sesquiterpene. These chemicals were known to be involved in the chemical protection of chicory against insect herbivores and therefore perhaps also offered disease resistance (Rees and Harborne 1985; Rumball et al. 2010). A continuing aim of witloof chicory plant breeders is to grow stable, high yielding agronomically-sound witloof chicory cultivars. The reasons for this goal are to optimize the amount of yield generated. The witloof chicory breeder must pick and grow these plants that have features leading to superior cultivars to accomplish this goal, for example, a novel chicory cultivar Witloof, named (Bobine), was announced. The discovery involves the seeds of (Bobine) witloof chicory cultivar, the plants and methods for growing it by crossing the (Bobine) cultivar with itself or with another witloof chicory section (Lecompte 2015). Thus, crossbreeding, gene farming and transgenic breeding are the key forms of seed development in contemporary agriculture. Such time-consuming, laborious and untargeted breeding systems cannot satisfy the increasing global demand for food. To solve this problem and improve crop selection efficiency, marker-assisted breeding and transgenic techniques were adopted, delivering desirable traits by exogenous transformation

into the elite variety. These genome editing systems are excellent tools for fast, selective mutagenesis and for defining the various plant molecular mechanisms for crop improvement. The development of next-generation breeding methods has revolutionized crop breeding. Given their usability, performance, high specificity, and amenability to multiplexing, genome editing technologies have several advantages over conventional agricultural methods. We infer that the pace of reproduction, in conjunction with genetic instruments and tools, enables plant biologists to scale up their research in the field of crop improvement (Ahmar et al. 2020).

1.4.2 Traditional Breeding Methodologies and Limitations

Plant breeding has mostly centered on increasing productivity, disease resistance, abiotic stress tolerance, longer shelf life, early or late growth and diversification of varieties (Kaushik et al. 2015). Traditionally, commercial varieties were developed by recurrent mass selection (Patella et al. 2019). Over time, Belgian farmers selected certain plants to develop for themselves; nearly every grower had one or more options. Collection and breeding were undertaken for purposes of human labor and plant profitability. At research stations, including Institut National de la Recherche Agronomique (INRA), France (De Coninck) and the Katholieke Universiteit Leuven (KUL), Belgium (Van Nerum), collection and reproduction first began. Subsequently, seed companies became interested and took over the breeding job. Selection research struggled with (more) efficiency and concerns in holding the endive head closed as soil pressure is responsible for this growth reaction (De Proft et al. 2003). Early winter cultivars were developed and later the very late cultivars were introduced for summer growth in northern France and seed in Flanders and The Netherlands. New F_1 hybrids were progressively created.

The production of both Witloof and Radicchio have long been based on populations held by farmers for their own use with very few selections, if any, preformed according to farmers requirements. All these genotypes, obtained by mass selection and maintained by intersecting selected, strongly heterozygous and genetically heterogeneous kin whose behavior and degree of adaptation to various environments and cultural conditions rely on the occurrence of advantageous genes or gene combinations. As interest in edible crops increased, farmers' selection criteria became increasingly attentive to the consumers preference and most of them formed their ideotype, resulting in a considerable amount of genetic and morphological distinction that was completely maintained before the advent of coordinated breeding programs, first by public agencies then, more recently, by private companies (Lucchin et al. 2008).

Endives are traditionally generated in a room by constricting the leaves within tubes. Field grown endive roots are submerged in a nutrient solution, under dark conditions, under room temperature conditions and correctly regulated hygrometry conditions. Forcing endive cultivation, therefore, involves significant financial expenditures in both equipment and labor to accomplish the different respective

phases of sowing, root picking and pushing them within a cultivation room to grow endives on a large scale (Lecompte 2015). In recent years, breeders have developed synthetics by crossing or poly-crossing a variety of maternal individuals of selected clonal lines based on their morphophenological and agronomic characteristics and eventually by carrying out progeny tests to assess their overall combination ability (Patella et al. 2019). The genetic base of the crop is quite narrow; thus, it can be predicted that negative characters will emerge as selection progresses. Most of the physiological complications of endive (browning of the flowering stem, the red color of the leaves) have been proposed to derive from inbreeding stress, also a successful cultivar can last 10 years, although a typical lifetime of 3–5 years is common. This means that the breeding activities are very high relative to the crop's possible financial return (De Proft et al. 2003). The self-incompatibility physiological process is not final. There are three compatibility types i.e., self-incompatibility, self-compatibility and pseudo-self-compatibility (Coppens d'Eeckenbrugge 1990). In the case of pseudo-self-compatibility, one pollen germination is delayed, no self-fertilization occurs while foreign compatible pollen is present.

Indeed, the strong structure of self-incompatibility (SI), which hinders the obtaining of extremely homozygous parents and the absence of a male sterility factor within the species or in sexually viable plants, makes it difficult to suggest a seed production scheme for F_1 and above all, to accept these new commercial varieties as true F_1 hybrids. Male sterility in hybrid seed production may play an important role in chicory breeding, particularly because SI of the parent lines is inadequate for reliable hybrid growth. Male sterile mutants that cannot produce fertile pollen or usable anthers cause breeders of many agricultural and horticultural crops to exploit heterosis in F_1 hybrid varieties (Lucchin et al. 2008).

Conventional plant breeding has made a huge contribution to feeding the world and has played vital roles in the creation of every modern human cultivar. Conventional breeding approaches rely on utilizing old techniques such as natural selection, but the creation of a new variety takes a lot of time. This technique can not alter any gene in the genome. So, new research is required to produce new variants and to remove any unwanted genes (Kumar and Sandhu 2020).

1.4.3 Role of Biotechnology

Plant biotechnology provides a wide variety of seed genetic enhancement solutions. For chicory, this involves DNA marker-assisted selection (MAS) in breeding programs (Moreno-Vázquez et al. 2004; Patella et al. 2019; Singh and Singh 2015). In addition, cell and tissue culture to enhance genetics and embryo rescue for interspecific hybrid recovery (Maisonneuve et al. 1995; Van der Veken et al. 2019); somaclonal variation (Brown et al. 1986; Zhang et al. 2011), somatic cell selection (Sellin et al. 1992); somatic protoplast fusion (Deryckere 2013; Matsumoto 1991); plant transformation (Dinant et al. 1997) and gene editing (Bernard et al. 2019; Bertier et al. 2018). Chicory yield production is greatly constrained by environmental

conditions despite its high productive capacity (Baert and Van Bockstaele 1992). Recently, it was considered one of the most significant origins of inulin which, from a biological point of view, is a root type of chicory plant (*Cichorium intybus* var. *sativum*) (Černý and Javor 2004; Van Laere and Van Den Ende 2002). Via backcross breeding or biotechnology methods, it is possible to make the inulin pathway in root type chicory cultivars more efficient (Shoorideh et al. 2018). In vitro techniques, tetraploids can be formed (De Roo 1967; Gobbe et al. 1986) and crossed by diploids to create triploids (Baert and Van Bockstaele 1992). Strategies for developing crops based on molecular markers and biotechnology techniques have addressed the shortcomings of conventional breeding activities in recent years (Nair and Schreinemachers 2020).

1.5 Molecular Breeding

1.5.1 Molecular Marker-Assisted Breeding

Associated or linked markers are changes, with population and generation validation required before application. Recently, traditional methods have been integrated with biotechnological methods to speed up breeding programs. Marker-assisted selection (MAS) is widely used by firms and research institutes to develop improved lines, allowing breeding activity based not only on phenotype assessment but also on plant genotype (Patella et al. 2019). The first genetic association maps were developed utilizing a large-scale implementation of molecular marker techniques, including amplified fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD) of *Cichorium intybus* (De Simone et al. 1997; Van Stallen et al. 2003). In 2010, a revised diagram of hereditary relations for *C. intybus* was developed using SSR markers (Cadalen et al. 2010). This genetic consensus diagram, comprising nine homologous linkage groups (LGs), was obtained after combining and ordaining molecular marker data from one witloof chicory and two commercial chicory progenies (Ghedina et al. 2015).

Seed industries commonly utilize marker breeding to create new hybrid varieties that manifest genetic distinctiveness, uniformity and durability. For the genotyping of 504 Red of Chioggia biotype samples, in total 29 localized microsatellite markers were used. Then, 2 synthetics, 4 F_1 hybrids and 2 derived F_2 populations were assessed to assess the distinctive essence of their gene pool and structure, instead, the uniformity and quality of 3 years of commercial F_1 variety production were also investigated (Patella et al. 2019). It has employed 27 microsatellites (SSRs) markers distributed among the linkage groups (Table 1.9).

Data on genetic variation through molecular markers and accessions have been effectively analyzed along with concise data on both marker loci and inbred lines. This approach has proven effective in evaluating the observed degree of homozygosity in inbred lines as an indicator of their genetic stability. Moreover, the specific

Table 1.9 Microsatellite markers for *Cichorium*

Locus	Series NCBI	Alleles	Repeat trend in library	Firstly series (5′–3) (5′–3′)	Set of Sizes (bp)
5291	CCIL5291.b1_F04.ab1	24	$(AAG)_{16}$	F: M13-GCATCCACTCAAGCTCATTG R: TGGATTTCTAGGCCACACCT	156–273
3984	CCIM3984.b1_P11.ab1	8	$(AAG)_{11}$	F: M13-GCAGCAACAACCCTTTCTTT R: GGTGGCGATTGAATTGAAGA	204–225
5055	CCIS5055.b1_M15.ab1	15	$(CAA)_{10}$	F: M13-TGTGAGACGTGGGATTCTGA	213–291
				R: GCTTTGGCTCCCTATGTCAC	
12,770	CCIM12770.b1_D01.ab1	15	$(CTT)_{18}$	F: M13- CATAAAGGCCCTCCATTCCAC R: GTAAAGCCAAGCGAGACAGG	168–237
6865	CCIL6865.b1_B14.ab1	10	$(GAT)_{10}$	F: M13-AAATGGTTCTGCATCAAAGGA R: CGATGGGGCTTGTTTCTTTA	231–258
1385	CCIL1385.b1_A12.ab1	26	$(GAT)_{11}$	F: M13-TTGCCTCTTGCTCCAATACC R: GGGTCCCTTTGTGTCATCAT	144–225
11,019	CCEL11019.b1_E20.ab1	11	$(ATTA)_5$	F: M13-CAATCGGTTA-ATCAATCAAATCAA R: GGTATCGTAAGCCAGCCAAA	219–291
13,676	CCEL13676.b1_G12.ab1	14	$(CAC)_{10}$	F: M13-TCAACGTGCTTCAAGACGAC R: GTGGTGGTGGTTCGACTTTT	225–270
2050	CCIS2050.b1_D09.ab1	9	$(CTT)_{10}$	F: M13-GCAACGGATGAAGGGTTACA R: GGAAATTAACCCCGGAAAAA	186–210
3899	CCEL3899.b1_E15.ab1	9	$(AATC)_5$	F: M13-CCTCGACAGAAAACCCTCTTC R: AGGTGCGGAAGCGTAAGTT	207–228
7179	CCIS7179.b1_E20.ab1	11	$(CTT)_{10}$	F: M13-GGCAGGACGTCTTTTTGGTA R: CCGAAGAATTTGAGGTTTG	186–225
8271	CCEM8271.b1_M04.ab1	10	$(ATG)_{11}$	F: M13-AACAATGGTGGGCAGAAAAC R: CAGGGGTAAATCGGGAAAAT	156–201

Source Závada et al. (2017)

combining ability (SCA) between maternal and paternal inbred lines was determined based on their genetic variation and the heterozygosity of their F_1 hybrids (Ghedina et al. 2015).

In the genus *Cichorium*, the most studied species is *C. intybus* due to the existence of a genome draft and other specific molecular assays linked to breeding in particular. For *C. intybus*, an insightful group of SSR markers is very successful in the genetic characterization of both hybrid parental and synthetic varieties (Patella et al. 2020). A genetic chicory map 1208 cM was produced using 247 plants with F_2

and 237 markers (170 AFLP, 28 SSRs, 27 EST-SNPs, 12 EST-SSRs) (Muys et al. 2014) (Fig. 1.8; Table 1.10). The illustration contains 84% of the chicory genome. In the Compositae genome project database, the corresponding sequences of chicory-genic-markers were used to identify possible etiologies in named lettuce ESTs (Muys et al. 2014).

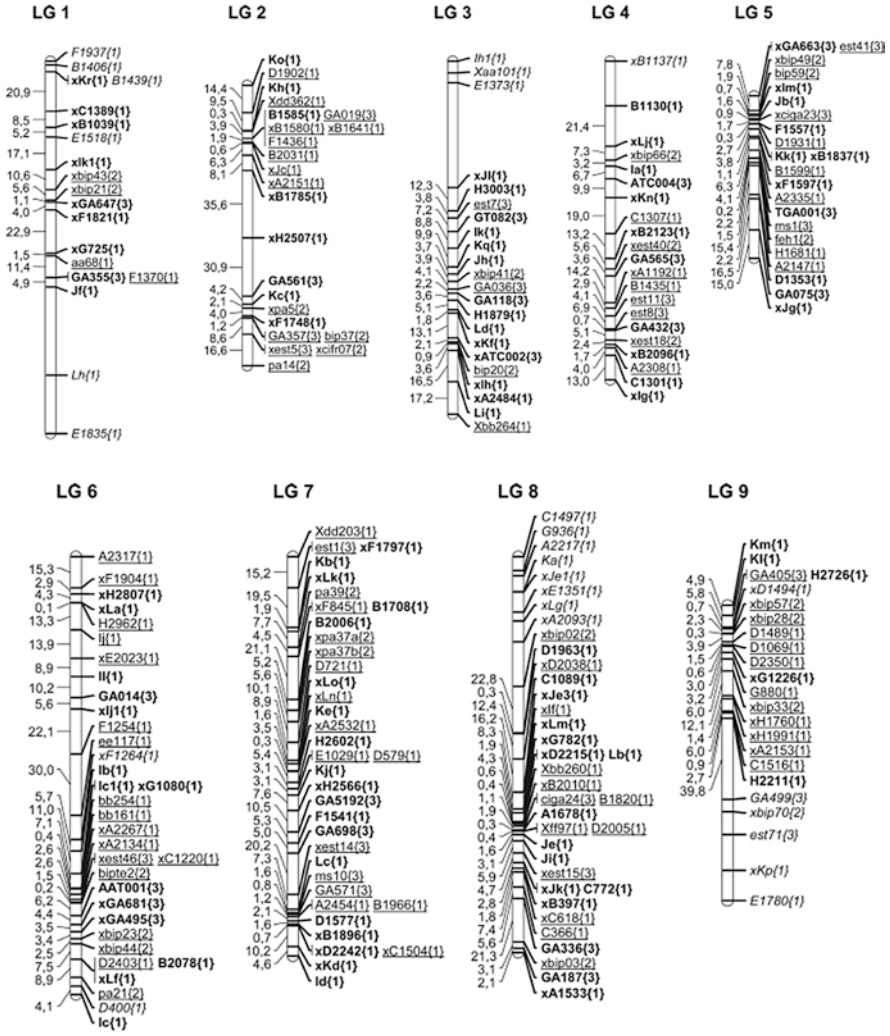

Fig. 1.8 Genetic map of industrial chicory (*Cichorium intybus* var. *sativum*). **Bold**: Framework markers placed at LOD 7.0 and a maximum distance of 40 cM. Underlined: other markers placed at LOD 7.0, max 40 cM. *Italicized*: markers placed at a LOD of 4.0, max 45 cM. (1) AFLP markers, (2) SNP markers, (3) SSR markers. (Source: Muys et al. 2014)

Table 1.10 EST-derived simple sequence repeats (SSRs) and single nucleotide polymorphism (SNPs) markers on the scientific advice mechanism of chicory (SAMCHIC) linkage map

Name of marker	Type of marker	Accession by GenBank	Name of marker	Type of marker	Accession by GenBank
Est 5	SSR[a]	FL672278	Bip 20	SNP[b]	EH691568
Est 7	SSR	FL673198	Bip 66	SNP	EH691923
Est 11	SSR	FL675360	Est 40	SNP	EH700985
Est 8	SSR	EH696752	Est 18	SNP	EH705156
Est 41	SSR	EH692953	Bip 59	SNP	EH682796
Ms 1	SSR	FL677058	Bip 49	SNP	EL353500
Est 46	SSR	EH687457	Feh 1	SNP	AJ242538
Est 14	SSR	EH683296	Pa 21	SNP	EL368712
Est 1	SSR	FL675136	Bip 2	SNP	EH676972
Ms 10	SSR	FL672898	Bip 44	SNP	EH689663
Est 15	SSR	EH691306	Bip 23	SNP	EH682079
Est 71	SSR	EH675186	Pa 39	SNP	FL677172
Bip 43	SNP[b]	EH707518	Pa 37a/b	SNP	FL677179
Bip 21	SNP	EH705277	Bip 02	SNP	EH672777
Pa 5	SNP	FL675412	Bip 03	SNP	EH698135
Pa 14	SNP	FL675436	Bip 70	SNP	FL679098
Bip 37	SNP	EH684149	Bip 57	SNP	EH692696
Cifr 07	SNP	JQ082518	Bip 33	SNP	EH672649
Bip 41	SNP	EL345653	Bip 28	SNP	EH709102

Source: Muys et al. (2014)
[a]*SSR* simple sequence repeat
[b]*SNP* single nucleotide polymorphism

1.5.2 Functional Genomics

Functional markers involve gene isolation and the identification of the functional analysis of allelic variation by mining. It will be necessary to encourage the selection of germplasm and wild chicory along with initiatives to investigate and preserve genetic diversity in situ and on the farm (Maggioni 2004). The chloroplast genome of chicory includes 127 functional genes, including 74 genes for protein-coding, 29 genes for tRNA and 24 genes for rRNA. However, 15 protein-coding genes formed 1 intron, 2 genes (*ycf3* and *clpP*) possessed 2 introns and the chloroplast genome had a GC value of 37.3% (the complete chloroplast), the first genome sequencing of the leaf chicory with a total annotation of more than 18,000 unigenes (Galla et al. 2016) and a high-density map of leaf chicory was successfully developed and the ms1 locus was precisely mapped (Palumbo et al. 2019). Such findings together help to establish the foundation for continuing *Cichorium* evolutionary and ecological research. The Genebank format sequence files were submitted to Organellar Genome Draw (OGDraw) (Lohse et al. 2013).

Chloroplast genome inverted repeat regions (IR), arge-single-copy (LSC) and a small single-copy (SSC) position were labeled. A total of 152,975 bp has been

observed for chicory chloroplast double-stranded circular DNA, including the reverse repeat region (IR,50,038 bp), a medium single-stranded region (SSC,18,561 bp) and a large one-copy region (LSC,84,376 bp) (Yang et al. 2019). Clustered regularly interspaced short palindromic repeat (CRISPR) aligned with protein CAS9 (CRISPR/Cas9) is a genome-editing method commonly utilized over the last 5 years owing to its simplicity, affordability and viability. This genome editing method can be used successfully with chicory, which will promote and speed genetic and functional biology improvements, to drive sgRNA expression. A U6 promoter (CiU6-1p) has been selected from among eight predicted U6 promoters in the chicory. A binary vector engineered to cause selective mutations was then built in the fifth exon of the chicory phytoene desaturase gene (CiPDS) and used to convert chicory (Bernard et al. 2019) (Figs. 1.9 and 1.10).

Both expressed sequence tags (ESTs) and formally defined with the public sequence repositories have been correlated (Dauchot et al. 2009). From another point of view, it is important from both a scientific and agro-economic point of view to cloning practical chicory 1-FEH I, but also into fructan-metabolizing plants by additional 1-FEH incorporation (Van den Ende et al. 2000). Nowadays, there are many tools used for genome editing such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), oligonucleotide-directed mutagenesis (ODM) and clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9). Such types of new tools create the point mutation, deletion and insertion of new nucleotide or substitution of a nucleotide in the targeted genome (Gaj et al. 2013; Gao et al. 2015; Kumar and Jain 2015; Lowder et al. 2016; Perez-Pinera et al. 2012).

1.5.3 Bioinformatics

Bioinformatics and data processing exploit the strength of genotype, phenotype and other data by providing access to advanced bioinformatics resources and support for biometrics; many reports on bioinformatics provide knowledge on phenotypes, enviro types and other details on cells. The proliferation in genetic and genomic evidence has not yet made its way into conventional plant production for a large range in plant organisms (Xu 2019). Ten root libraries were developed from one single chicory cultivar (Arancha) harvested during the 2002 growing season, providing a seasonal analysis of the genes expressed in the chicory roots (Fig. 1.11). There were also two repositories of cDNA created from leaves and nodules. The nodule library was collected from witloof chicory tissue culture. Around 1000 clones from their 3′ end were sequenced out of each cDNA catalog, to a total of 12,524 3’ EST sequences. Of the 12,524 chicory ESTs, 12,226 sequences existed after repetitive sequences, vector and organelle sequences that still reflect more than 7106 bp were deleted. Those sequences should be named the PHYTOMOL dataset in the future. All the EST sequences generated are accessible at NCBI under accession numbers [GenBank: FL670599] to [GenBank: FL682824]. The EG assembler

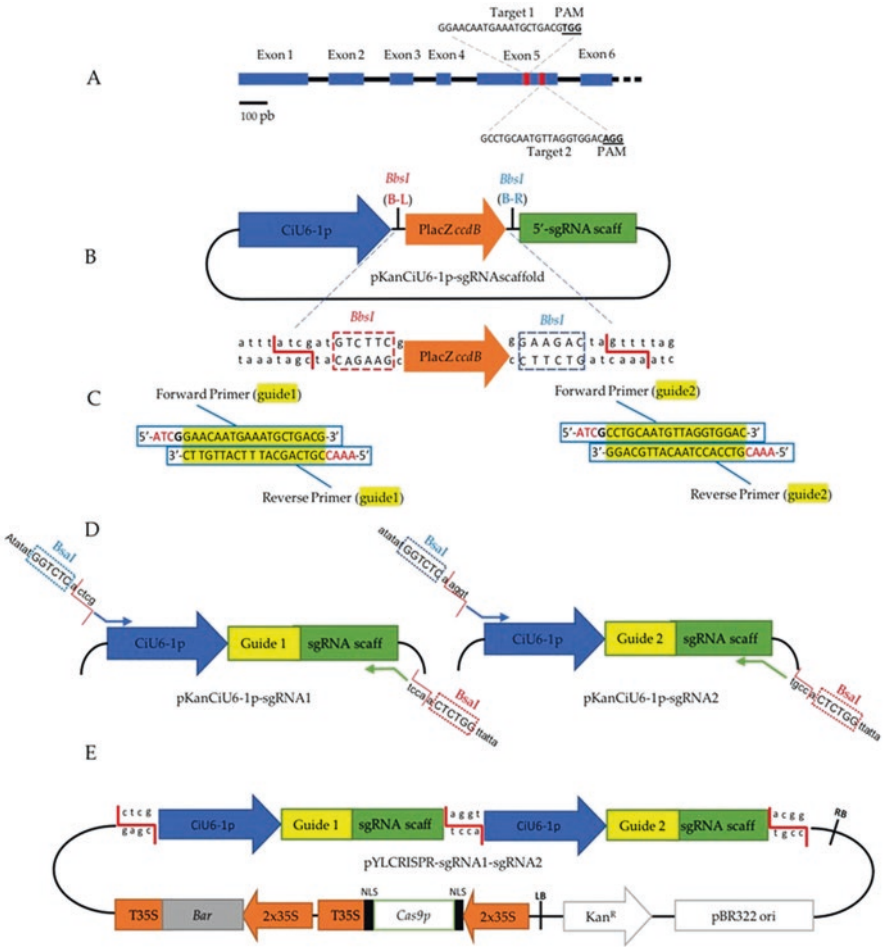

Fig. 1.9 Overview of the experimental design for CiPDS disruption. (**a**) Schematic position of the two guide RNAs (red boxes) targeting the fifth exon of CiPDS. The **blue** boxes indicate exons; the black lines indicate introns. (**b**) Schematic view of pKanCiU6-1p-sgRNAscaffold. Between the CiU6-1p and the sgRNA scaffold (scaff), a ccdB gene driven by the LacZ promoter (counter-selection marker) is surrounded by BbsI (second-generation enzyme) recognition sites, which allows the ligation of the hybridized guide adaptor shown in **c**. Red B-L depicts the orientation of the BbsI recognition site which achieves the cleavage on the left side. **Blue** B-R depicts the orientation of the BbsI recognition site which achieves cleavage on the right side. (**c**) Sequences of the complementary guide adaptors with the 19 pb guide sequence (yellow), the transcription initiator nucleotide G (bold), and the binding sites necessary to insert the hybridized guide adaptor into the pKanCiU6-1p-sgRNAscaffold (red). (**d**) Representation of the vector resulting from the insertion of the hybridized guide adaptors in the pKanCiU6-1p-sgRNAscaffold, (note that one vector is constructed for each guide). The primers used for the preparation of CiU6-1p-guide-sgRNA cassettes for Golden Gate Cloning are also shown. (**e**) Representation of the final plasmid pYLCRISPR-sgRNA1-sgRNA2 resulting from the cloning of the two cassettes into the pYL CRISPR/Cas9P35S-B. RB = Right Border, LB = Left Border, NLS = Nuclear Localization Signal. (Source: Bernard et al. 2019)

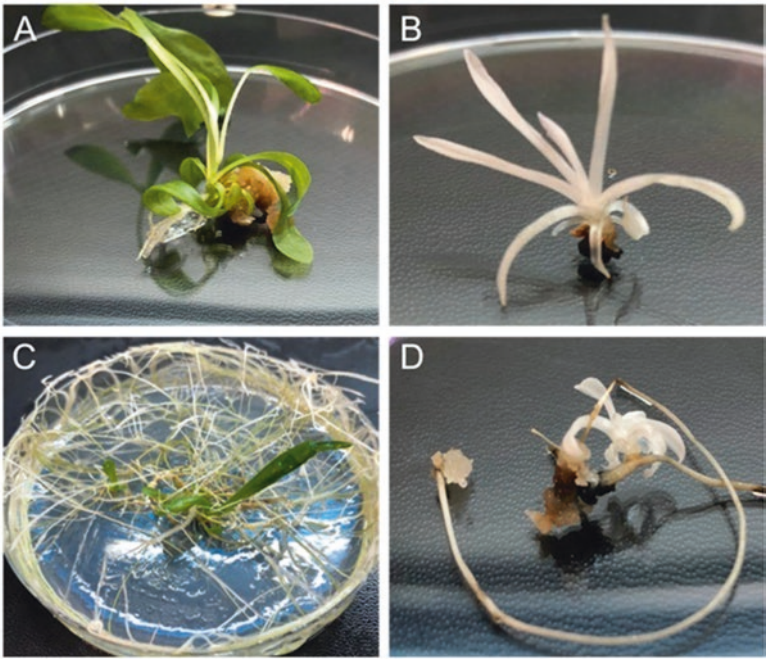

Fig. 1.10 Phenotype of genome-edited plants. (**a**)Wild-type (WT) shoot emerging from a WT callus, (**b**) CiPDS edited albino shoots emerging from a callus, (**c**) Hairy root line transformed with wild-type *Agrobacterium rhizogenes* strain 15,834 with the emerging shoot, (**d**) Albino shoot emerging from hairy root line engineered to knock out CiPDS. (Source: Bernard et al. 2019)

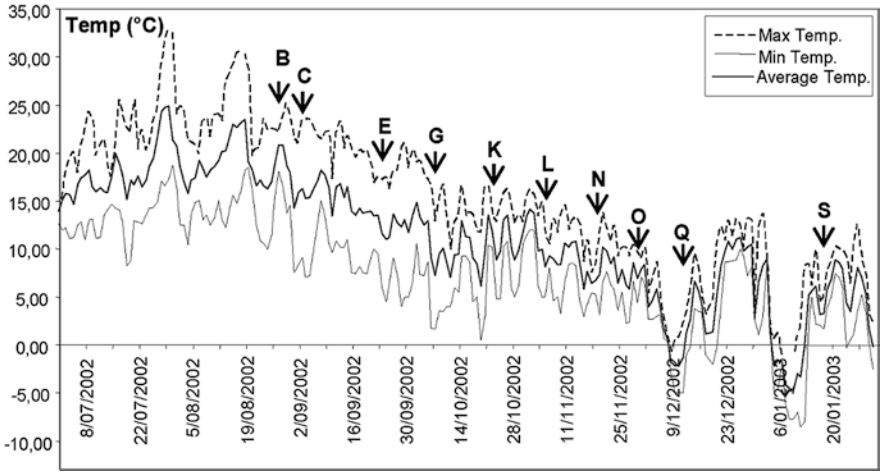

Fig. 1.11 Sampling times for the chicory root tissues. Evolution of mean, average and maximum temperatures in Gembloux (5030-Belgium) increasing season 2002. The sampling dates used to generate cDNA libraries are marked with arrows and corresponding letters of reference. (Source: Dauchot et al. 2009)

bioinformatics pipeline (SNP/INDEL) was used to create a unigene dataset (Dauchot et al. 2009) from the Compositae Genome Project Database (https://compgenomics.ucdavis.edu/) (Muys et al. 2014).

1.6 Tissue Culture Application

1.6.1 Micropropagation Approaches

Micropropagation of chicory *Cichorium intybus* is an important technique to counteract specific problems in propagation, such as incompatibility of pollination and producing haploid plants for plant breeding programs. Chicory micropropagation can produce several plantlets in a very short period. Most of the Compositae species such as chicory tissue culture are not successful when in vitro premature bolting takes place. Consequently, there is non-successful acclimatization. To counteract this problem, plants are rejuvenated through micropropagation of cauline leaves and hence successfully acclimatized (Conner et al. 2019). Also, these impediments can be countered by natural products through callus. The liquid culture of chicory in vitro can be used to obtain natural products such as antimicrobial agents and phenolic antioxidants compounds. In this regard, Al Khateeb et al. (2012) found that the ethanolic and methanolic extract of chicory reduced bacteria growth by 50 and 70%, respectively. The obtained phenolic antioxidants are rich in the methanolic extract as compared to the ethanolic extract. However, antioxidants in chicory and total phenol content of callus cultures and in vitro plantlets are less than in the wild plants (Fig. 1.12).

Another method of wild chicory micropropagation is through callus production using chicory leaf explants. The best concentration of IAA (0.5 mg dm^3) and 2-isopentenyl adenine (2-iP) (4 mg dm^3) to produce 97% shoots from callus (Dakshayini et al. 2016; Doliěski and Olek 2013). For root culture, 25-day-old in vitro raised seedlings were used for both hypocotyl and leaf as explants using half-strength (MS) medium (Murashige and Skoog 1962). The best combinations of 0.5 mg/L NAA and 0.1 mg/L indole-3butyric acid (IBA) have been found to cause the largest percentage of rooting from mature leaf explants under absolute dark conditions in liquid culture. Further enhancement of root production through culturing 0.5 g roots on half-strength MS liquid culture supplemented with 0.2 mg/L NAA and 0.5 mg/L IBA in liquid medium under total dark condition. The obtained biomass was increased to 5.820 g of root after 6 weeks (Nandagopal and Kumari 2007).

1.6.2 Embryo Rescue

Another means of micropropagation of the Indian chicory plant is somatic embryogenesis. Indeed, 1 month after pollination, plantlets can be obtained ready to select the desired agronomic traits (Varotto et al. 2000). Nodal stem and petiole were used

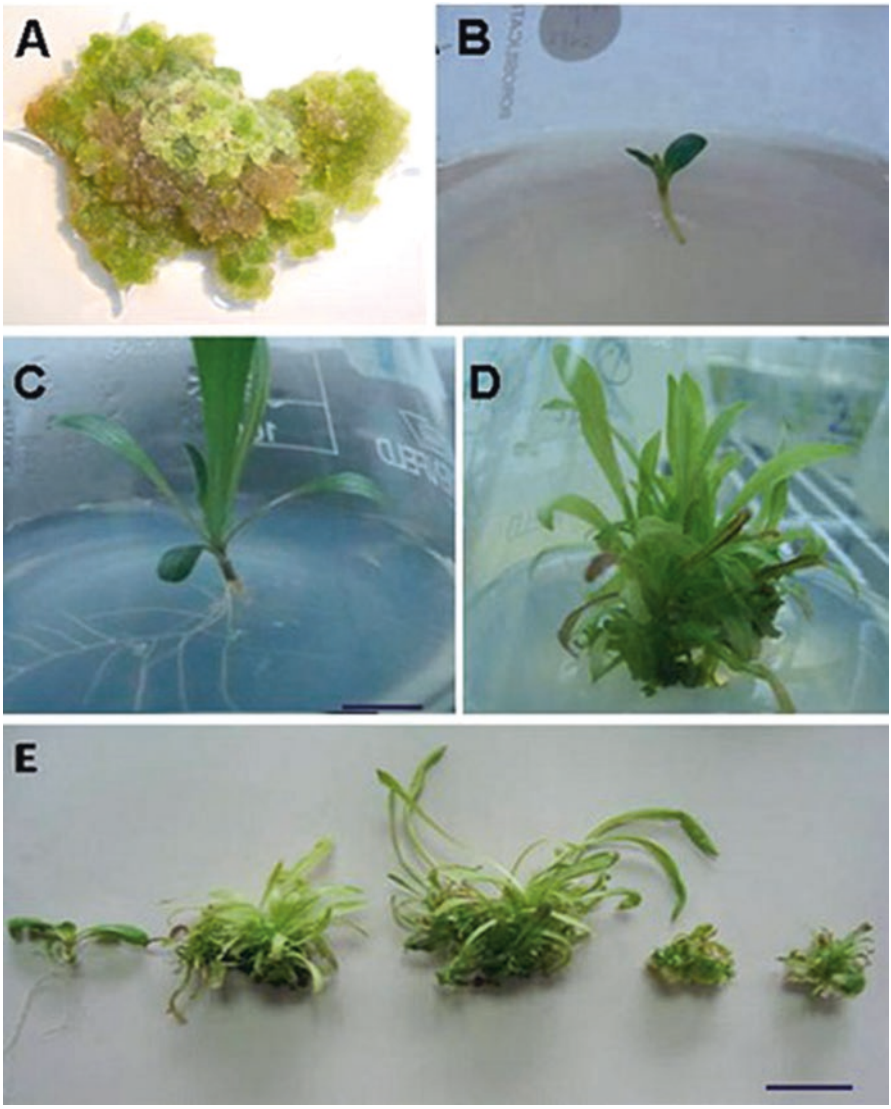

Fig. 1.12 Propagation *C. pumilum* in vitro. (**a**) Medium callus of 1.5 mg/L BA and 0.5 mg/L NAA. (**b**) Small shoot formed on an MS medium containing 1.5 mg/L BA, (**c**) Rooting of explants after 6 weeks in average enriched with 1.0 mg/L NAA, (**d**) Shoot proliferation in average 1.5 mg/L BA, (**e**) Various stages of shoot development; all scale bars reflect 2 cm. (Source: Al Khateeb et al. 2012)

as explants using Murashige and Skoog (1962) MS medium supplement with 2,4-dichlorophenoxyacetic acid (2,4-D), α-Naphthalene acetic acid (NAA) and indole-3-acetic acid (IAA) to produce callus after 3 weeks. Higher somatic embryogenesis was produced by using 1.5 mg/L Kinetin and 0.5 mg/L IAA as well as

500 mg/L casein hydrolysate (Abdin and IIah 2007). However, immature embryos of Italian red chicory collected in vitro after pollination within 24–72 h can successfully recreate plantlets acclimatized culture of zygotic embryos in vitro is an effective technique to improve the breeding cycle of Red Italian chicory (Van der Veken et al. 2019).

1.6.3 In Vitro Pollination

A system for in vitro pollination of chicory ovules has been established. The purpose is to avoid self-incompatibility after the in vitro self-pollination of ovules isolated from flower buds before anthesis and flower buds. A limited number, 0.76% of seedlings have been collected (Castan and De Proft 2000). Sometimes there is a need to maintain in vitro maintenance of specific mother plants until there are full evaluation and pollination. In vitro plant maintenance is subject to full loss due to contamination and human error. Plant cryopreservation is thus another method of mother plant maintenance (Rasmussen and Ekstrand 2014; Towill 1988).

1.6.4 Synthetic Seed

Although chicory seed was introduced to India, it became successfully produced locally in wide areas of India for successful planting. Chicory became widespread all over the world. In this case, there is no need for the production of synthetic seed of the chicory plant (Gangopadhyay et al. 2011; NISCAIR 1992).

1.7 Genetic Engineering and Gene Editing

Natural ancestors and genetic tool families are used as a source of tolerance to biotic and abiotic causes and as a source of selection traits in genetic engineering (FAO 2010). The editing of genomes targets traits in organisms to create new genetic variation. There are two methods for determining gene function, namely forward and reverse genetics. The main goal in forward genetics is to change a specific genotype to obtain desired phenotypes, while in reverse genetics we target the phenotype and then move towards its genotype. With exposure to map-based cloning and the T-DNA tag, mutations in the gene of interest may be identified and screened to help in forward genetics (Page and Grossniklaus 2002), such modifications include point mutation, chromosomal mutation, translocation and deletion in the organism genome (Kao and Michayluk 1974) and became a more effective hybridization method to improve yield, disease resistance and herbicide tolerance (Kumar and Sandhu 2020).

Genetic innovation was introduced to commercial chicory but remains limited to labs and closely-regulated laboratory plots. Wild species of *Cichorium intybus* exist in farm fields, posing the chance of potential gene transfer to wild relatives from future genetically-engineered varieties (Van Cutsem et al. 2003). Genome editing, which consists of targeting and digesting DNA at a single genome location, is an essential method for studying gene activity and enhancing chicory (Bortesi and Fischer 2015), where three specific genome technologies have been developed: zinc finger nucleases (ZFNs) (Kim et al. 1996), transcription activator-effector nucleases (TALENs) (Christian et al. 2010) and frequently clustered protein (CAS)-related short palindromic repeat (CRISPR) structures. Such strategies cause targeted DNA to have double-strand breaks (DSBs). These splits can be repaired in eukaryotic cells in two separate ways: the recombination of non-homologous end-to-end (NHEJ) and the homologous (HR). NHEJ is the most commonly used DSB repair mechanism in many organisms and can lead to insertions or deletions that could induce a knockout gene (Cao et al. 2016). The CRISPR/Cas9 method uses CRISPR RNA (crRNA) and a tiny transactivating CRISPR RNA (*trans*-RNA) which can hybridize to shape a mature dual crRNA, that distinguished simplicity, low cost, versatility and high efficiency. Chicory genome editing with CRISPR/Cas9 method can be used effectively to promote and speed genetic and functional biology development (Bernard et al. 2019).

There is another way of using chicory-mediated *Agrobacterium* transformation dependent on shoot regeneration medium which does not involve specific compounds (a non-selection approach), the goal is to generate transgenic chicory plants on a shoot-induction medium without selective compounds and to investigate the inheritance of the incorporated transgene, thus producing marker-free genetically-modified plants in the context of a non-selection transformation method (Maroufi 2015).

Male sterile lines of chicory are produced through the inclusion of the barnase gene. The important characteristic of this genetically modified plant is the tolerance gene for herbicides (Bais and Ravishankar 2001). There is proof suggesting no alteration occurs as a result of genetic manipulation. Once chicory is cultivated as a vegetable crop, it is picked until it flowers naturally. When some plants are vernalized, leading to premature flowering, no pollen will be produced. The usage of *Agrobacterium rhizogenes* mediated hair root development as an alternate form of root biomass growth in a bioreactor would be suitable for regulated cultivation without environmental health concerns. The hairy root can be produced environmentally free from pesticides and phytohormones (Bais and Ravishankar 2001) (Table 1.11). Cytoplasmic male sterility (CMS) may lead to 100% true hybrids. CMS does not occur naturally in chicory (Deryckere 2013). Chikkerur et al. (2020) found through their studies of the production of short-chain fructooligosaccharides from the inulin of chicory root using fungal endo-inulinase, the pathway for higher inulin yield from chicory roots followed by its conversion into short-chain fructooligosaccharides (SC-FOS) with fungal endoinulinase treatment.

Table 1.11 Characteristics of genetic transformation research of chicory species (*Cichorium intybus* L.)

Varieties	*Agrobacterium* strain/ plasmid	Explant source	Selective agent	Genes transferred	Trait	Reference
C. intybus cv. Belgian endive hybrid Flash	*Agrobacterium rhizogenes* A4RSII and 8196	Roots, floral stems	Rifampicin and spectinomycin	T_L and T_R- DNA	Converted from biennial to annual flowering	Sun et al. (1991)
C. intybus Witloof	*Agrobacterium tumefaciens* LBA4404/pGH6	Leaf	Kanamycin	*csrl-l/nptII	Resistance to chlorsulfuron	Vermeulen et al. (1992)
C. intybus cv. Rossa di Chioggia	*Agrobacterium tumefaciens* pGV3850/pKU2 *Agrobacterium tumefaciens* LBA4404/pBI121 *Agrobacterium tumefaciens* pGV2260/pGUSIN	Leaf	Kanamycin	**nptII uidA/nptII ***uidA	Transfer of gus gene to radicchio	Genga et al. (1994)
C. intybus L.var. Witloof cv. Flash	*Agrobacterium tumefaciens* pGV22601/pGSGLUC1 *A.tumefaciens* pGV2260/ pTDE4	Tap-root, leaf, Cotyledon	Kanamycin	nptIIluidA nptII/uidA	Transfer of gus gene to radicchio	Abid et al. (1995)
C. intybus var. *sativum* cv. Hicor, Inula, Tilda, VBF, VBG, VAX	*Agrobacterium tumefaciens* pGV2260/35SGUSIN pGV1531	Seedling buds	Kanamycin	nptII/uidA	Obtaining an offspring with complete genetic stability	Frulleux et al. (1997)
C. intybus cv. Pala Rossa	*Agrobacterium tumefaciens* GV3101)/pCB124	Cotyledonous and leaves	Kanamycin	nptII, ifn-a2b	Obtaining transgenic chicory plants with interferon-*a2b* gene.	Matvieieva et al. (2009)

(continued)

Table 1.11 (continued)

Varieties	*Agrobacterium* strain/ plasmid	Explant source	Selective agent	Genes transferred	Trait	Reference
C. intybus cv. Pala Rossa	*Agrobacterium tumefaciens strain GV3101*	Cotyledons	Kanamycin	*nptII* and target *esxA* genes	The synthesis of transgenic plants with tuberculosis antigene (*ESAT6*)	Matvieieva et al. (2011)
C. intybus	*Agrobacterium tumefaciens* pRI101-Mgfp LBA4404	Leaf explants	Kanamycin	*Mgfp-5* gene	Synthesis of transgenic plants with the foot protein type five (*Mgfp-5*) gene	Lv et al. (2019)
C. intybus Blue (Samen Mauser, Switzerland)	*Agrobacterium rhizogenes* A4M70GUS/pRiA4	Roots	Kanamycin	*CiGASlo* and *CiGASsh*	Varying sesquiterpene lactones (STLs) content and reduced bitter compounds for inulin production	Bogdanović et al. (2020)

**csrl-l* is *Arabidopsis thaliana* mutant acetolactate synthase gene, ***nptll* neomycin phosphotransferase II conferring resistance to kanamycin, ****UidA* the encoding gene *β-glucuronidase,* PGUSIN includes an intron in the *uidA* gene-spliced correctly into the eukaryotic cells. Source: Abid et al. (2001)

1.8 Mutation Breeding

1.8.1 Mutation Breeding

The essential and special function for mutation breeding is the production of new mutated alleles. In 1900 Hugo de Vries used the term *mutation* to define inheritable phenotypic shifts. The use of induced mutants for the development of crops is regarded as mutation breeding. Mutation breeding uses a variety of physical (ionizing radiations such as X-rays, ultra-violet (UV) light and gamma rays (Gupta 2019) and chemical agents such as nitrous acid, ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS) and diethyl sulfate (DES) to explore the potential for the production of new varieties (Khan and Ansari 2014). It causes spontaneous changes in DNA sequences throughout the genome, especially in crops with a small genetic base like chicory.

The self-pollinated chicory grows wildly and also has limited genetic diversity, which provides ample space for mutant breeders to boost crops (SAM 2017). Overall, ionizing radiation is favored due to its ease of use, strong penetration, reproducibility, high level of mutation and less problem of disposal. While all chemical mutagens react with DNA by alkylating the phosphate groups as well as the purine and pyrimidine bases. Mutagenesis is an essential element in each mutagenesis system (Gupta 2019). Compared with radiological approaches, chemical mutagens appear to induce shifts in single base pairs (bp) (Till et al. 2004, 2007). Although still limited to endogenous genome potential and mutagenesis, high-resolution sequencing will provide a fairly effective complement to recombinant DNA technology and genetically modified organisms (GMOs) in the further development of better-adapted crops to climate change and the increasing global population (Sikora et al. 2011).

Changes caused by conventional mutagenesis are typically more severe than those arising from gene-editing methods and changes to conventional breeding techniques (CBT), especially through mutation breeding in plants, frequently require sampling a large population of random changes (SAM 2017). The chemical mutagen methyl methanesulfonate (MMS) (monofunctional alkylating agent) was used as the most effective method for inducing morphological and genetic variability in plants, especially those with limited genetic variability during microsporogenesis (Khan et al. 2009).

As the *Cichorium intybus* genotype is homozygous, with a minimal genetic variation, sowing is widely practiced. Mutagenesis is important for a broad genetic base (Khan et al. 2009). Mutation breeding is mostly used in traditional plant breeding combined with modern biotechnology (Bado et al. 2013; Matijevic et al. 2013; Shu et al. 2012). Conventional mutations have been widely utilized in plant breeding since the 1960s (SAM 2017). It is possible to change the genome of an organism at several locations through contact with a chemical mutagen like alkylating agents or irradiation like UV radiation or in an untargeted manner (Eur Comm 2018).

1.8.2 In Vitro Mutagenesis and Selection

Pamidimarri et al. (2009) and Khan et al. (2012) analyzed the impact of 6-aminopurine (6-AP) in 3 consecutive generations on mutation induction *Cichorium intybus* (M_1 to M_3) (Table 1.12) (Ateto 2019). There are different single nucleotide polymorphisms (SNPs) and indel polymorphisms in 3 fructan exohydrolase (FEH) genes when the candidate-gene approach was successfully applied to determine the phenotype/genotype interaction in the non-model chicory species. A statistical association was found between a 47 bp indel located in the 3′ UTR of 1-FEH IIa (Dauchot et al. 2014). Hamid et al. (2015) found that proline and lipid peroxidation increased as the concentration of ethyl methanosulphate (EMS), soluble protein, and sugar content increased (Table 1.12).

Dickeya dadantii (*Erwinia chrysanthemi*) (EC16) strains were developed with several mutations affecting three bacterial virulence mechanisms, namely (pel) coding for the main pectate lyase (pelABCE), hypersensitive reaction and pathogenicity (HRP) and sensitivity to antimicrobial peptides (SAP) (López-Solanilla et al. 2001). A process of *Agrobacterium*-mediated chicory transformation based on a shoot regeneration medium that does not generate particular compounds (a non-selection approach) (Maroufi 2015). Recent in vitro controlled mutagenesis approaches using CRISPR/Cas9 technologies as an effective tool for investigating gene activity and generating new varieties in chicory (Bernard et al. 2019).

1.8.3 Molecular Analysis

Since the early 1990s, when taxonomists became more familiar with the study of DNA polymorphisms, some researchers have tried to examine the relationships between cultivated *Cichorium intybus* and its wild relatives. This probably explains the use of mitochondrial restriction fragment length polymorphism (RFLP) markers and the use of other markers. More detailed molecular approaches, such as internal nuclear transcription spacers (ITS) and simple sequence repeats (SSRs) markers are also used (Barcaccia et al. 2016).

Table 1.12 Effect of different ethyl methanosulphate (EMS) concentrations on proline, protein and soluble sugar content in *Cichorium intybus* L.in vitro raised shoots

EMS concentrations (%)	Proline (µg/g fresh weight)	Protein (mg/g fresh weight)	Sugar (mg/g fresh weight)
0	14.05 ± 0.61	4.10 ± 0.23	24.40 ± 1.44
0.2	19.45 ± 0.40	5.31 ± 0.41	30.02 ± 0.44
0.4	32.51 ± 0.70	5.43 ± 0.35	32.0 ± 1.58
0.8	46/02 ± 1.58	5.46 ± 0.70	35.72 ± 0.79
1.2	59.17 ± 0.76	4.24 ± 0.27	19.01 ± 0.70
1.6	63.43 ± 0.53	3.91 ± 0.30	11.04 ± 1.22

Source: Hamid et al. (2015)

The 6-aminopurine (6-AP) mutants were further analyzed using RAPD markers, which distinguish the mutants genotypically, based on the occurrence of new bands and the absence of old bands in combined RAPD markers (Khan et al. 2012). To evaluate the mutations, the PCR products were sequenced with the Sanger sequencing process using the same primers (E5-F, E5-R). The chromatograms were analyzed using the codon code Aligner software. A unique priming pair (C9-F, C9-R) amplified the CAS9 and insulated the PCR products (around 410 bp) into a 1% agarose gel (Bernard et al. 2019). RAPD research was performed on 24 industrial varieties of F_1 used in hydroponic pushing (*Cichorium intybus* var. foliosum) (Van Stallen et al. 2001). Analysis of reverse transcription PCR (RT-PCR) has confirmed the right expression of the transgene in metamorphic plants resulting from the leaf in vitro grown cultivar Melci seedlings inoculated with *Agrobacterium* and thus safe incorporation into the genome (Maroufi 2015). Using a CaMV 35S field priming pair on genomic DNA, PCR verified the integration of the expression cassette (T-DNA) into the plant genome (Maroufi et al. 2018).

1.8.4 Enhanced Traits and Improved Cultivars

Treatment with large 6-AP concentrations resulted in base addition and replacement, resulting in *Cichorium intybus* mutations. From an economic point of view, three morphological mutants were developed to support the production of new varieties with improved commercial properties appropriate for the Indian landscape (Khan et al. 2012). But In fact, the selection by farmers has traditionally paid attention to morphological characters, on which the market value mainly depends, while other important characteristics such as biotic or abiotic stress resistance, have been given little attention (Barcaccia et al. 2016).

The details found in the analysis of the development of the chicory flower could help evaluate the generation of genes involved in flower growth in general and male sterility in *Cichorium intybus* in particular and be a starting point for reviewing the 524-CMS fertility restoration which may lead to an understanding of cytoplasmic male-sterility (CMS) in chicory (Habarugira et al. 2015). Radicchio has useful genes that control some of the key quality characteristics or resistance to major pathogens. Farmer selection has traditionally paid attention to morphological characteristics, on which the market value mainly depends. Other important characteristics such as resistance to biotic or tolerance to abiotic stress have been given little attention (Barcaccia et al. 2016).

Chicory (*Cichorium intybus* var. Witloof) called R10K, was regenerated into fertile herbicide-resistant chlorsulfuron plants with a resistance 1500–2000 higher than that of the wild type (Dewaele et al. 1997). New and special Witloof were produced when the witloof chicory breeder selects and crosses the haploid cultivars accompanied by diploid parental varieties (Dauchot et al. 2014; Van der Veken et al. 2019).

The use of the transformation mechanism has the potential for routine use in the development of selectable marker-free transgenic chicory plants for research or

commercial projects, with no selection of a transgene expressed in T_0 and T_1 generations (Maroufi 2015). The CRISPR/Cas9 genome editing method can be used effectively with chicory, which will promote and drive genetic development and practical biology (Bernard et al. 2019).

1.9 Hybridization

1.9.1 Conventional Hybridization

Rick (1953) who described chicory hybridization with a wild relative, endive *Cichorium endivia*, as a problem for commercial seed growers in California, suggested hybridization between cultivated and wild chicory (Kiær et al. 2007). The genetic basis of the crop is rather narrow. Therefore, as selection progresses, negative characters should be anticipated for appearance. The majority of endive physiological complications e.g. browning of the flowering stem and the red color of the leaves was observed as a result of inbreeding distress. Early and winter cultivars were produced. The very late cultivars were later introduced for summer growth. Ellstrand (2003) noted that chicory is not on the list of multiple lines of evidence for hybridizing crop species indicating that spontaneous hybridization with wild plants is possible (Kiær et al. 2007). The system self-incompatibility in chicory provides scope for growing F_1 hybrid cultivars; however, the full use of self-incompatibility in the production of F_1 creates problems for breeders (Castafio et al. 1997). New F_1 hybrids were developed slowly (De Proft et al. 2003).

The inbred-hybrid theory, on which chicory breeding is based, demands that homozygous line production be checked as parents of new potential hybrids as soon as possible. To achieve the optimal degree of homozygosity, the development of these lines typically requires 6–8 generations of inbreeding. It is a complex and long-term technique because chicory (*Cichorium intybus*, 2n = 18) is cross-pollinated and biennial. Various efforts have been made to increase the homozygosity of inbred lines by coercive selfing and brother-sister family mating. The later only uses up three times as much energy as selfishness. Plant development and production programs are requiring increasingly effective breeding methods. Haplodiploidization will be of great importance between them in the rapid growth of chicory pure lines (Doré et al. 1996).

On the other hand, improving the development of inulin in the root of industrial chicory through traditional breeding has reached it is maximum. Therefore, it was important to increase the genetic diversity of industrial chicory *Cichorium intybus* to increase its inulin output (Deryckere et al. 2012). Embryo rescue hybridization can be accomplished by inducing interspecific hybrids and haploid chicory plants by chicory *C. intybus* and other species such as blue sow thistle (*Cicerbita alpina*). These haploids can then be regenerated by doubling the chromosome using

antimitosis inhibitors such as trifluralin and oryzalin and one-week exposure to 0.05 g/L (Van der Veken et al. 2019).

1.9.2 Somatic Cell Hybridization

The goal of somatic chicory hybridization of this plant was to induce or transfer cytoplasmic male sterility (CMS). To this end, conditions were identified for effective plant regeneration from the Pevele cultivar protoplasts. It was observed that the second stage, the preconditioning and age of donor plants, the source of nitrogen, sucrose, auxin and the rapid transition to an agarose medium were crucial in achieving good regeneration yields. Male sterile of chicory plants were obtained by fusion of chicory mesophyll protoplasts with male-sterile sunflower plants associated with the hypocotyl. The protoplasts were connected via the polyethylene glycol (PEG) process. The goods were manually picked and grown in a liquid medium at a very low density. Rambaud and Vasseur (2001) reported that micro calli were formed by 3–20% of separate heterokaryocytes, than calli that may cause budding. Van Huylenbroeck et al. (2012) found that a single plant cell can fuse nuclear and cytoplasmic DNA with an asymmetric fusion of industrial chicory *Cichorium intybus* protoplasts and other *Cichorium* organism protoplasts. The main goal of this fusion approach is to put in our industrial chicory a cytoplasmic male sterile (CMS) trait. However, somatic hybridization by protoplast fusion must be optimized in chicory. The scientific aspects of the separation, fusion and regeneration of protoplast as well as the successful screening of putative hybrid fusion materials are under investigation. Industrial chicory *C. intybus* var. *sativum*, endive *C. endivia* var. *crispum* and wild chicory *C. intybus* var. *foliosum* were used for hybrid partners. After industrial chicory and endive fusion, up to 30% of regeneration are hybrids. On average, the fusion of industrial chicory with wild chicory has produced 4% hybrid regenerates (Deryckere et al. 2012).

Deryckere (2013) demonstrated that protoplast regeneration is important for somatic hybridizations. The optimized bead technique with low melting point agarose (LMPA) allowed complete regeneration of plantlets from *Cichorium* for the first time. The protoplast of *C. endivia* and increased protoplast regeneration capacity of other *Cichorium* species. Therefore, this fine-tuned LMPA bead technique can be used for protoplast regeneration after protoplast fusions of the genus *Cichorium*.

1.9.3 Hybrid Cultivars

Radicchio F_1 hybrids can show an increase of 25–30% in leaf yields per plant compared to varietal cycle length open-pollinated (OP) synthetics (unpublished data, Blumen Group SpA). That is why the F_1 hybrid production methods have recently been introduced by private breeders and seed companies (Patella et al. 2019).

The production of somatic hybrids and cybrids by fusion with protoplast will contribute to the production of new cultivars which are economically valuable. The expertise gained from the work on the chicory genetic elements may be useful for further breeding. The protoplast fusion technique indicates its importance for revolutionary chicory breeding (Van Huylenbroeck et al. 2012). A novel witloof chicory cultivar, called Bobine was revealed. The discovery involves the seeds of Bobine witloof chicory cultivar, the plants of Bobine witloof chicory cultivar and methods for growing a witloof chicory plant by crossing the Bobine cultivar with itself or another section of witloof chicory. Also, the invention relates to cultivar-derived methods of Bobine for the production of other witloof chicory lines. It has acquired traits such as male sterility, herbicide resistance, pest resistance, bacterial, fungal or viral disease resistance, decreased number of seeds, increased shelf life, accelerated senescence and water or heat stress tolerance (Lecompte 2015).

1.10 Conclusions and Prospects

Chicory has economic value and is widely cultivated worldwide for various uses such as root biomass for making a coffee substitute, as a vegetable crop, fodder for improving protein and, recently, to obtain essential phytochemicals and pharmacologicals. Since chicory has three forms of compatibility, root chicory seems to have a limited generic basis which might hamper breeding progress. For that, there is the need to achieve integration between traditional breeding and biotechnology to developing commercial varieties. The sowing/planting material availability for the production of chicory and absence of an alternative are also problems. The other problem in the export of chicory is represented by the multiple harmonized system (HS) code classifications. The Chicory Innovation Consortium (CHIC) was established in 2018 and its goal is to introduce new plant breeding techniques (NPBTs) in chicory and to grow it as a multifunctional crop.

Climate change is one of the most serious challenges facing our world today. Most experts believe that the earth is warming up more rapidly than before due to the large volume of greenhouse gasses injected into the atmosphere by humans, involving many practices such as the combustion of fossil fuels (coal, oil, gas), running vehicles and tree clearing, as well as other human operations, such as rainforest logging, forestry, livestock and chemicals processes. In general, climate change results in major shifts in weather variables such as rainfall rates, surface temperatures, heat waves and global carbon dioxide (CO_2) or ozone levels, sea-level fluctuations, in addition to the emergence of new weed flora and insect pests or pathogens.

Climate change is expected to be the primary cause of the numerous abiotic and biotic stresses which have had a major impact on agricultural production; besides CO_2, there is methane (CH_4) gas which accounts for 2/3 of all agricultural emissions. Also, CH_4 emissions from forage diets typically account for 6–9% of gross energy intake. As part of the global climate change crisis, the adverse atmospheric impacts of livestock production are reduced by the adoption of new grazing systems and the use of plants with high nutritional value and bioactive compounds. Many pasture plants, such as chicory, are well suited to temperate climatic conditions and can provide many benefits for livestock production, animal health and environmental protection, such as nitrogen (N) leaching and methane pollution. Their mitigation will minimize greenhouse gases (GHG) emissions and potentially provide more resources for meat and milk production. The sulfur hexafluoride (SF6) tracer technique has calculated that chicory is useful in reducing tannin containing CH_4 emissions from grazing animals by up to 30% compared with perennial ryegrass. By contrast, bioactive compounds in condensed tannins help shift N from urine to feces. This is especially important in reducing the problems with N leaching in pastures. Chicory will be enhanced as a versatile multipurpose crop, tolerant of adverse environmental conditions and high tolerance of insect pests and providing dairy farmers with the potential to extract bioactive compounds, leading to sustainable agriculture and a bio-based economy.

Some varieties of chicory, apart from the narrow genetic base, suffer from pollination incompatibility. Only very limited breeding efforts have been directed towards improving root chicory relative to other field crops. Although it is of industrial importance and major research gaps remain in its pharmacological activities and discoveries. Haploid production for plant breeder programs must be attentive to the fertility of the resulting diploid plant and optimize somatic hybridization by protoplast fusion in chicory. Studying the relationship between root strains, harvest period and yield components is needed to develop resistant and early-maturing varieties. This also facilitate solving production problems such as early stalk development (bolting) that reduce dramatically marketable products. Moreover, research is needed for the estimation of genetic diversity and heritability within the root reservoir of chicory, as well as evaluation of heterotic influences in root and leaf of chicory species. Similarly, quantitative trait loci (QTLs) should be defined to enhance those traits and apply synthetic signaling strategies to plants to reduce the worst impacts of climate change and provide a way to engineer crops for drastically different climates and technological models that can accelerate the cycle of design-build-test-learn.

Appendixes

Appendix I: List of Major Institutes Engaged in Chicory Research

Institutes Name	Country	Website
Department of Biological Sciences, Monash University Clayton, Victoria	Australia	https://www.monash.edu/science/schools/biological-sciences
Institute for Agricultural and Fisheries Research (ILVO)	Belgium	https://www.Ilvo.Vlaanderen.Be/Language/En-US/EN/Home.Aspx
Institute of Botany, Bulgarian Academy of Sciences Sofia	Bulgaria	http://www.bio.bas.bg/botany/
Institute of Botany, Academy of Sciences Průhonice	Czech Republic	https://www.ibot.cas.cz/en/
Kunming Institute of Botany, Chinese Academy of Sciences	China	http://english.kib.cas.cn/au/bi/
Institute Charles Violette (ICV)	France	https://Institutcharlesviollette.Univ-Lille.Fr/
Crops Research Institute Kumasi	Ghana	https://www.Cropsresearch.Org/
Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben	Germany	https://www.ipk-gatersleben.de/en/institute/about-us/s/
Bundes Forschungs Institut für Kulturpflanzen	Germany	https://www.Julius-Kuehn.De/
Systematic Botany, Justus-Liebig-Universität	Germany	https://www.uni-giessen.de/faculties/f08/departments/botany/systematic-botany-group
Botanic Garden and Botanical Museum Berlin, Freie Universität Berlin, Germany	Germany	https://www.fu-berlin.de/en/sites/botanischergarten/index.html
Crops Research Institute Kumasi	Ghana	https://www.Cropsresearch.Org/
Departamento de Biología Vegetal y Ecología, Universidad de Sevilla Sevilla	Spain	http://area.us.es/bioeco/
Medicinal Plants Research Unit, College of Agricultural Engineering, Baghdad University	IRAQ	https://Coagri.Uobaghdad.Edu.Iq
Wageningen University	Netherlands	https://www.Wur.Nl/En/Newsarticle/Breeding-Chicory-For-Medicine.Htm
Instituto de Biologia Experimental e Tecnológica (Ibet)	Portugal	https://Eatris.Eu/Institutes/Instituto-De-Biologia-Experimental-E-Tecnologica-Ibet/
Institute for Systematics and Ecology of Animals	Russia	http://www.eco.nsc.ru/
Korea Research Institute of Bioscience and Biotechnology Daejeon,	South Korea	https://www.kribb.re.kr/eng/main/main.jsp
Oregon State University (OSU)	USA	https://bpp.oregonstate.edu/

Appendix II: World List of Varieties and Wild Types of Chicory

Accession No.	Species	Origin	Material type
PI 651946	*Cichorium intybus*	North Holland, Netherlands	Cultivar
PI 651930	*C. intybus*	USA	Cultivar
NSL 69921	*C. intybus*	Pennsylvania, USA	Cultivar
PI 651954	*C. intybus*	Germany	Cultivar
PI 652015	*C. intybus*	France	Cultivar
PI 651955	*C. intybus*	Baden-Wurttemberg, Germany	Line
PI 652024	*C. intybus*	Saxony-Anhalt, Germany	Wild
PI 652048	*C. intybus*	Italy	Cultivar
PI652007	*C. intybus*	Poland	Wild
PI652019	*C. intybus*	Switzerland	Wild
PI652020	*C. intybus*	Hungary	Wild
PI652026	*C. intybus*	Mazandaran, Iran	Wild
PI652028	*C. intybus*	Russian Federation	Wild
PI652033	*C. intybus*	Coimbra	Wild
PI 432336	*C. intybus*	Cyprus	Line

Source: U.S. National Plant Germplasm System (https://npgsweb.ars-grin.gov/gringlobal/search.aspx)

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Chapter 2
Chinese Cabbage (*Brassica rapa* L. var. *pekinensis*) Breeding: Application of Molecular Technology

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Abstract Chinese cabbage (*Brassica rapa* L. var. *pekinensis*) is an economically important vegetable providing nutrients such as fiber, calcium and vitamins. Most cultivars of Chinese cabbage are F_1 hybrids with vegetative heterosis, and harvesting of commercial F_1 hybrid seeds makes use of self-incompatibility or cytoplasmic male sterility. Production of Chinese cabbage is always threatened by abiotic and biotic stress; climate change and increasing numbers of races and varieties of pathogens are also serious problems. The demand for abiotic or biotic resistant cultivars

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is growing year by year. An effective breeding method is desired, and marker-assisted selection (MAS) is a leading candidate. To apply MAS, identification of the causative gene or the locus linked to the causative gene controlling a trait for breeding is required. We review the recent research using molecular biology approaches and discuss how this information can apply to Chinese cabbage breeding.

Keywords Brassica · Disease resistance · Heterosis · Marker-assisted selection · Self-incompatibility · Vernalization

2.1 Introduction

The Brassicaceae is a diverse family of angiosperms containing 338 genera and 3709 species (Warwick et al. 2006); among them *Brassica rapa* L. originated from the highlands near the Mediterranean Sea and spread northward into the Scandinavian peninsula and westward from eastern Europe and Germany, and then to Asia (Mizushima and Tsunoda 1969; Tsunoda et al. 1980). Now *B. rapa* is an economically important species and shows extreme morphological divergence (termed morphotypic). With selection by plant breeders, *B. rapa* is an important vegetable crop consumed worldwide, especially in Asia and Europe. *B. rapa* comprises leafy vegetables (Chinese cabbage (var. *pekinensis*), komatsuna (var. *perviridis*), pak choi (var. *chinensis*)), root vegetables (turnip (var. *rapa*)) and oilseed (var. *oleifera*) (Fig. 2.1) (Cheng et al. 2014, 2016). Chinese cabbage, which forms a head with large pale-green colored leaves and wide white midribs, is an important vegetable in Asia. The non-heading vegetables, komatsuna and pak choi, are also important vegetables in Asia. Turnip develops enlarged hypocotyls, and there are variations of both shape and color. There are morphotypes of oilseed in *B. rapa*, and seeds are used for oil extraction.

It is hypothesized that Chinese cabbage was domesticated from non-heading plants about 6000 years ago in China. Chinese cabbage later expanded to other countries such as Japan and Korea (Sun et al. 2018b). There are the wrapped-over and joined-up heading types of Chinese cabbage. The wrapped-over type (also known as heavy-leaves type) is early maturing and round-headed, and adapted to warmer climates. The joined-up type (also known as many-leaved type) is late maturing and longer-headed and adapted to cool climates. Heading types have some merits such as long-term storage stability, cold tolerance and high yielding relative to non-heading types (Sun et al. 2018b).

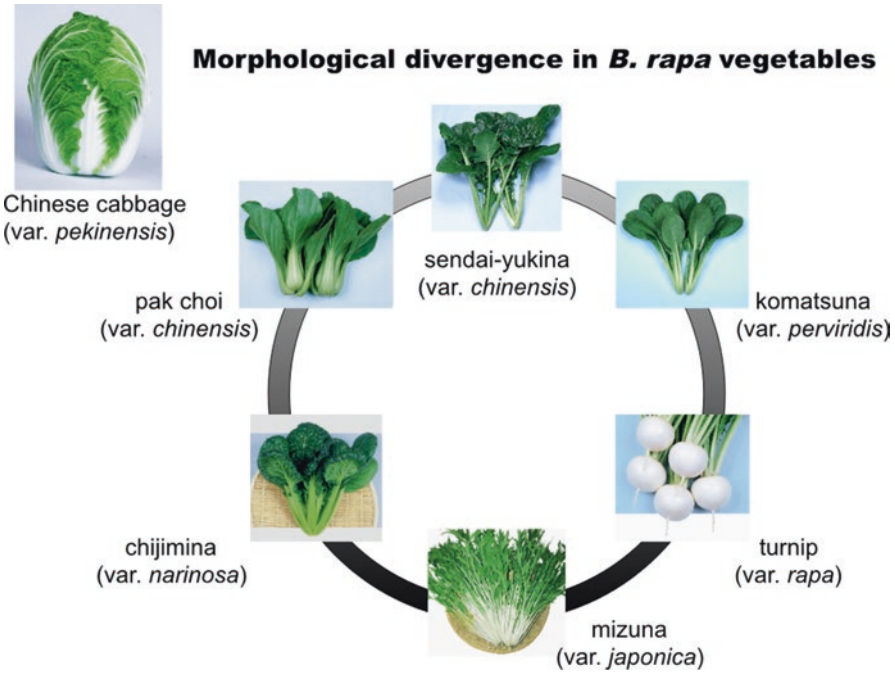


Fig. 2.1 Variations of *Brassica rapa* vegetables

2.2 Important Traits for Breeding and Cultivation

Traditional breeding of Chinese cabbage is selective breeding; breeders select lines that have desirable characteristics such as shape or yield from the offspring. There are many types of open-pollinated cultivars released, and they are preferred in specific local areas or by local farmers. Due to the agronomic benefits of F_1 hybrids, such as uniformity of phenotype, stress tolerance, disease resistance and increase in yields, most commercial cultivars of Chinese cabbage sold today are F_1 hybrids (Fujimoto et al. 2018), even though the price of seeds of F_1 hybrid cultivars is higher than that of open-pollinated cultivars. Hybrid breeding came from the discovery of heterosis (hybrid vigor), which is defined as the superior performance of hybrid plants over the parents, because heterosis is observed in yield-related traits (Fig. 2.2) (Crow 1998; Fujimoto et al. 2018). Open-pollinated cultivars were replaced by F_1 hybrid cultivars. The breeding of F_1 hybrid cultivars starts with the development of pure elite lines (inbred lines). The selection for desired traits such as disease resistance is carried out, and about five to seven generations of selfing are required for developing inbred lines as parental candidates. The suitability of elite lines for use as parents in an F_1 hybrid breeding program is tested by examining the level of heterosis in the offspring that result from all possible combinations of crosses of such inbred lines. Self-incompatibility or cytoplasmic male sterility is used for the

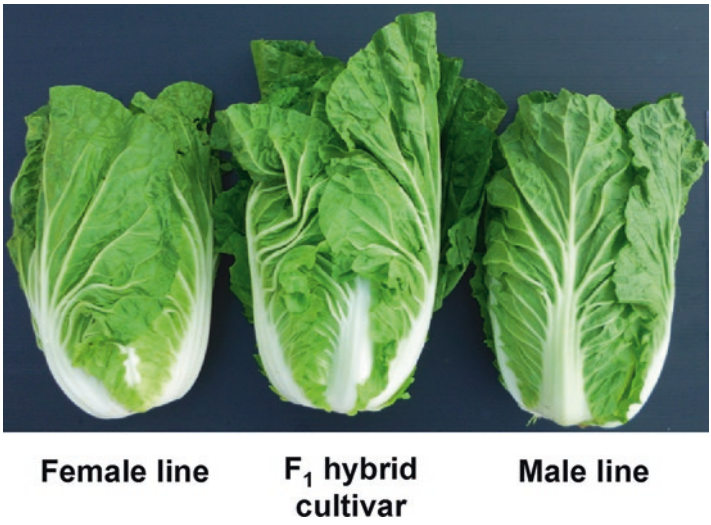


Fig. 2.2 Yield heterosis in a commercial F_1 hybrid cultivar of *Brassica rapa*

production of F_1 hybrid seeds in Chinese cabbage to avoid contamination by non-hybrid seeds (Fujimoto and Nishio 2007; Yamagishi and Bhat 2014). The strength of self-incompatibility or stability of male sterility is important for harvesting highly pure F_1 seeds.

Unlike other organisms, plants are sessile and thus, incapable of migratory behavior within a generation; plants are highly sensitive to environmental conditions. In general, Chinese cabbage cultivars need prolonged cold treatment for flowering, and this induction of flowering by exposure to prolonged cold is called vernalization. In plants, there are two types of vernalization according to the age of the plant that senses low temperature, the seed-vernalization and the plant-vernalization types. Seed-vernalization type plants can sense low temperatures during seed germination, while plants need to reach a certain developmental stage before they become sensitive to low temperatures in plant-vernalization-responsive type (Itabashi et al. 2018). Chinese cabbage is a seed-vernalization type, thus when Chinese cabbage faces the conditions of the cold at any stage, it will start bolting before reaching harvest time. Thus, premature bolting is a problem for the Chinese cabbage, and high bolting resistance is an important character for F_1 hybrid cultivars (Shea et al. 2018a). In some warm areas, heat stress tolerance is an important breeding factor, and this trait will become more important because of global warming. The need for heat tolerant cultivars of Chinese cabbage is increasing as decrease in productivity by heat stress has become apparent. To solve this issue, it is important to understand the molecular mechanism of heat stress tolerance, and various researches have been conducted in Chinese cabbage (Song et al. 2016; Wang et al. 2016, 2019; Wang et al. 2011a; Yu et al. 2012). Furthermore, biotic factors such as disease and insects are of concern. For example, the anticipated increase in humidity and soil temperatures in some regions poses an increased danger from soil-borne

pathogens such as *Plasmodiophora brassicae* (clubroot) and it is difficult to control these soil-borne pathogens by chemical treatment. Chinese cabbage cultivars are continuously threatened by problems of loss of productivity caused by various types of diseases. Thus, it is essential to have disease resistance in cultivars, and resistance against multiple pathogens is desired.

2.3 Molecular Breeding

In the process of breeding, selection by field trials is an important step, but it takes much labor and time. Furthermore, field trials always have the risk that plant phenotypes are affected by environmental conditions. Marker-assisted selection (MAS) could lead to more efficient breeding systems. MAS has been applied to many crops, and some DNA markers have been applied for MAS in Chinese cabbage. However, the variety of DNA markers are still limited for using MAS in Chinese cabbage. To apply MAS to Chinese cabbage, we need to identify genes that control important traits, and to understand the molecular mechanisms of these traits.

2.3.1 Basic Genetic Information

Whole genome sequence information is very important for molecular biological research. Starting with the model plant species, thale cress, *Arabidopsis thaliana* (L.) Heynh., which is in the Brassicaceae, whole genome sequences have been determined in many plant species, and recent innovation of sequence technology is accelerating the number of species or accessions within a species. *B. rapa* was the first species within the genus *Brassica* to be sequenced, and a double haploid (DH) line of Chinese cabbage, Chiifu-401-42, was used for sequencing (Wang et al. 2011b). Comparison of whole genome sequence between *B. rapa* and *A. thaliana* revealed that many orthologous genes are conserved and the *B. rapa* genome undergoes a whole genome triplication (WGT) after speciation between the genera *Brassica* and *Arabidopsis* (Wang et al. 2011b). This WGT results in multiple copies of paralogous genes. Three subgenomes, the least fractioned subgenome (LF) and two more fractionated subgenomes (MF1 and MF2), are present within the *B. rapa* genome (Cheng et al. 2012). Recently pangenomes, which refers to a full genomic (genic) makeup of a species, and resequence of other lines of the reference genome were constructed in *B. rapa* and other related species using more than one hundred lines within the species (Bayer et al. 2018; Cheng et al. 2016; Golicz et al. 2016). Genetic diversity has been examined among *B. rapa* accessions, and Chinese cabbage was found to be recently diverged (Cheng et al. 2016). Among Chinese cabbage accessions, the genetic diversity of spring Chinese cabbage accessions was found to be lower than that of autumn or summer Chinese cabbage accessions (Su et al. 2018).

In eukaryotes, the genome is compacted into chromatin, and the chromatin structure plays a key role in expression of genes: gene expression can be controlled by changes in the structure of chromatin without altering the DNA sequence. Changes of gene expression regulated in this manner are termed *epigenetic* control (Fujimoto et al. 2012a). Two well-known epigenetic modifications are DNA methylation and histone modifications. DNA methylation is the addition of a methyl group (CH_3) to a cytosine nucleotide at the fifth carbon position. In plants, such methylation is not only observed at CG sites, but also at CHG and CHH sites (where H is A, C or T). DNA methylation plays a role in transcriptional regulation of genes or transposon silencing. Histone lysine residues are methylated through the addition of methyl groups at the lysine's amine to form mono-, di- or tri-methylated state and each methylation state can be associated with different functions (Fuchs et al. 2006; He et al. 2011). In plants, histone deacetylation, di-methylation of the ninth lysine of H3 (H3K9me2) and H3K27me3 are associated with gene repression, and histone acetylation, H3K4me3 and H3K36me3 are associated with gene activation (Fuchs et al. 2006; He et al. 2011). Epigenetic states such as DNA methylation or histone modification were identified at the whole genome level in Chinese cabbage (Takahashi et al. 2018a, b).

DNA markers have been developed for detecting genetic diversity and making genetic linkage maps. There are various types of DNA markers such as randomly amplified polymorphic DNA (RAPD), cleaved amplified polymorphic sequences (CAPS) / restriction fragment length polymorphism (RFLP), amplified fragment length polymorphisms (AFLP), simple sequence repeats (SSRs), insertion/deletion polymorphism (InDel) and single nucleotide polymorphism (SNP) markers. SSR markers have been widely used, and many SSR markers are available in *B. rapa* (Guo et al. 2014; Hatakeyama et al. 2010; Pino Del Carpio et al. 2011; Ramchiary et al. 2011; Suwabe et al. 2002, 2006). Sequencing technology enables us to identify SNPs easily, and SNPs are widespread in the Chinese cabbage genome (Cheng et al. 2016; Shea et al. 2018b). Restriction-site associated DNA sequencing (RAD-seq) where the flanking region is sequenced from a specific restriction site, is useful for developing DNA markers and high-throughput genotyping (Baird et al. 2008; Kawamura et al. 2016).

2.3.2 Self-Incompatibility

As self-incompatibility is used for harvesting the F_1 hybrid seeds, it is an important trait for Chinese cabbage. Detailed understanding and precise control of self-incompatibility is therefore important for improving schemes used for the generation of hybrids and the production of pure hybrid seeds at a commercial scale. Several review articles are helpful for understanding the self-incompatibility in Brassicaceae (de Nettancourt 2001; Doucet et al. 2016; Suwabe et al. 2010; Watanabe et al. 2012).

2.3.2.1 Regulation of Self and Non-self Recognition

Many plant species are self-incompatible, a mechanism that rejects *self-* or *self-related* pollen to prevent inbreeding depression and for the maintenance of genetic variations in populations. At the time of self-pollination, the stigma recognizes the pollen as *self*, and self-pollen is rejected by the inhibition of pollen germination and/or pollen tube penetration into papillar cells on top of stigma tissue. The self-incompatibility mechanism of the Brassicaceae shares the following common characteristics; the pollen self-incompatibility phenotype is determined sporophytically and self-incompatibility recognition is regulated by a single multi-allelic *S*-locus (Bateman 1955; Hatakeyama et al. 1998; Nou et al. 1991, 1993a, b; Thompson and Taylor 1966). In the *S*-locus, one gene, the *S-locus receptor kinase* (*SRK*), is expressed in the stigma and determines the stigma-side of self-incompatibility specificity (Fig. 2.3) (Stein et al. 1991; Takasaki et al. 2000; Watanabe et al. 1994). *SRK* encodes a transmembrane type receptor serine/threonine protein kinase (Stein et al. 1991). The second gene, the *S-locus protein 11* (*SP11*, also designated *SCR*) is expressed in the anther tapetum tissue and determines pollen-side self-incompatibility specificity (Schopfer et al. 1999; Shiba et al. 2001; Takayama et al. 2000a). *SP11* encodes a small cysteine rich peptide (Suzuki et al. 1999; Watanabe et al. 2000).

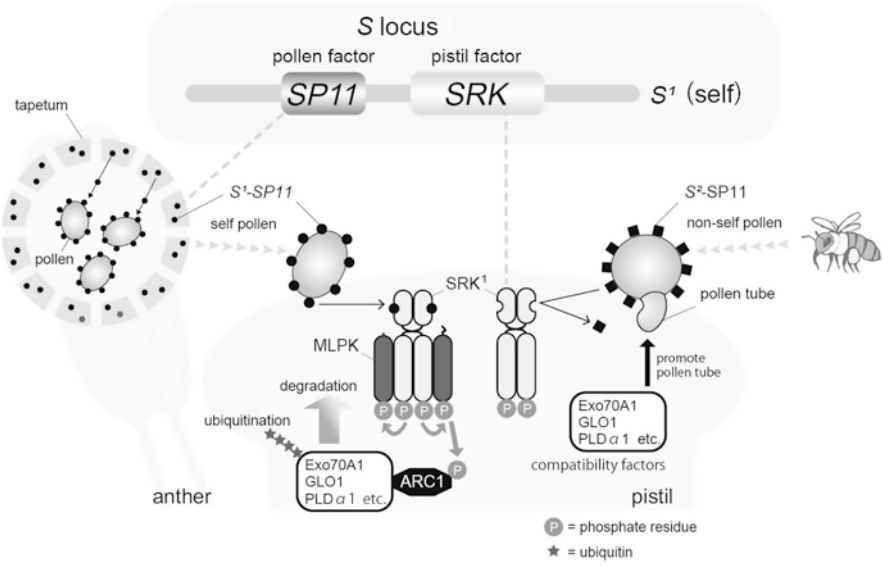


Fig. 2.3 Molecular model of self-incompatibility in *Brassica*. The stigma specific SRK, female determinant and the pollen specific SP11/SCR, male determinant of self-incompatibility in *Brassica* physically interact by *S* haplotype specific manner. During a self-incompatible pollination, auto phosphorylation of SRK itself and a downstream component MLPK are induced. Both MLPK and SRK can activate ARC1. Then, ARC1 promotes ubiquitination of compatibility factors such as Exo70A1, GLO1, PLDα1 and possibly other factors for degradation by proteasome pathway. Degradation of these compatibility factors will result in rejection of self-pollen

These two highly polymorphic and tightly linked genes, which are inherited as one set, thus, this allelic type of *S*-locus is called an *S haplotype* (Nasrallah and Nasrallah 1993). *S*-locus glycoprotein (*SLG*), which closely resembles the extracellular domain of *SRK*, is also linked to the *S*-locus (Stein et al. 1991; Watanabe et al. 1994). The *S* haplotypes are divided into two groups, class-I and class-II, based on the sequence homology of *SLG, SP11* or *SRK* (Fig. 2.4) (Fujimoto and Nishio 2007; Nasrallah et al. 1991). It is known that the sequence diversity within each class is less than the diversity between class-I and -II (Fig. 2.4) (Hatakeyama et al. 1998). The genome structure of the *S*-locus is highly polymorphic; gene placement, distance between *SP11*, *SRK* and *SLG*, and the orientation of these genes are different between *S* haplotypes (Fujimoto et al. 2006a). The *S* haplotype specific physical interaction between SP11 and a receptor domain of SRK is thought to trigger a signal cascade leading to the rejection of self-pollen on the stigma surface (Kachroo et al. 2001; Shimosato et al. 2007; Takayama et al. 2001). Several studies have been conducted about the recognition/interaction state of SP11-SRK complex (Boggs et al. 2009; Chookajorn et al. 2004; Kusaba et al. 1997; Mishima et al. 2003; Sato et al. 2002). Recently, a three-dimensional crystal structure of the extracellular domain of SRK^9 in the complex with S^9-SP11 in *B. rapa* was reported (Ma et al. 2016). Both $eSRK^9$ (extracellular domain of SRK^9) and S^9-SP11 form a stable 2:2 tetrameric complex. Inactivation of self-incompatibility genes described here can make *Brassica* crops become self-compatible. Furthermore, by understanding the epigenetic regulation of the *SP11* gene described below, it is thought that precise control of self-incompatibility can be possible in accordance with various seed

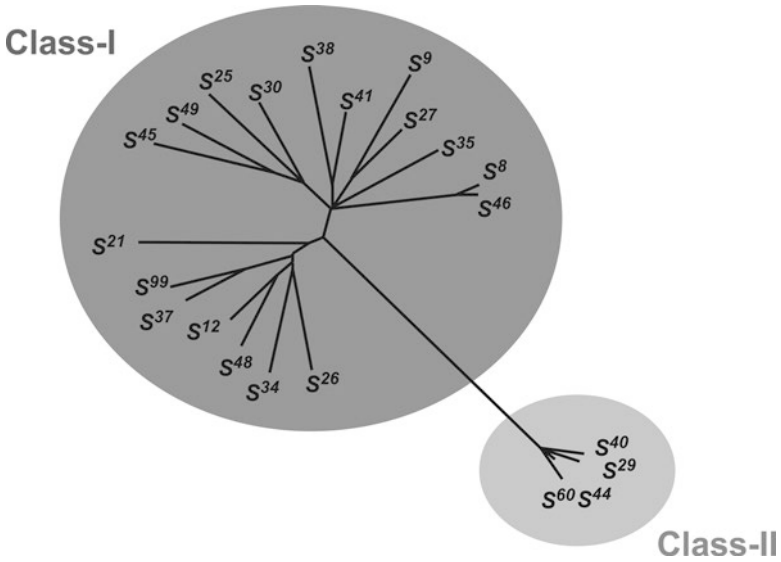


Fig. 2.4 *S* haplotype diversity in *Brassica rapa*. Phylogenetic tree was constructed by using the sequence of *SLG* or *SRK*

producing methods. For such purposes, genome editing technology and transformation techniques may allow artificial control of self-incompatibility in the future.

2.3.2.2 Dominance Relationship of *Brassica*

Since the self-incompatibility of cruciferous plants is regulated in a sporophytic manner, in *S*-locus heterozygotes, transcription and translation products of two alleles are expressed on both the stigma side and the pollen side. On the pollen side, Thompson and Taylor (1966) and Hatakeyama et al. (1998) found that there are two relationships; class-I *S* haplotypes are codominant between the other class-I *S* haplotypes and class-I *S* haplotypes are dominant over class-II *S* haplotypes. Molecular analysis in *Brassica* demonstrated that a plant heterozygous for class-I *S* haplotype and class-II *S* haplotype failed to accumulate the transcripts derived from the class-II *SP11* although transcripts derived from the class-I *SP11* were detected (Shiba et al. 2002). The self-compatible cv. Yellow Sarson has a non-functional class-I *S* haplotype because of being a deletion in the promoter region of *SP11*. In heterozygote derived from crossing cv. Yellow Sarson and a homozygous class-II *S* haplotype line, expression of *SP11* in the allele of class-II *S* haplotype was suppressed (Fujimoto et al. 2006b). This result indicates that a factor other than the expression of the *SP11* governs the suppression of class-II *SP11* expression. Subsequently, Shiba et al. (2006) demonstrated that the suppression of recessive class-II *SP11* expression was induced by *de novo* DNA methylation of the promoter region of class-II *SP11*. Further analysis for this mechanism identified that DNA methylation of the promoter region of the recessive *SP11* was induced by an anther specific *trans*-acting small noncoding RNA (sRNA) (Tarutani et al. 2010). This sRNA is called *SP11 methylation inducer* (*Smi*). The 24 nucleotide (nt)-sequences of *Smi* were similar to target sequences of the recessive *SP11* promoter region (Fig. 2.5) (Tarutani et al. 2010).

However, this sRNA model illustrated only dominance relationship between class-I and class-II, and it cannot explain dominance relationship among class-II *S* haplotypes. Four class-II *S* haplotypes have been reported (S^{29}, S^{40}, S^{44}, S^{60}) in *B. rapa* and dominance relationships of them are linear, $S^{44} > S^{60} > S^{40} > S^{29}$, in which S^{44} is the most dominant and S^{29} is the most recessive in class-II *S* haplotypes (Kakizaki et al. 2003). It was suggested that small noncoding RNA, which is called *SP11 methylation inducer 2* (*Smi2*) and produced from a different position to *Smi*, induces *de novo* methylation and suppression of expression of recessive *SP11* (Fig. 2.5) (Kakizaki et al. 2006; Yasuda et al. 2016).

The dominance relationship of stigma appears to be a posttranscriptional modification of *SRK* and is different from the dominance relationships of the male (Hatakeyama et al. 2001). However, the molecular mechanisms are not understood. Further genetic and biochemical studies are required to understand the dominance relationship of the stigma.

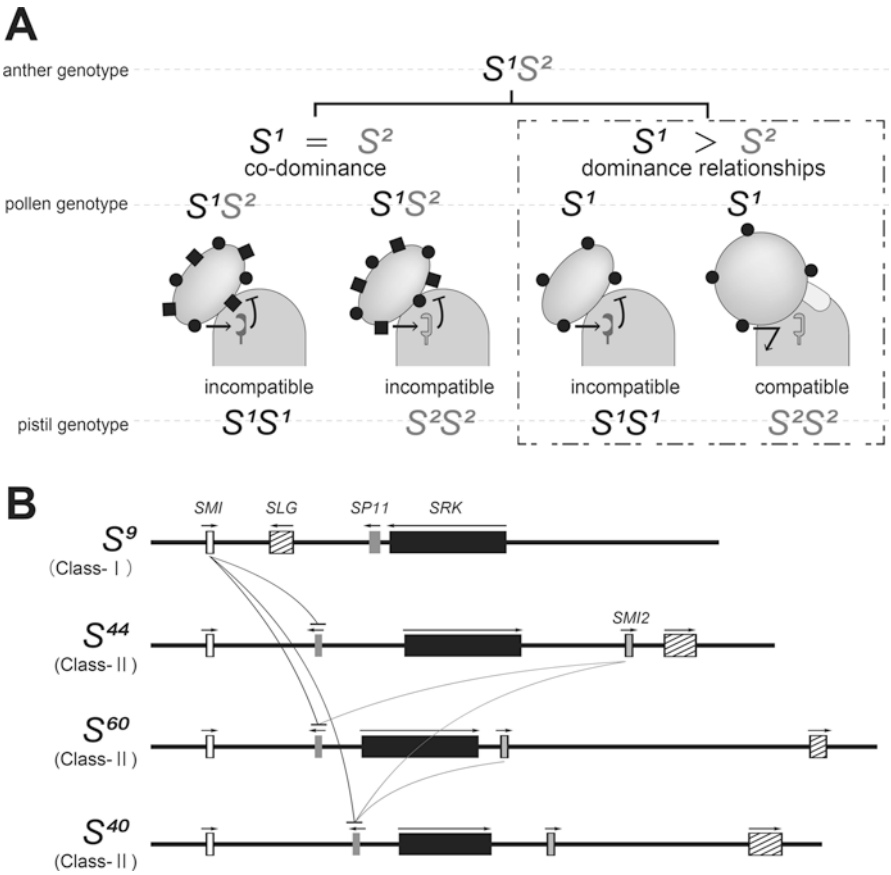


Fig. 2.5 Schematic diagram of dominant-recessive interaction of self-incompatibility in *Brassica rapa*. A: In the S^1/S^2 heterozygous plant, pollen phenotype is determined by the interaction between *S* haplotypes. In case of co-dominant interaction ($S^1 = S^2$), the pollen from S^1/S^2 represent both of S^1 and S^2 phenotype and rejected by the pistil both of S^1 and S^2. When the S^1 is dominant over S^2, pollen exhibit only the S^1 phenotype. B: Class I *S* haplotype, S^9 is dominant over class II *S* haplotypes (S^{44}, S^{60}, S^{40}). The dominant S^9 haplotype suppresses the expression of class II *S* haplotypes by the small RNA produced from *SMI* region. Linear dominant-recessive interaction such as $S^{44} > S^{60} > S^{40}$ in class II *S* haplotype is explained by *polymorphic dominance modifier* model. Allelic small RNA from *SMI2* region and their target sequences control the dominance hierarchy. The small RNA from dominant *S* haplotype induces DNA methylation of *SP11* promoter region of relatively recessive *S* haplotypes and repress *SP11* gene expression

2.3.2.3 Disruption of Self-incompatibility for Pollination Control

Self-incompatibility can be overcome by pollinating immature flower buds. Stigmas are self-compatible early in their development and their ability to reject self-pollen is acquired just prior to flower opening. This feature could be due to insufficient expression of *SRK* in the stigma. Most commercial F_1 hybrid seed production uses

the self-incompatibility mechanism. To develop elite parental lines, harvesting the selfed seeds is essential and bud pollination using immature flower buds is the typical technique used. Because bud pollination is laborious, it is not suited for maintenance of selfed seeds of parental lines. Self-incompatibility can be overcome under some physiological and environmental conditions, such as plant age (Horisaki and Niikura 2008; Ockendon 1978), NaCl treatment (Tantikanjana and Nasrallah 2015), high temperature (Matsubara 1980; Okazaki and Hinata 1987) and CO_2 gas (3–5%) treatment (Nakanishi et al. 1969). The NaCl treatment is simple and adequate for small-scale seed production. But it is not used frequently because it is detrimental to pollinating insects. Localization of SRK at the plasma membrane and in close juxtaposition to the cell wall is critical for SRK function. Hence, breakdown of self-incompatibility by NaCl treatment has been explained by plasmolyzed stigma epidermal cells (Tantikanjana and Nasrallah 2015). CO_2 gas treatment has been used uniformly on a large scale for harvesting the selfed seeds for many years. From physiological analysis of CO_2-induced self-incompatibility breakdown, accumulation of Ca^{2+} at the pollen-stigma interface occurs. Pre-treatment of pollen or pistil with CO_2 gas before pollination did not cause self-incompatibility breakdown. CO_2 gas treatment is just after pollination (Lao et al. 2014). Little is known about the molecular mechanism of self-incompatibility breakdown by CO_2 gas treatment. In radish (*Raphanus sativus* L.), it has been reported that there is genetic variation in the reaction level of self-incompatibility to CO_2 gas treatment. A high degree of self-incompatibility breakdown under the CO_2 gas treatment is controlled by one stigma-side recessive locus, which is different from the *S*-locus (Niikura and Matsuura 2000). In the case of *B. rapa*, several quantitative trait loci (QTL) have been shown to control the CO_2 gas response (Lao et al. 2014).

2.3.2.4 Downstream of SRK and Strength of Self-Incompatibility

Allele specific binding of SP11 to SRK induces autophosphorylation of the SRK kinase domain (SRK-KD). This promotes the association of another membrane-immobilized protein kinase, *M*-locus protein kinase (MLPK). MLPK is a serine threonine kinase with autophosphorylation activity, and the absence of MLPK can result in the disruption of self-incompatibility in *B. rapa* (Fig. 2.3) (Kakita et al. 2007; Murase et al. 2004). The binding partner of SRK-KD, an arm-repeat containing 1 (ARC1) was isolated as an important interactor (Gu et al. 1998). ARC1 acts as a positive regulator for self-incompatibility reaction, because downregulation of *ARC1* results in partial breakdown of self-incompatibility response in *B. napus* (Stone et al. 1999). ARC1 possesses arm-repeat domains that interact with the SRK-KD and has a U-box for an E3 ligase interaction (Stone et al. 2003). And also it has been shown that ARC1 ubiquitinates compatible pollination factors Exo70A1, GLO1 and PLDα1 to reject self-pollen (Fig. 2.3) (Samuel et al. 2009; Sankaranarayanan et al. 2015).

To harvest F_1 hybrid seeds using the self-incompatibility system, strength and stability of the self-incompatibility reaction is one of the critical breeding traits in

parental lines because weak self-incompatibility leads to low quality of F_1 hybrid seeds. Several QTL analyses on the strength and stability of self-incompatibility have been reported in *B. rapa* (Hatakeyama et al. 2010; Isokawa et al. 2010). However, causative genes have not been identified and developing DNA markers for selection of strong self-incompatible lines are still in the future. Given future global climate change, stable and strong self-incompatibility is required for F_1 hybrid breeding, and for the development of DNA markers related to the strong self-incompatible is desired.

2.3.2.5 Other Genes Affecting Pollen-Stigma Interaction

Other factors affect pollination and pollen tube penetration to the stigma. One of the *SLG*-like genes, *S locus-related glycoprotein 1* (*SLR1*), which is expressed specifically in the stigma, is involved in the adhesion of pollen grains to the stigmatic surface. Its interaction partner SLR1-binding protein (SLR1-BP) has been also identified (Takayama et al. 2000b).

Recently, it has been found that the specific interaction of duplicated *S*-locus genes, *stigmatic unilateral incompatibility 1* (*SUI1*) for stigma side and *pollen unilateral incompatibility 1* (*PUI1*) for pollen side induces pollen-stigma recognition and pollen rejection (Takada et al. 2017). *SUI1* and *PUI1* are located on chromosome A04 and tightly linked to each other in *B. rapa*. This specific interaction occurs between some Japanese lines as stigma parent and Turkish line as pollen parent, but the reciprocal combination, Japanese line as pollen parent and Turkish line as stigma parent is compatible (Takada et al. 2005, 2013). This one-way incompatibility (unilateral incompatibility, UI) reaction is carried out by the MLPK-dependent pollen rejection pathway as in self-incompatibility (Takada et al. 2013). A large number of SRK-like proteins and SP11-like small peptides in the genome may cause a specific incompatibility phenomenon such as unilateral incompatibility (Takada et al. 2017). It would be necessary to pay attention to these genes, which can affect the pollen-stigma interaction with incompatibility, when selecting the parental lines for F_1 hybrid cultivars.

2.3.2.6 Determination and Discrimination of *S* Haplotypes

In *B. rapa*, it has been estimated that there are about over 100 *S* haplotypes (Nou et al. 1993b). Identification of *S* haplotypes is important for the selection of parents when self-incompatibility is used to cultivate F_1 hybrid seeds. As the method of test cross is time-consuming, DNA marker-based selection is desirable. There are several methods for determining and discriminating between different *S* haplotypes. The simplest one is using sequence diversity of *SLG*, *SRK* and *SP11*. The PCR-RFLP method is well known and easy to use for both breeders and researchers (Nishio et al. 1994, 1996; Park et al. 2001, 2002). Using this method, *S* haplotypes in many cultivars in Chinese cabbage or other *B. rapa* vegetables have been

identified (Sakamoto and Nishio 2001). By using class-I *SLG* specific primer pair and class-II *SLG* specific primer pairs, it is possible to amplify most *S* haplotypes of Chinese cabbage or other *B. rapa* vegetables (Kawamura et al. 2015). Another method for *S* haplotype discrimination, the dot-blot method has been developed using the sequence of *SP11*, highly variable between *S* haplotypes (Fujimoto and Nishio 2003). In this method, the *S* haplotype of many samples can be determined easily without electrophoresis.

The purity of seeds is an important aspect of F_1 hybrid seed production. Confirmation of seed purity can be conducted by a field grow-out trial, but it is time consuming and laborious. Therefore, a DNA-marker based testing can avoid the need for costly and arduous field trials. DNA markers designed to identify *S* haplotype may be used to confirm the purity test (Fujimoto and Nishio 2007). SSR markers are able to distinguish the parental alleles of F_1 hybrid cultivars, and such markers may also be used to access seed purity of an F_1 hybrid. Additionally, SSR markers can discriminate multiple markers thereby increasing the accuracy of seed purity testing. Highly polymorphic SSR markers have been identified in *B. rapa* (Kawamura et al. 2015, 2016).

2.3.3 Heterosis and Hybrid Vigor

Breeding of F_1 hybrid cultivars of Chinese cabbage is based on heterosis, which produces high yields. Several genetic approaches such as QTL or genome-wide association study (GWAS) have been performed in many plant species (Fujimoto et al. 2018), but the molecular mechanism of heterosis is still unknown.

For the crossability test for candidates of parental lines, all possible combinations of the inbred lines are used to identify suitable parents for F_1 hybrid generation. This is expensive, time consuming and labor intensive. Thus, an efficient method for predicting hybrid performance in the parental generations is desired. One candidate method is the genetic distance between parental lines because it is believed that there is a positive correlation between genetic distance and heterosis; crosses between more genetically divergent parental lines lead to greater heterosis in maize (Moll et al. 1965). However, a positive correlation is not always observed between genetic distance and heterosis in plants (Barth et al. 2003; Girke et al. 2012; Yang et al. 2017). Using 32 F_1 hybrids of Chinese cabbage, genetic distance between parental lines and heterosis levels at three developmental stages, cotyledon area at 6 days after sowing (DAS), leaf length x width of largest leaf at 21 DAS and harvested biomass, were examined (Kawamura et al. 2016). Heterosis is quantified via the use of two indices. The first index is the mid-parent heterosis (MPH), which measures the performance of a hybrid against the mean value of its parental lines. The second is the best-parent heterosis (BPH), which measures the performance of hybrids in comparison to the parent having the best value for the trait (Springer and Stupar 2007). There was no correlation between genetic distance and MPH or BPH of the parameter examined (Kawamura et al. 2016), indicating that the hybrid

performance in Chinese cabbage cannot be predicted from the genetic distance between parental lines.

There are few reports showing heterosis levels in Chinese cabbage. In the commercial cultivar of Chinese cabbage, W39, a heterosis phenotype is seen at 4 DAS with hybrids having increased cotyledon size, and leaf size in first and second leaves in F_1 hybrids were larger than that in the best-parent. Growth speed evaluated by counting leaf number in F_1 hybrids was not faster than parental lines (Saeki et al. 2016). This early developmental heterosis is similar to F_1 hybrids in *A. thaliana* (Fujimoto et al. 2012b). Yield also shows heterosis (25% greater than the better-parent) (Fig. 2.2). Focusing on the early developmental heterosis phenotypes, transcriptome and hormone profiling have been performed using the F_1 hybrid cultivar W39 and its parental lines. Plant hormone profile of 43 derivatives in 2-day cotyledons and 10-day first and second leaves were similar in parental lines and the F_1 hybrid of Chinese cabbage (Saeki et al. 2016). Transcriptome analysis by RNA-sequencing using 2-day cotyledons showed genes categorized into *photosynthesis* and *chloroplast part* tended to be upregulated, but this upregulation is stochastic (Saeki et al. 2016).

Resequencing the genome of parental lines of the F_1 hybrid cultivar of Chinese cabbage, W77, was performed and SNPs were identified (Shea et al. 2018b). Not only moderate-impact SNPs, nonsynonymous mutations without changing the framework of amino acid sequence but also high impact variants causing frameshifts, nonsense mutations, or other mutations that could possibly result in the loss of gene function were identified in parental lines (Shea et al. 2018b). Heterosis research in Chinese cabbage has just started, and it will take more time to understand the molecular mechanisms and apply MAS for choosing the best parental combination.

2.3.4 High Bolting Resistance

A major goal of plant breeding to produce novel varieties is by shifting the seasonal timing of reproduction better adapted to local environments and changing climatic conditions. In higher plants, the flowering transition represents a crucial developmental trait from the vegetative to reproductive stage in the life cycle. In the breeding of Chinese cabbage, late-flowering or late-bolting is especially important because premature bolting triggered by low temperature leads to decreased yield and quality of harvested products. Chinese cabbage is generally cultivated in autumn and spring, the autumn from August to November in the Northern Hemisphere when the temperature declines, but spring cultivation has developed in response to the demand for year-round supply (Akter et al. 2018). However, in the early spring there is a risk that low temperature (0–13 °C) acting on the germinating seeds or seedlings induces flower bud differentiation and can cause early bolting before reaching the harvesting stage. Therefore, plant breeders now focus on developing bolting-resistant Chinese cabbage cultivars for the spring. To ensure bolting occurs

in favorable conditions, many plants bolt only after a period of vernalization, which is defined as *the acquisition or acceleration of the ability to flower by a chilling treatment*. The regulation of flowering time and its associated network has been extensively studied in *A. thaliana* (Blümel et al. 2015; Fornara et al. 2010; Whittaker and Dean 2017).

In *A. thaliana, FRIGIDA* (*FRI*) activates *FLOWERING LOCUS C* (*FLC*) expression, which acts as a floral repressor by inhibiting the activation of a set of genes required for transition of the apical meristem to a reproductive state. Prolonged exposure to cold decreases the *FLC* expression. During cold, the lowered *FLC* expression is mediated or maintained by *VERNALISATION INTENSIVE3* (*VIN3*) a plant homeodomain (PHD) finger protein and *VERNALISATION* (*VRN*) genes. VIN3, VRN5, VIN3/VRN5-like 1 (VEL1) interact with VRN2 protein and form plant homeodomain-Polycomb Repressive Complex 2 (PHD-PRC2) (Berry and Dean 2015; Whittaker and Dean 2017). *FLC* repression is associated with the enrichment of H3K27me3 at the locus catalyzed by the PHD-PRC2. During cold, H3K27me3 accumulation occurs at the transcription start site of *FLC* and spreads across the whole *FLC* gene when plants are returned to a warm temperature. The spreading of H3K27me3 maintains the stable repression of *FLC* expression. Moreover, LIKE HETEROCHROMATIN PROTEIN 1 (LHP1), associated with H3K27me3, and VRN1 are also required for the maintenance of stable *FLC* repression (Berry and Dean 2015; Whittaker and Dean 2017). The first intron, promoter region, and exon 1 are important for the regulation of *FLC* expression by prolonged cold treatments (Sheldon et al. 2002). In addition, cold induced long noncoding RNAs (lncRNAs) such as COOLAIR, COLDAIR and COLDWRAP are also involved in vernalization (Heo and Sung 2011; Kim and Sung 2017; Swiezewski et al. 2009). COLDAIR and COLDWRAP play a role in the recruitment of the PRC2 complex to *FLC* following cold exposure.

There are four *FLC* paralogs in Chinese cabbage (*BrFLC1, BrFLC2, BrFLC3, BrFLC5*) (Schranz et al. 2002) and three have been confirmed to be floral repressors (Kim et al. 2007). A genome variation map of 194 accessions of Chinese cabbage described that incorporation of elite alleles of *BrVIN3.1* and *BrFLC1* is a source of variation during selection and the quantitative response of *BrVIN3.1* to cold due to variations in the *cis* elements of the promoters, which significantly contributes to bolting-time variation in Chinese cabbage (Su et al. 2018). QTL analysis showed co-localization of flowering time QTL and the *BrFLC* gene (Itabashi et al. 2018; Lou et al. 2007; Zhao et al. 2010). For example, an F_2 population was derived from the cross of an early bolting commercial F_1, Early, and an extremely late bolting line (Tsukena No. 2) where QTLs for bolting time with vernalization co-localized with the late bolting alleles of *BrFLC2* and *BrFLC3*. Tsukena No. 2 has large insertions in the first intron of *BrFLC2* and *BrFLC3* with weak repression by vernalization, suggesting that this insertion may cause the late-bolting phenotype (Kitamoto et al. 2014). These two alleles of Tsukena No. 2 are transferred into Chinese cabbage by backcrossing with MAS, and the new late-bolting F_1 hybrid of Chinese cabbage was developed (Kitamoto et al. 2017). Although a long insertion in the first intron causes weak repression of *BrFLC2* and *BrFLC3* transcripts by vernalization, sequence

similarity to the vernalization response element (VRE) in the first intron or to the COLDAIR of *A. thaliana* are not detected of any of the *B. rapa* paralogs (Kitamoto et al. 2014). In addition, COLDAIR-like transcripts were not detected in two Chinese cabbage lines following vernalization (Li et al. 2016). Now, the regulatory element response to cold exposure in *FLC* paralogs has not been identified in Chinese cabbage, thus identification of the sequences important for vernalization will be important for using MAS breeding for bolting resistance Chinese cabbage cultivar.

2.3.5 Clubroot Disease Resistance

Clubroot disease caused by the soil-borne obligate parasite *P. brassicae* is one of the major diseases of Chinese cabbage. The disease causes loss of fine roots and the development of clubs (galls) in the infected roots leading to a swollen and distorted root system, resulting in purplish discoloration in foliage, stunted growth, wilting and in severe infection, death of the plant (Hwang et al. 2012). One of the earliest European accounts of the clubroot disease comes from fourth century in Italy (Crisp et al. 1989). The next case was described in the fifteenth-sixteenth centuries in Spain, followed by England in the eighteenth century. By the nineteenth century, clubroot disease had spread across most of the European countries and as far as the United States and Japan (Yoshikawa and Buczacki 1978). In Asia, the clubroot disease was first reported in 1892 on turnip and cabbage, and in 1978, on Chinese cabbage in Japan (Yoshikawa 1983) followed by Korea and China (Cho et al. 2003b). Recently, clubroot disease has become a severe, year-round threat to Chinese cabbage production in East Asia. Clubroot disease is estimated to be present in approximately 10% of all areas where host plants are cultivated (approximately 6 million ha of *Brassica* crops) (Dixon 2009; Wallenhammar 1998). In China, there is 20–30% loss of Chinese cabbage yield. Cultural and chemical control of clubroot disease may reduce the severity, but the longevity of the resting spores of this pathogen in soil makes the practice ineffective (Rahman et al. 2011). Development of resistant varieties is considered the most effective way to control clubroot disease and has been a major research objective for breeding of Chinese cabbage cultivars in the last few decades.

2.3.5.1 *P. brassicae* and Its Life Cycle

P. brassicae is an obligate biotrophic protist in the Plasmodiophorids within the eukaryote supergroup Rhizaria, which is distinct from other plant pathogens such as fungi or oomycetes (Burki et al. 2010). There are two main characteristics shared by all Plasmodiophorids, one is cruciform nuclear division and the others are biflagellate zoospores, multinucleate protoplasts (plasmodia) and long-lasting resting spores (Braselton et al. 1975).

Plasmodiophorids have a complex life cycle consisting of different zoosporic stages that include the formation of plasmodia inside host cells, and formation of resting spore (Fig. 2.6). Its life cycle can be divided into three main stages: (i) primary infection of root-hair and development of secondary zoospores that are released into the soil; (ii) secondary infection of host cortex by secondary zoospores leading to gall formation and (iii) maturation of resting spores, which is released into the soil upon the death of the plant and disintegration of root tissues (Ingram and Tommerup 1972; Kageyama and Asano 2009; Olszak et al. 2019).

This pathogen forms primary plasmodia in root hairs during the primary infection stage. Then a number of synchronous nuclear divisions occur in the plasmodia, which lead to the formation of zoosporangia. The zoosporangia form clusters in the root hair and sometimes in epidermal cells. This is followed by formation of secondary zoospores (4–16 in number) in each zoosporangium. After releasing these zoospores, the empty zoosporangia remain in the root hairs. The secondary zoospores cannot be visually differentiated from the primary zoospores (Fig. 2.6). Zoospores formed by the fusion of two distinct zoospores, as opposed to the division of nuclei, are known as binucleate zoospores and occur occasionally (Ingram and Tommerup 1972; Tommerup and Ingram, 1971). During the secondary infection stage, infected cells act as a proliferation site as the pathogen develops into

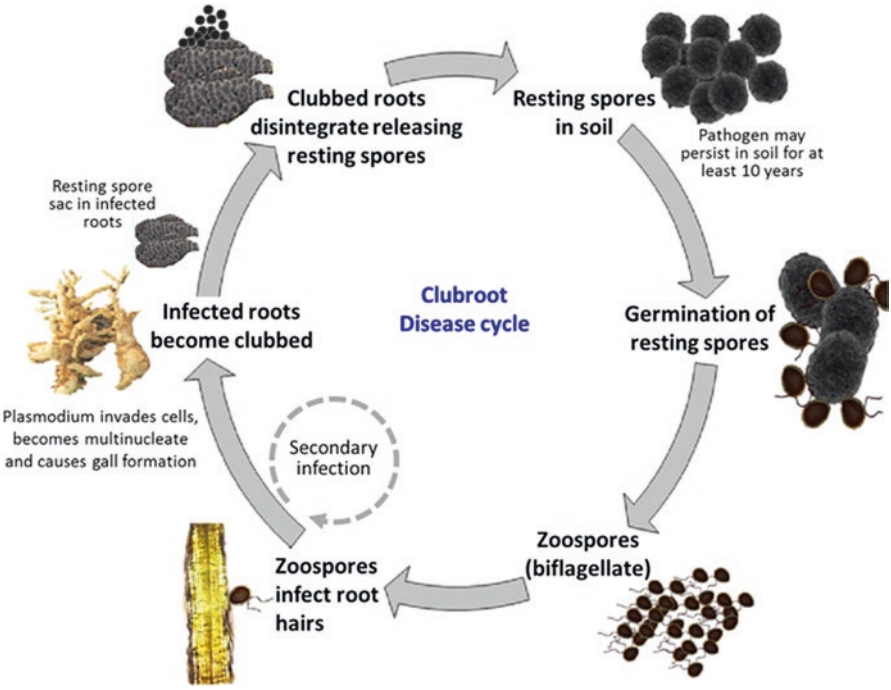


Fig. 2.6 The life cycle of *Plasmodiophora brassicae* leading to the formation of clubs/galls in the infected root. (Modified from Hirani and Li 2015)

secondary plasmodia. This secondary plasmodia then propagate, leading to cellular hypertrophy and formation of galls in the root tissues. The secondary plasmodia then further develops into multinuclear plasmodia over the course of several nuclear divisions (Garber and Aist 1979) before finally developing into resting spores. These resting spores are released into the surrounding soil (Fig. 2.6). The genetic diversity of pathogen is increased by the complex cleavages that produce the resting spores (Kageyama and Asano 2009).

2.3.5.2 Population Diversity and Pathotype Determination of *P. brassicae*

Populations of *P. brassicae* vary in terms of pathogenicity and virulence in different *Brassica* crops, and this often leads to breakdown of clubroot resistance in cultivars (Hatakeyama et al. 2004; Holtz et al. 2018; Tanaka et al. 1998). Understanding the pathogenic diversity and their crop-wise virulence are necessary for success in breeding clubroot resistance cultivars. Various host systems have been proposed to identify pathotypes (or races) of the clubroot pathogen including the differential cultivar sets of Williams (Williams 1966), Some (Some et al. 1996) and the European clubroot differential (ECD) Set (Buczacki et al. 1975). All three systems have been used to characterize *P. brassicae* populations, and the pathotype designation of Williams is frequently employed because of its comparative ease in practical use. The classification system of Williams is used to identify 6 pathotypes of *P. brassicae* including pathotypes 1, 2, 3, 5, 6 and 8, and the genetic diversity in pathogenicity of *P. brassicae* is determined using Japanese clubroot-resistant (CR) lines of *B. rapa* (Cho et al. 2003b; Kuginuki et al. 1999; Strelkov and Hwang 2014; Strelkov et al. 2006).

Molecular detection of the pathogen is difficult as the pathogen cannot be cultured in the lab (Siemens et al. 2009). However, release of the *P. brassicae* genome in recent years enables a genome-wide comparison of diverse species (Rolfe et al. 2016; Schwelm et al. 2015). Based on sequence polymorphism in nuclear ribosomal DNA sequences of *P. brassicae*, Laila et al. (2017) developed DNA markers that can distinguish the nine different Korean geographical isolates into two distinct groups, and genetic variants that are unique in the virulent populations and virulent isolates were identified (Holtz et al. 2018).

2.3.5.3 Sources and QTLs for Clubroot Resistance

Sources of resistance to clubroot disease have been mainly found in *B. rapa* and *B. nigra* (Buczacki et al. 1975; Hasan et al. 2012). Mapping based approaches identified at least 20 QTLs in *B. rapa* (Fig. 2.7) (Diederichsen et al. 2009; Hatakeyama et al. 2013; Hirani et al. 2018; Nguyen et al. 2018; Piao et al. 2009). European turnips carrying strong resistance to clubroot are major sources of currently known *CR* genes (Matsumoto et al. 1998). The resistance to clubroot disease in Asian Chinese cabbage lines has mainly been introduced from different European turnip

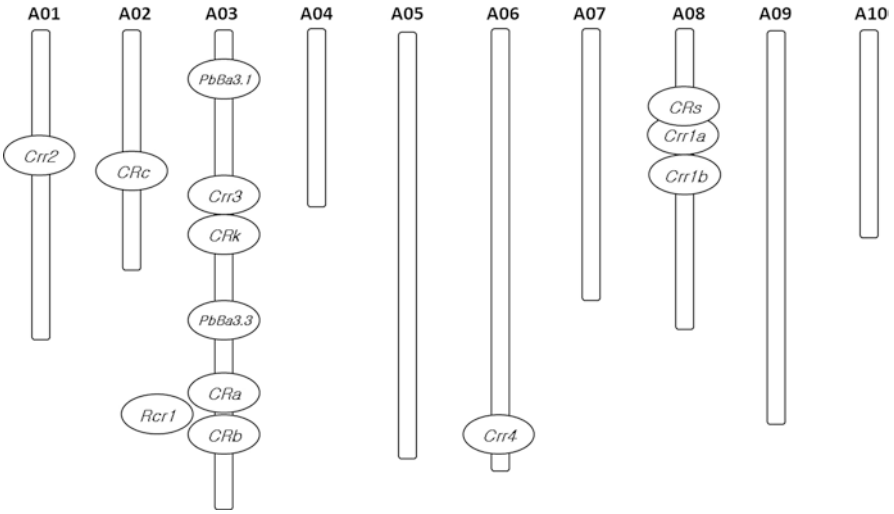


Fig. 2.7 Chromosomal locations of the clubroot resistant QTLs identified so far in *Brassica rapa*

accessions such as Siloga, Gelria R, Milan White, and Debra (Hirani et al. 2018; Matsumoto et al. 2005). Three *CR* genes (*Crr1*, *Crr2*, *Crr4*) were introduced from Siloga (Kuginuki et al. 1997; Suwabe et al. 2003), two genes (*CRk*, *CRc*) were introduced from Debra (Matsumoto et al. 2012; Sakamoto et al. 2008) and *Crr3* was introduced from Milan White (Hirai et al. 2004; Suwabe et al. 2006). *CRb* (Piao et al. 2004) and *CRa* (Matsumoto et al. 1998) were introduced from Gelria R and ECD02 (136–8), respectively. The gene *Rcr1* was identified in pak choi cv. Flower Nabana, which showed resistance against five pathotypes of *P. brassicae* in Canada (Chu et al. 2014; Yu et al. 2016).

Advances in sequence-based technology led to the development of genome-wide markers, which enabled identification of some novel *CR* genes. For example, genome-wide SNP markers derived from bulked segregant RNA sequencing (BSR-Seq) were used to identify *Rcr2* in Chinese cabbage cv. Jazz (Huang et al. 2017). SNPs identified by genotype by sequencing (GBS) techniques were used to detect three QTLs (*Rcr4, Rcr8, Rcr9*) that conferred resistance against six pathotypes in the German turnip cv. Pluto (Yu et al. 2017). Double digest restriction site-associated DNA sequencing (ddRAD-seq) analysis was used to detect a novel locus *CR*s on chromosome A08 against a Korean *P. brassicae* isolate, Seosan (Laila et al. 2019). *Crr3*, *CRa*, *CRb*, *CRc* and *CRk* are dominant genes, while *Crr1* and *Crr2* are recessive genes. Upon transmission of the cabbage *CR* genes, *Crr3*, *CRa*, *CRb*, *CRc* and *CRk* move as single genes, while *Crr1*, *Crr2*, *Crr4* and *CRb* (*CRa*) move as QTL (Jang et al. 2019).

2.3.5.4 Prospects of Marker-Assisted Breeding in Developing Clubroot Resistant Chinese Cabbage

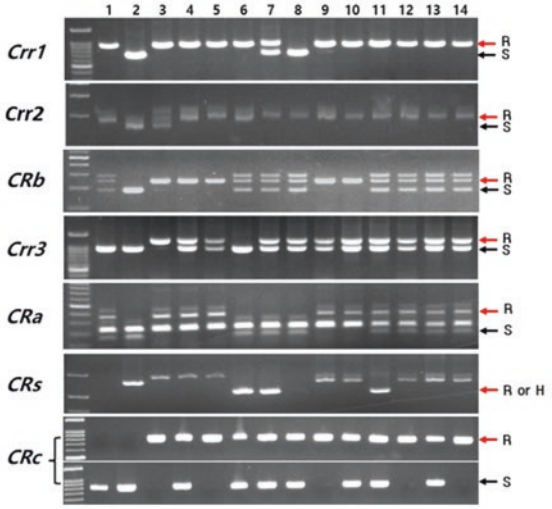
Chinese cabbage production in Korea is greatly affected by clubroot disease. Breeding for clubroot resistance by seed companies has mainly focused on Chinese cabbage due to its economic importance. A number of commercial clubroot resistant Chinese cabbage cultivars were developed using the resistance genes from European turnip cvs. such as Siloga, Gelria R, Milan White, and Debra (Jang et al. 2019). However, there are several reports of breakdown of resistance, especially upon successive cultivation for several consecutive seasons (Kuginuki et al. 1999; Tanaka et al. 1998).

Long survivability of the resting spores in soil (10–20 years) and diversity in population makes it difficult to control *P. brassicae* by cultural practices or chemical treatments (Voorrips 1995). Therefore, the long-term management of clubroot disease is most effectively carried out through rotation of resistant cultivars (Niemann et al. 2017). This necessitates the development of breeding materials containing multiple *R* genes to ensure stable resistance against a wide spectrum of pathogenic isolates. The QTLs and the closely linked markers identified can be exploited in marker-assisted breeding.

Fine mapping and molecular characterization of the clubroot resistance loci identified several genes having roles in clubroot resistance. *Crr1* and *CRa* encoding Toll-Interleukin-1 receptor/nucleotide binding site/leucine-rich repeat (TIR-NBS-LRR, TNL) proteins have been isolated from Chinese cabbage (Hatakeyama et al. 2013; Ueno et al. 2012). *CRb* was isolated from the Chinese cabbage cv. CR Shinki. It has six open reading frames similar to an NBS-LRR encoding gene (Hatakeyama et al. 2017). Jang et al. (2019) developed a CAPS marker based on a SNP at Bra016021 encoding a GMC oxidoreductase family protein detected via GWAS and this marker successfully differentiated resistant and susceptible genotypes against Korean *P. brassicae* strain Yeoncheon. These functional markers can be useful for screening global germplasms and breeding materials (Kawamura et al. 2015), and developing clubroot resistant cultivars. Indeed, through MAS, Matsumoto et al. (2012) successfully pyramided three *CR* genes, (*CRa, CRk*, *CRc*) in four homozygous inbred lines and in one F_1 hybrids, and they exhibited high resistance against six field isolates *P. brassicae* in Japan. Several markers have recently been used to screen promising Korean Chinese cabbage lines that successfully detected clubroot resistant lines (Fig. 2.8) (Park et al. unpublished data).

2.3.6 Fusarium Wilt Disease Resistance

Fusarium oxysporum causes Fusarium wilt disease and is a soil-borne fungus. The fungus usually invades the host plants through young roots and wounds in older roots (Walker 1930). The fungus passes through water-conducting xylem tissue and spreads to root, stem and leaves. *Brassica* vegetables are infected by two forma



Molecular Marker	Chinese cabbage lines													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Crr1	R	S	R	R	R	R	H	S	R	R	R	R	R	R
Crr2	R	S	H	R	R	R	R	R	R	R	R	R	R	R
CRb	H	S	R	R	R	H	H	H	R	R	H	H	H	H
Crr3	S	S	R	H	H	S	H	H	H	H	H	H	H	H
Cra	H	S	R	R	R	S	S	S	R	R	H	H	H	H
CRs	S	R/H	S	S	S	R/H	R/H	S	S	S	R/H	S	S	S
CRc	S	S	R	H	R	H	H	H	R	H	H	R	H	R

Fig. 2.8 Marker-assisted screening of a few Korean promising Chinese cabbage lines for clubroot resistance using molecular markers in PCR based assays. *R* Resistant, *S* Susceptible, *H* Heterozygous

specialis of *F. oxysporum*. The first one is *F. oxysporum* f. sp. *conglutinans* (*Foc*) that can infect *B. rapa* and *B. oleracea*, and is more virulent on *B. oleracea* than on *B. rapa*. The second is *F. oxysporum* f. sp. *rapae* (*For*) that infects only *B. rapa* (Enya et al. 2008). When Chinese cabbage is inoculated by *F. oxysporum* f. sp. *conglutinans* or *rapae*, in the beginning of the infection process, the parenchyma tissue between the veins of their leaves changes the color from green to yellow, and the yellowing spots will spread and cover the whole leaf (Walker 1930). At the same time or after losing the green color from the infected leaves, the vascular elements in the infected tissue become brown, and finally, the plants die. Because the infected plants show these severe symptoms, the yield is decreased. *F. oxysporum* f. sp. *conglutinans* favors hot temperatures and heavy rainfall. This fungus can live without host plants for more than 10 years, and the traditional methods for fungus control such as seed treatment, crop rotation and fungicides are not effective in preventing the fungus from spreading. When the fungus is found in the field, there is no way to maintain crop yield except by using Fusarium wilt resistant cultivars.

There are two races in *F. oxysporum* f. sp. *conglutinans* reported as type A and B. Type A resistance is stable under high or low temperature, and shows a single dominant inheritance pattern reported in *B. oleracea* (Farnham et al. 2001; Pu et al. 2012). Type B resistance is not stable under high temperature (above 24 °C), and shows a polygenic inheritance pattern reported in *B. oleracea* (Farnham et al. 2001). Microscopic analysis of *B. oleracea* showed that fungus development was faster in the susceptible cultivars compared with the resistant cultivars and that the resistant cultivars limited the fungus development and spreading (Li et al. 2015; Pu et al. 2016).

Resistance genes to Fusarium wilt have been isolated from *B. rapa* and *B. oleracea*. In *B. rapa*, a single dominant resistance gene to *F. oxysporum* f. sp. *conglutinans* was identified using an inoculation testing an F_2 population of Chinese cabbage (Shimizu et al. 2014). Transcriptome analysis using Fusarium wilt resistant and susceptible inbred lines of Chinese cabbage identified adjacent genes, Bra012688 and Bra012689 (*FocBr1a*, *FocBr1b*, respectively), as the resistance genes to *F. oxysporum* f. sp. *conglutinans* (Shimizu et al. 2014). These genes are typical type resistance genes with TIR, NBS, and LRR domains. In the susceptible lines, these two genes have been deleted. In *B. oleracea*, *FocBo1* or *FOC1* was isolated as the single dominant resistance gene to *F. oxysporum* f. sp. *conglutinans* type A (Lv et al. 2014; Shimizu et al. 2015). FocBo1/FOC1 has TIR, NBS, and LRR domains, and is an ortholog of Bra012688. *B. oleracea* does not have the functional gene of Bra012689 ortholog, suggesting that Bra012688 is the Fusarium wilt resistance gene in *B. rapa*. Two dominant DNA markers from Bra012688 and Bra012689 were developed for MAS in *B. rapa*. Kawamura et al. (2015) assessed these two markers in 20 inbred lines of Chinese cabbage.

2.4 Genetic Engineering

No commercial transgenic Chinese cabbage cultivar has yet been released, although a number of other commercial transgenic vegetable crop cultivars are available. *Agrobacterium tumefaciens*-mediated transformation is commonly used in Chinese cabbage. However, success in making transgenic plants is line dependent (Kuginuki and Tsukazaki 2001). There are some reports of *Agrobacterium*-mediated transgenic Chinese cabbage (Baskar et al. 2016; Cho et al. 2003a; Min et al. 2007; Zhang et al. 1998, 2012). Transformation of *N-acyl-homoserine lactonase* (*AHL-laconase*) into the Chinese cabbage inbred line Kenshin enhances tolerance to soft rot disease (Vanjildorj et al. 2009). Transformation of microRNA, MIR319a2, into Chinese cabbage cv. Bre reduces the expression levels of the target gene, *PCF transcription factor 4–1* (*BrpTCP4–1*), and head shape changed from round to cylindrical, suggesting manipulation of this gene could improve the head shape in Chinese cabbage (Mao et al. 2014). Overexpression of MIR156a in Chinese cabbage cv. Bre delays the time of leaf folding, and overexpression of *SQUAMOSA PROMOTER BINDING-LIKE 9–2* (*BrpSPL9–2*), which is a target of miR156a1, in Chinese cabbage results

in early heading, suggesting that miR156 and *BrpSPL9* genes are potentially important for genetic improvement of earliness of Chinese cabbage (Wang et al. 2014). Transformation of *A. thaliana HEAT-INDUCED TAS1 TARGET2* (*HTT2*) showed thermotolerance in Chinese cabbage (Jiang et al. 2018). Transformation of protease inhibitor encoding gene *sporamin* into Chinese cabbage cvs. Youdonger and Shanghaiqing showed more resistance to insects like diamondback moth (*Plutella xylostella* L.) (Cui et al. 2017; Qiu et al. 2013).

Konagaya et al. (2013) studied the effects of selectable markers in Chinese cabbage cv. Chihiri 70. An *acetolactate synthase* (*ALS*) gene from Chinese cabbage was cloned and mutated to herbicide resistant and used as a selective marker. Transformation using this selective marker gave a similar transformation frequency to antibiotic selective markers. Transgene inheritance and herbicide resistance in the first generation of transgenic plants were confirmed (Konagaya et al. 2013).

Genome editing introduces targeted mutations into a genome with immense precision. Clustered regulatory interspaced short palindromic repeats (CRISPR)/CRISPR associated protein9 (Cas9) is a revolutionary genome editing tool commonly used in crops (Bao et al. 2019). There are some reports of gene editing by the CRISPR/Cas9 system in Chinese kale or cabbage (*B. oleracea*) (Lawrenson et al. 2015; Ma et al. 2019; Sun et al. 2018a) or *B. napus* (Okuzaki et al. 2018). Genome editing will be a powerful tool for identification of gene function in Chinese cabbage and may be used in the development of new cultivars of the crop.

2.5 Mutation Breeding

Ethyl methanesulfonate (EMS) is widely used for mutagenesis in crops as it provides a high mutation rate. Using this chemical mutagen, only mutations of a dominant phenotype can be identified in the M_1 generation. To identify the recessive phenotype, the M_2 generation is used for selection. Thousands of seeds are used for EMS treatment (making the M_1) and the selfed seeds (M_2) from each M_1 line or bulked M_1 population are harvested. As Chinese cabbage is self-incompatible, it is laborious to harvest seed and maintain lines, indicating that mutation breeding is not suitable for this crop. Indeed, there are few reports of developing new cultivars by mutation breeding. However, a few self-compatible Chinese cabbage lines have been identified (Fujimoto et al. 2006c), and these lines could be better for making the mutagenized population. Using the self-compatible line of *B. rapa* (R-O-18), EMS-induced mutagenesis populations have been generated (Stephenson et al. 2010). In a modified method, EMS was used to treat flower buds, and doubled haploid lines generated by microspore culture. This method can produce homozygous mutants quickly, and 142 mutants with changed leaf shape, leaf color, corolla size, flower color, bolting time or downy mildew resistance have been identified (Lu et al. 2016). Even if a mutagenized population is generated effectively, it is another issue whether the phenotype is appropriate for the cultivars seen. As Chinese cabbage has paralogous genes caused by a whole genome triplication, the chance of phenotypic

change by mutation is lessened because of functional complementation between paralogous genes.

2.6 Hybridization

The genus *Brassica* includes allotetraploid species having two different genomes, i.e., *B. juncea* (AABB, 2n = 36), *B. napus* (AACC, 2n = 38) and *B. carinata* (BBCC, 2n = 34). These allotetraploid species could be generated by interspecific hybridization between three basal species, *B. rapa* (AA, 2n = 20), *B. nigra* (BB, 2n = 16) and *B. oleracea* (CC, 2n = 18) (U 1935).

Interspecific crossing may result in traits that could not be obtained by intraspecific hybridization; hybridization breeding by interspecific crossing introduces agriculturally-valuable traits into existing cultivars such as biotic or abiotic stress tolerance. However, there are reproductive barriers such as pre- and post-zygotic isolation in interspecific crossing (Osabe et al. 2012; Tonosaki et al. 2016). There is different cross ability in interspecific crossing, i.e., crosses between *B. napus* and *B. rapa* are successful, while crosses between *B. napus* and *B. oleracea* are not easy. A cross between *B. rapa* and *B. oleracea* is more successful than a cross between *B. oleracea* and *B. rapa*. The two lines of *B. rapa*, Shogoin-kabu and Chiifu have different ability to cross to radish, *R. sativus*; Shogoin-kabu can produce several seeds when crossed with *R. sativus*, whereas Chiifu does not produce any seeds (Tonosaki et al. 2013).

Artificial techniques to overcome the reproductive barriers by embryo rescue techniques (embryo culture, ovary culture, ovule culture) have been developed for producing interspecific hybrids. One example of an artificially generated interspecific hybrid is HAKURAN, which is obtained by interspecific crossing between HAKU-sai called Chinese cabbage (*B. rapa*) in Japanese and kan-RAN called cabbage in Japanese. Some other cultivars produced by interspecific hybridization between *B. rapa* and *B. oleracea* have been released in Japan (Kaneko and Bang 2014).

Recently clubroot disease has spread into oilseed crops (*B. napus*), thus clubroot resistant canola/rapeseed cultivars are needed. However, there are limited resistance sources available in *B. napus*. Clubroot resistant Chinese cabbage cultivars have been developed by transferring clubroot resistance genes from European turnip (Matsumoto et al. 2005). As interspecific hybrids between canola and *B. rapa* are able to be produced by embryo rescue, transformation of resistance genes of *B. rapa* into canola could develop resistant canola/rapeseed cultivars (Liu et al. 2018).

BoFLC2 is a key factor of vernalization in cabbage (plant-vernalization-responsive type) (Itabashi et al. 2018). This *BoFLC2* is transferred into Chinese cabbage (seed-vernalization type) by interspecific hybridization and backcrossing of Chinese cabbage. The BC_3F_2 offspring did not show the plant-vernalization-responsive type, however, the duration of cold required for successful vernalization leading to flowering was increased (Shea et al. 2017, 2018c).

2.7 Conclusion and Prospects

MAS is a useful tool for effective breeding of Chinese cabbage. We describe some examples of DNA markers used for breeding such as identification of *S* haplotypes, Fusarium wilt resistance gene and clubroot disease resistance genes. However, some traits are not applicable for MAS such as those related to environmental factors (stability of self-incompatibility, vernalization, abiotic stress tolerance). Molecular biological study can be used for some traits, but there are other traits whose molecular mechanism has not yet been clarified. Breeding for abiotic or biotic stress tolerance will be needed to widen the current resistant germplasm resources, possibly by screening germplasm more broadly, and exploring resistance in wild relatives. Molecular characterization of key genes leads to the development of novel gene based DNA markers. Pyramiding of factors in elite lines via MAS can confer broad resistance against stress. The recent innovation of high-throughput sequencing technology enables precise genetic study. Genome editing technologies may also be useful in manipulation of key genes. Further research on agriculturally-important traits will be helpful for the establishment of effective breeding methods to produce advanced cultivars of Chinese cabbage.

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Appendices

Appendix I: Research Institutes Relevant to Chinese Cabbage

Institution	Specialization and research activities	Contact information and website
Beijing Vegetable Research Center (BVRC), Beijing Academy of Agriculture and Forestry Science (BAAFS)	Genetic analysis on agronomical important traits	https://bresov.eu/network/partners/bvrc
Chungnam National University	Genetics and molecular marker development in Chinese cabbage	http://horti.cnu.ac.kr/
Graduate School of Agricultural Science, Kobe University	Epigenetics, heterosis, vernalization	http://www.ans.kobe-u.ac.jp/
Graduate School of Life Sciences, Tohoku University	Self-incompatibility	https://www.lifesci.tohoku.ac.jp/

(continued)

Institution	Specialization and research activities	Contact information and website
Graduate School of Sciences and Technology, Niigata University	Disease resistance	https://www.gs.niigata-u.ac.jp/~gsweb/index.html
Henan Academy of Agricultural Sciences	Creation of germplasm resources and QTL mapping for different traits in Chinese cabbage	http://www.hnagri.org.cn/index.php
Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences (IVF, CAAS)	Whole genome sequencing	https://www.gfar.net/organizations/institute-vegetables-and-flowers-chinese-academy-agricultural-sciences
John Innes Centre	Whole genome sequencing, germplasm resources	https://www.jic.ac.uk
National Institute of Horticultural and Herbal Science	Germplasm collection, molecular marker and cultivar development in Brassicaceae	https://www.nihhs.go.kr
RIKEN BioResource Center	Germplasm resources	https://epd.brc.riken.jp/en/
Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences	Leafy heads of Chinese cabbage	http://english.sibs.cas.cn/
State Key Laboratory of Crop Genetics and Germplasm Enhancement, Nanjing Agricultural University	Flowering	https://www.researchgate.net/institution/Nanjing_Agricultural_University/department/State_Key_Laboratory_of_Crop_Genetics_and_Germplasm_Enhancement/members
Sunchon National University	Genetic inheritance and molecular breeding of clubroot resistant Chinese cabbage	https://www.scnu.ac.kr/horti/main.do
The National Agriculture and Food Research Organization in Japan	Development of parental lines for clubroot resistant Chinese cabbage using MAS	http://www.naro.affrc.go.jp
Tohoku university Brassica seed bank	Seed bank of Bressicaceae germplasm	http://www.agri.tohoku.ac.jp/pbreed/Seed_Stock_DB/Stock_English_top.html
Wageningen UR Plant Breeding, Wageningen University and Research Centre	Genetic analysis on agronomical important traits	https://www.wur.nl/en.htm

Appendix II: Genetic Resources of Chinese Cabbage

Cultivar	Important traits	Cultivation location
Akimeki	Clubroot (Crr1, Crr2, CRb)	Norin seed Co., Japan
Bre	Inbred line of medium-cycling crop type	China
Chihiri 70	Transformable strain	Takii Seed Co., Japan
Chiifu-401	The first whole genome sequenced Chinese cabbage	Korea and Japan
CR gangsan	Clubroot (Crr2, CRb, CRa)	Nonghyeob seed Co., Korea
CR Shinki	Clubroot (CRb)	Takii Seed Co., Japan
Gokurakuten	High regeneration rate, creation of transgenic *Brassica rapa*	Takii Seed Co., Japan
R-o-18	Reverse genetics (EMS-induced mutagenesis population)	UK
RJKB lines	Inbred lines	Japan
W39	Biomass heterosis, early developmental heterosis	Watanabe seed Co., Japan
Wantai	Inbred line of slow-cycling crop type	China
Yellow sarson	Self-compatible, mutant for *S*-genes and *MLPK* gene	India

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Chapter 3
Breeding Advances and Prospects in Rocket Salad (*Eruca vesicaria* ssp. *sativa* Mill.) Cultivation

Pasquale Tripodi, Paula Santos Coelho, and Carla Guijarro-Real

Abstract Cultivated rocket salad (*Eruca vesicaria* Mill.) is a fast-growing annual herbaceous plant characterized by leaves with a peculiar pungent taste and strong flavor. The crop, native to the Mediterranean Basin and western Asia, is widespread in all habitable continents as a leafy vegetable. Its consumption can be fresh as a topping on many dishes or mixed in salad packages. Leaves can be an ingredient in preparations such as purees, sauces, pesto or liqueurs. Secondary uses in cosmetics and medicine occur due to related depurative and anti-inflammatory effects. Although it is not an intensive crop, attention must be given to increased productivity. Indeed, appropriate soil preparation and good water availability are necessary to improve the production and the qualitative profile. Rocket salad contains a range of health-promoting compounds including glucosinolates, flavonoids carotenoids, vitamins, fiber and polyphenols. The richness of these compounds suggests a role for rocket in the prevention of common degenerative diseases in human. The content of these phytochemicals is influenced by genotypic factors, cultivation and processing conditions. The overall increase in cultivated area and related consumption requires a concerted effort to create new varieties which are well suited to face the challenges of climate change, emerging diseases or novel trends in consumption. Thus, breeding targets are addressed for the improvement of nutritional properties and shelf life, and to increase resistance to biotic and abiotic factors like diseases, insects, drought and salinity. In this chapter, we present an overview of the origin, economic and nutraceutical importance, genetic resource characterization and con-

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servation, resistance to biotic and abiotic stresses and breeding objectives of rocket salad.

Keywords Antioxidants · Breeding · Cultivated rocket salad · *Eruca* · Resistance

3.1 Introduction

Cultivated rocket salad (genus *Eruca*), also called arugula, is a leafy vegetable, belonging to the large Brassicaceae family, which includes several economic crop species, and is consumed fresh as a salad green. The crop, characterized by leaves with a distinct pungent taste and strong flavor, is a fast-growing vegetable native to the Mediterranean Basin and western Asia, and dispersed to southern Europe, North Africa, Iran, India and Pakistan (Hall et al. 2012; Tripodi et al. 2017). Today, it is grown in all habitable continents in both marginal areas and/or fertile soils. The scientific name of cultivated rocket salad is *Eruca vesicaria* (L.) Cav. Four subspecies are recognized including ssp. *vesicaria* (L.) Cav., *sativa* (Mill.) Thell., *longirostris* (Uechtr.) Maire and *pinnatifida* (Desf.) Emberger & Maire (Gómez-Campo 2003). Subspecies *sativa*, also called *Eruca sativa*, is the most consumed and economically relevant. For this reason, the cultivated rocket salad uses abbreviated name *E. sativa,* which refers to *E. vesicaria* ssp. *sativa*. Based on conserved protein genes among the Brassicaceae mitochondrial genomes, *Eruca* is quite close to species belonging to the *Brassica* genus (Wang et al. 2014) (Fig. 3.1).

Eruca sativa is a diploid having 11 pairs of chromosomes (2n = 22) (Padulosi and Pignone 1997) and an annual life cycle. The species shows a variable degree of self-incompatibility and allogamy. Under natural conditions, it begins to flower in early spring when temperatures are high and the photoperiod is long, ending with the production of seeds in late spring/early summer. A high growth rate is also observed resulting in increased leaf size and early flowering, and resulting in high biomass production.

Eruca sativa plants are characterized by rosette leaves at the ground level, width to 60 mm and length to 200 mm; rather thick and with rib central well, shallow lobes, toothed or incised with short petioles. The stem, with a variable height of up to 1 m, is generally slightly hairy at the bottom with the rest hairless, although variability can be observed. The cauline leaves are almost sessile and increasingly thin as they approach the apex, with lobes that become increasingly marked and longer. The flowers, with a diameter of about 25 mm, are arranged in spiciform racemes and carried by very short peduncles. Flowers are characterized by a caduceus calyx and elongated vertical stylus typical of this crop (Gómez-Campo 2003). Each flower consists of 4 rounded petals with a white, cream, or yellow color and thin veins with a color ranging from brown to the purple (Fig. 3.2).

The calyx is made up by 4 lanceolate violet-green sepals. The fruit is a siliqua with a diameter of 5–7 mm and length of 20–25 mm. It is almost always glabrous,

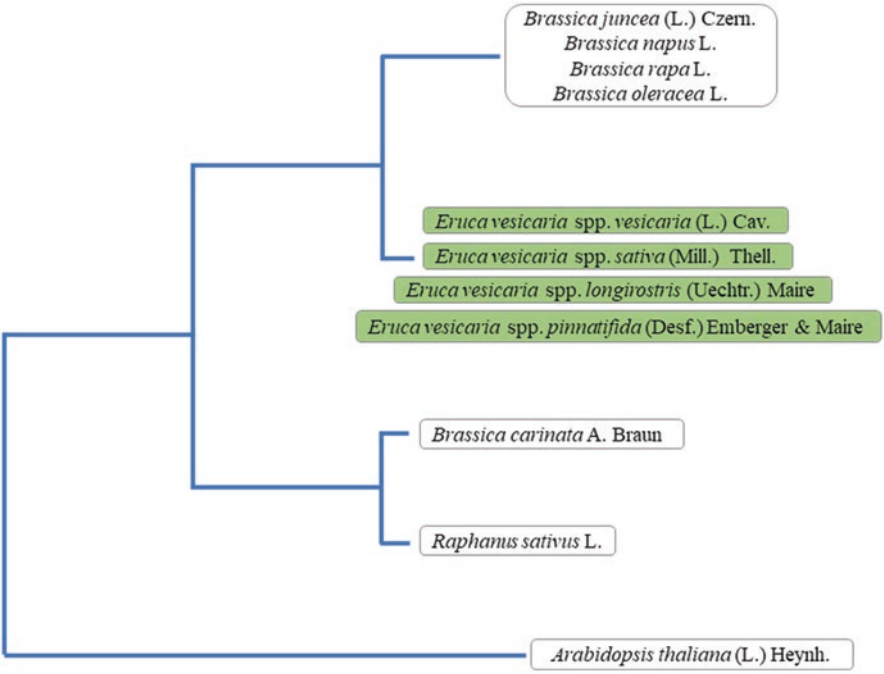

Fig. 3.1 Phylogenetic tree showing the relationship between *Eruca* and closely-related species, drawn using mitochondrial conserved genes. (Source: Reconstructed from Wang et al. 2014)

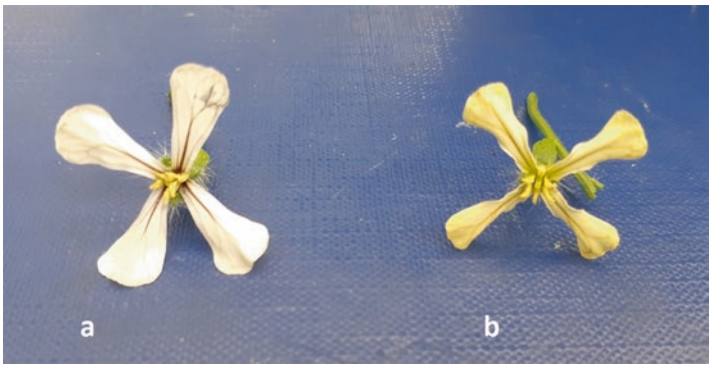

Fig. 3.2 Details of *Eruca vesicaria* ssp. *sativa* flowers. (**a**) Detail of a flower with white petals and yellow stamens, (**b**) Detail of a yellow flower

with 2 valves which enclose 20–35 seeds with elliptical ovate shape, dimensions of about 1–1.5 mm, and variable color from more or less yellow to brown dark. The weight of 10,000 seeds is variable, 15–20 g. Seeds have a fast germination, requiring a temperature range of 25–28 °C.

3.1.1 Uses and Economic Importance

Consumption of rocket salad dates back to Greek and Roman times and included food and non-food uses such as cosmetics, oil and medical purposes due to its purported anti-inflammatory and depurative effects (Hall et al. 2012; Padulosi and Pignone 1997; Tripodi et al. 2017). Traditionally, it was consumed in the Middle East and southern Italy, mainly for fresh consumption (D'Antuono et al. 2008), and also used as an oilseed crop in South Asia, mostly for industrial purposes as a lubricant (Garg and Sharma 2014). In recent years, the demand for prepared and ready-to-use leafy vegetables, the increased attention of a healthy diet by consumers, as well as the growing interest in new culinary products, has resulted in an expansion of the cultivated area in several world regions. In particular, Mediterranean countries are major producers and exporters given favorable climate conditions. Today, cultivated rocket salad is mainly commercialized within mixed salad packages and the spicy leaves are eaten fresh in salads, as topping on various recipes, or added to soups. Furthermore, several preparations are made such as purees, sauces, pesto and liqueurs. Other cosmetic uses concern the production of body lotions or creams. Raw leaves are rich in several health-promoting compounds, including fiber, carotenoids, flavonoids, vitamins and glucosinolates (Bell and Wagstaff 2019). Many of these compounds have an important role in nutrition ensuring beneficial effects to human health and are thought to reduce the risk of common degenerative diseases.

3.2 Current Cultivation Practices and Challenges

3.2.1 Soil Preparation and Cultivation Methods

Proper soil preparation is needed because *Eruca* is a direct sown crop. Although a wide variety of soil conditions can be tolerated, a well-drained and humus-rich soil is preferred. Generally, in clay-loam soils a deep digging of 0.30–0.35 m in advance on the date of sowing is performed. This operation is preferred when any residues from the previous crop and/or organic fertilizers are buried.

Subsequent harrowing and/or milling works are performed in order to break up clods, and avoid the presence of dust in the most superficial area that can be the cause of subsequent formation of crusts after sprinkler irrigation. In sandy soils, a mechanical dig is carried out or milling at 0.20–0.30 m.

Soil preparation proceeds with surface leveling in order to guarantee the uniformity of the planting depth. Finally, wide rows on which sowing is carried out are made. In addition to the correct soil preparation, it is recommended that repeated cultivation be avoided in the same field soil over seasons, since it can lead to a buildup of parasites damaging to the plants.

Sowing takes place in late summer for the fall/early-winter harvest and in late winter for spring-summer harvest. Crop density can range from 50–200 plants/m^2 (<0.5 g of seeds) according to the variety grown. In general, sowing 1 cm in depth is required. After sowing, a uniform wetting of the soil is required in order to guarantee a prompt and uniform emergence. Seeds germinate in a few days.

Plants are grown in both open fields and protected environments. For the latter, the ideal standard structure is a multi-tunnel of galvanized iron, 7.20 m wide, eaves height of 2.2–4 m and a length of 40–50 m. A greenhouse with double opening is preferred (one at the head, another in the arch) to facilitate ventilation. The cover is generally made of thermal plastic with anti-drip additives to facilitate the flow of condensation collected by an internal gutter all along the tunnel, so as to prevent it from dripping on the seedbed. The duration of the crop cycle may vary in relation to the period of cultivation: shorter in the summer and longer in winter.

Rocket salad plants are grown on bines, the sizes of which vary but in general, in the above described 7.20 m tunnel, can be 4–5 bines, with a width of 120–180 cm, and a depth of about 10 cm. These dimensions ensure an adequate airing and facilitate the passage of agricultural machine wheels. Sowing takes place with precision mechanical seed drills. Very clayey soils impose low plant densities per meter square since the soil retains water for a long time, especially in winter, affecting the health of plants which are exposed to diseases of telluric origin. Controlled conditions allow air exchange in greenhouses preventing adversities of the aerial part of the plants in the case of high relative humidity. Harvest can be manual for bunches, with a sickle or machines. In general, 15–18 million seeds per hectare are sown. It is also possible to cultivate rocket salad on plastic mulch, destined exclusively for the first-class market where only hand-picked bunches are made. The planting layout includes 250,000 holes per hectare arranged on 6 rows spaced 18 cm from each other and with a distance of 18 cm between plants. Differences in latitude also appear substantial; in the Northern Hemisphere rocket salad is grown mainly from spring to autumn, while in the Southern Hemisphere it is concentrated in the winter-spring period. These circumstances make it possible to obtain production for the entire year. In general, from 4–10 harvests can be obtained, according to the cultivation cycle.

3.2.2 Irrigation

To improve the production and the qualitative profile of rocket salad, with crunchy and not very dark leaves, it is necessary to have soils characterized by good water availability. The choice of the irrigation system must guarantee uniform water distribution. The most widespread systems are micro-irrigation systems with full coverage for sprinkling with static (sprayer) or dynamic (sprinkler) sprayers, with medium-low flow rates (70–120 L h^{-1}), and modest ranges (3–5 m). Irrigation sprinklers normally come equipped with anti-fog and anti-drip devices to avoid the drift effect and dripping at the end of the operation, thus improving the performance in

terms of uniformity. For proper distribution of the water supplied, it is important to pay attention to the size of the irrigation system, in particular the spacing between the wings and between the dispensers and the flow rate of the nozzles. In the case of tunnels, the irrigation systems can consist in two lines of sprayers along the greenhouse and/or with a line of perimeter sprays. This scheme allows a proper irrigation on the heads where it is easier for the air currents to act on the light drizzle, deviating the fall and leaving the ground uncovered. Only in the case of using plastic mulch, for the cultivation of the rocket salad packed in bunches, it is possible to use tubes with drippers on the ground. Spraying of plants is also used, especially in the post-sowing phase, to facilitate germination. In the case of heavy soils, mobile bars with low spraying and low flow rate (10–15 mm h^{-1}) are used. In this case, a better uniformity of distribution is observed. The system can be also used for pesticide treatments. Frequent irrigation interventions up to the complete emergence of the seedlings are needed. The largest volume of watering needed is immediately given after sowing. On soil easily forming a superficial crust, at this stage it will be appropriate to decrease volumes and increase the frequency of distributions up to total emergence of the crop. Sprinkler irrigation, if not well managed, can cause serious damage to the crop because, with the high density adopted, the plants grow with very tender leaves which, remaining wet for long times, can be easily attacked by pathogenic fungi, mainly downy mildew. In soils where sufficient water conditions are found (due to previous irrigation), high quantities of water are not needed and the cycle between emergence and collection can be rather short. During the period between the complete distension of the cotyledons, one irrigation intervention, often aimed at supplying nutrients, can be sufficient for rocket salad. However, a careful observation of the crop is fundamental in order to determine whether to reduce or add more irrigations than those planned. In a state of water scarcity, in fact, it is possible to identify plants with stunted growth, thick, dark green leaves within the crop. Irrigation operations are preferably carried out in the morning, which also corresponds to the period of greater crop water consumption, and especially to allow rapid drying of the leaves thus preventing the onset of fungal diseases.

3.2.3 Fertilization

As in any crop, soil chemical analysis is recommended to know the micro- and macroelement composition. Typically, rocket salad can grow in poor nutrient soils because it is a plant with minimal needs. A major concern relates to nitrate accumulation. In order to prevent it, organic fertilizers with a C/N ratio greater than 8 can be used. To this end, the nitrogen quantity must be divided into two or three interventions within the cycle, avoiding distributions near harvest to reduce concentration of nitrates in the edible plant parts. Different strategies can be applied in fertilization, from pre-sowing bottom fertilizer to granular products directly on the ground during cultivation. Moreover, fertigation with water-soluble products is often practiced. The latter method allows delivering the elements with irrigation

according to plant needs, thus reducing nitrate accumulation. In this way, the crop will be maintained at optimal nutritional levels. Moreover, an excess of nutrients must be avoided. In this regard it could be conceivable to use a basic fertilizer which, in addition to the organic substance, containing NPK in 4:1:4 ratio. Finally, it must be taken into account that the contributions of the individual nutrient elements must be such as not to cause an increase in the soil salinity over time.

3.2.4 Current Agricultural Challenges

An intensive method of cultivation, the absence of rotations and a system characterized by high investments in protected environment are the main pillars for rocket salad cultivation. These conditions, in combination with high relative humidity and temperatures, contribute to the favorable development of pathologies.

Pathogen resistances are described below (Sect. 3.4). Other constraints are represented by weeds (*Chenopodium* spp., *Portulaca oleracea* L., *Solanum nigrum* L.), which can seriously affect cultivation, taking over the culture. The only methods for the containment of parasites and weeds are chemical control (pesticides, fumigants), the use of natural enemies or agronomic techniques (false seeding for weeds). Moreover, soil disinfestation with different systems such as steam, fumigation or solarization allow a good control of soil borne pathogens and weeds.

3.3 Germplasm Biodiversity and Conservation

Investigation of the genetic diversity of crops is fundamental for management and conservation of germplasm resources and for genetic improvement purposes. Both breeders and germplasm curators benefit from the information assembled on plant genetic resources. Unlike other plant species, in *Eruca* spp. few efforts have been performed so far. Therefore, genetic information (e.g., genetic and physical maps, resistance genes, etc.) as well as established mapping populations are not yet fully available for this crop.

3.3.1 Germplasm Diversity

The first step in breeding programs involves assessing the variability available. In rocket salad it is possible to find several works in pursuit of this goal, mainly based on a morpho-agronomic characterization and nutritional traits. In addition, several authors have also evaluated rocket salad genetic diversity by means of molecular markers (Garg and Sharma 2015; Guijarro-Real et al. 2020; Zafar-Pashanezhad et al. 2020).

The traits selected for analysis in the different works depend on the ultimate use expected for the data collected. Rocket salad is commonly known by consumers as a minor leafy vegetable of bitter, hot flavor. However, it is also of relevance in some regions as an oilseed crop. Thus, it is grown in India for the extraction of oil that is high in erucic acid; the crop commonly being known as *taramira*. Unlike other oilseed Brassicaceae crops, taramira is drought tolerant. In fact, Fallahi et al. (2015) found that rocket salad germplasm had a good response under high salinity levels and osmotic stress conditions, with germination percentages above 70% even at 150 mmol NaCl and −14 bars of osmotic level. Taramira is, therefore, a good option for marginal lands with low fertility.

Several authors have evaluated the germplasm of rocket salad due to its interest as an oilseed crop using a combination of morphological and molecular techniques (Guijarro-Real et al. 2020; Taranto et al. 2016; Warwick et al. 2007).

Warwick et al. (2007) investigated a large collection (~180) of *Eruca* accessions mainly belonging to the ssp. *sativa* and including some samples of ssp. *vesicaria* (14) and ssp. *pinnatifida* (1). In their study, two strategies of assessment were performed: a) analysis of morpho-agronomic variation in a set of 159 ssp. *sativa* genotypes and b) analysis of the genetic diversity of a subset of 49 accessions, including representatives from all three subspecies by means of amplified fragment length polymorphisms (AFLP) markers. The collection revealed a wide diversity explained by almost 67% in the first two components. Interesting differences were also detected between the Mediterranean and Asian groups in terms of plant height, days to maturity and oil content. The analysis with AFLP revealed 234 polymorphic bands, well separated into the three subspecies and clearly showed that accessions of the ssp. *sativa* from Morocco were closer to the ssp. *pinnatifida*, while the Spanish native of the ssp. *vesicaria* were more similar to *pinnatifida* than to *sativa*. The results also indicated a separation of Mediterranean and Asians accessions (Warwick et al. 2007). Another study aimed at the evaluation of the variability of morphological, agronomic and molecular features was performed in 50 individuals from 5 accessions of Spanish arugula (Egea-Gilabert et al. 2009). Agronomic traits involved the characterization of leaf morphological and qualitative traits while genetic diversity was assessed using 9 inter simple sequence repeat (ISSR) markers producing 247 polymorphic bands among the accessions. Results revealed significant differences among the accessions for all the quantitative traits, indicating a high degree of phenotypic variability which was almost 50% in the first two principal components. Moreover, molecular analysis allowed a discrimination of three distinct groups although the variation observed was lower than the agronomic traits. Overall, the study proved local accessions could be good candidates for breeding programs. Bozokalfa et al. (2011) in a study addressed at both the characterization of cultivated rocket salad and its discrimination from wild rocket salad (*Diplotaxis* genus), evaluated 24 *Eruca* accessions for 18 quantitative agronomic traits including plant, leaf and siliqua characteristics, and 33 qualitative morphological traits including common descriptors for *Eruca* (IPGRI 1999). The investigation provided data to confirm that rocket salad is characterized by a wide diversity based on agronomic and morphological plant properties, confirming what was

underlined in previous works. Jakhar et al. (2010) evaluated yield-related traits with high interest for this purpose. They found variability among 146 accessions for traits such as the number of silique and seeds per siliqua, height of the plant or number of branches, thus suggesting the possibility of improving the crop for a higher production. Garg and Sharma (2015) found that the total oil content in seeds of 30 genotypes from India was 25–38% (w/w) and it was possible to identify accessions containing up to 47.5% of erucic acid (relative concentration from the total fatty acids profile). Their results suggest that using an adequate selection strategy may improve the crop for higher yield of erucic acid. In addition, the authors reported a great degree of genetic diversity among materials coming from different agroclimatic zones. The materials were evaluated using 15 ISSR markers (Table 3.1) and grouped in 6 clusters in the genetic dendrogram developed. Moreover, they found a correlation between the molecular analysis and the content in erucic acid, where the materials showing the highest content grouped together in the genetic dendrogram. These results suggest that using an adequate selection strategy might improve the crop for higher yield in erucic acid, and specific markers linked to the fatty acid biosynthesis pathway could be found.

Taranto et al. (2016) evaluated a collection of 40 rocket salad accessions one-half of which belong to *Eruca*. The multidisciplinary study involved the assessment of morphological, agronomic and biochemical traits as well as molecular diversity. The main findings showed that the cultivated rocket genepool is greater than the wild one, highlighting, furthermore, the potentiality of germplasm stored in gene banks. More recently, a comprehensive investigation of 155 cultivated rocket accessions retrieved from 30 countries across Europe, Asia, Africa and the Americas was carried out using common descriptors, automated tools for phenotyping and 15 ISSR markers (Guijarro-Real et al. 2020) (Table 3.1). The study, which to date is the most comprehensive in terms of number of accessions and characters evaluated, revealed the distinction of the germplasm analyzed, according to European and Asian origins contributing to identify sources of variation that could be exploited in arugula breeding programs. Zafar-Pashanezhad et al. (2020) evaluated 60 accessions from different origins (Europe, Asia, Africa) for plant morphology and seed yield-related traits, and also for molecular diversity by using 19 ISSR marker (Table 3.1). As for the Indian genotypes, the morphologic variability registered suggests that it could be possible to select among genotypes for improving the grain yield. Genetic variability among accessions was also found. However, in this case, there was a considerable lack of correlation between the molecular and morpho-agronomic diversity.

While molecular differentiation could derive from factors including mutation, genetic drift and gene flow, it would need natural selection under specific environmental factors for grouping geographical-related accessions by means of the morpho-agronomic traits. Despite the use of rocket salad as an oilseed crop, most research studies are focused on the edible use as a leafy vegetable. As a vegetable, there are two main concerns to be evaluated for crop improvement, the morphology of the leaves and their nutritional and the organoleptic traits, mainly related to the accumulation of glucosinolates.

Table 3.1 List of primers used and polymorphic amplified products in recent studies evaluating the genetic diversity in germplasm collections of *Eruca sativa*

Primer sequence	Number of polymorphic bands		
	Garg and Sharma (2015)	Zafar-Pashanezhad et al. (2020)	Guijarro-Real et al. (2020)
$(AG)_8T$	13	18	–
$(AG)_8G$	–[a]	13	–
$(GA)_8T$	14	19	–
$(GA)_8A$	7	–	–
$(CT)_8T$	6	–	–
$(GA)_8C$	–	–	4
$(CT)_8C$	2	–	–
$(CT)_8G$	–	15	4
$(TC)_8C$	10	–	–
$(AC)_8T$	13	13	–
$(AC)_8C$	15	–	–
$(AC)_8G$	15	15	–
$(TG)_8C$	11	–	–
$(AG)_8C$	–	16	–
$(CA)_8G$	–	11	–
$(ACC)_6$	15	12	–
$(CA)_6AG$	–	17	–
$(CA)_6GT$	–	12	–
$(GA)_8YT$	–	16	–
$(GA)_8YC$	–	20	–
$(AC)_8YA$	–	14	–
$(AC)_8YT$	–	20	–
$(AC)_8YG$	–	16	–
$(TC)_8RG$	–	8	–
$(CT)_8GC$	10	–	–
$(GA)_8CC$	6	–	–
$(GAC)_3GC$	13	–	–
$(GACA)_4$	–	20	–
$(GATA)_4$	–	11	–
$(AC)_{16}TG$	10	–	–
$(GAA)_{11}$	–	–	2
$(CT)_{14}$	–	–	5
$(TC)_{10}$	–	–	7
$(TC)_{11}$	–	–	5
$(GT)_{18}$	–	–	3
$(ATG)_8$	–	–	3
$(AT)_{19}$	–	–	3
$(TA)_{11}$	–	–	10
$(AT)_{12}$	–	–	4

(continued)

Table 3.1 (continued)

Primer sequence	Number of polymorphic bands		
	Garg and Sharma (2015)	Zafar-Pashanezhad et al. (2020)	Guijarro-Real et al. (2020)
$(AG)_9$	–	–	1
$(TC)_{20}$	–	–	3
$(GA)_5$	–	–	2
$(GATA)_2$	–	–	2

[a]Indicates not used in the study

The available rocket salad germplasm exhibits a high variability in terms of leaf morphology. Thus, Taranto et al. (2016) compared 20 accessions of rocket salad and found genotypes with morphologies ranging from elliptic to spatulate shape, showing several degrees of lobation and pubescence. Similarly, Bell et al. (2017a) reported differences in size, shape and hairiness among 7 accessions selected. Guijarro-Real et al. (2020) compared 151 accessions of *E. vesicaria* subsp. *sativa* obtained from the Centre for Genetic Resources (Netherlands), the Leibniz-Institut für Pflanzengenetik und Kulturpflanzenforschung (Germany) and USDA – Germplasm Resources Information Network. As in the previous works, this study reported a great level of variability for leaf qualitative and quantitative traits. According to these works, it would be possible to develop breeding programs aimed at obtaining several varieties differentiating in morphology, thus matching specific market purposes. In fact, Bell et al. (2017b) hypothesized, as derived from the results, that lobed varieties, similar to the wild rocket type, would have a greater acceptance by consumers; by contrast, varieties of larger leaf area and less lobing would be more appropriate for including in mixes of cut vegetables. Together with the morphology, the accumulation of glucosinolates and relationship with other compounds (sugars) is probably the second aspect of rocket salad improvement that deserves great attention from breeders. As already described, the accumulation of glucosinolates plays a key role in the flavor of rocket salad, as well as in its potential functionality. Since they are bioactive compounds with demonstrated health benefits for humans, increasing the content is initially desired and a goal for rocket salad improvement. The synthesis and accumulation of glucosinolates depends on several factors including the species and genotype as genetic factor, but also on the response to environmental factors such as biotic/abiotic stresses or fertilization. For the latter, some reviews exposing the topic can be found, such as the work of Bell and Wagstaff (2017). Regarding the genetic component of this synthesis, several works have reported the presence of diversity among accessions of rocket salad (Bennett et al. 2007; D'Antuono et al. 2008; Taranto et al. 2016). The information provided could be used for planning breeding programs to enhance the accumulation of glucosinolates as a nutritionally- interesting component of rocket salad. There is, however, a second aspect of glucosinolates to be considered, which might limit the development of materials high in glucosinolates. Glucosinolates are also related to the flavor of rocket salad and other Brassicaceae crops, or more particularly, to the bitter

and hot perceptions in these crops. Therefore, the selection for nutritional quality must also include the evaluation of consumer acceptance of its organoleptic quality. In fact, there are several cultivars which have been selected for the pungency attribute. Thus, in countries such as Italy, the pungent varieties are of high value; by contrast, non-pungent varieties are appreciated in countries such as Turkey and Egypt, where the species is used as a main ingredient for salad (Pignone and Gómez-Campo 2011).

Both the scientific studies focused on variability and the existence of different varieties with characteristic attributes indicate that rocket salad has been domesticated to some degree and breeding taken place. However, there are some limitations for rocket salad improvement, which may undermine the success. These constrains will be described in the section on conservation of germplasm. Special consideration is needed for the self-incompatibility described in *Eruca vesicaria*. Sharma et al. (1985) found that self-pollination in rocket salad did not produce seed; results were tested by 30 activities of self-pollination. Wang et al. (2009) found that there was variability for the degree of self-compatibility among 34 materials of rocket salad evaluated, with most of them (85%) classified as self-incompatible type. Moreover, even within accessions, different genotypes can show different degrees of self-compatibility. These studies show that, even when there is an opportunity for selecting genotypes of rocket salad to obtain stable lines, this approach may not be the most satisfactory. By contrast, the development of open-pollinated varieties seems more feasible. It is, in fact, a common approach followed by breeders in rocket salad, as reported by Pignone and Gómez-Campo (2011) and includes mass selection as a methodology for improvement.

3.3.2 Phylogeny and Genomic Diversity

A mitochondrial DNA (mtDNA) genome of *Eruca sativa* was sequenced for phylogenesis and evolutionary analysis with other *Brassica* species (Wang et al. 2014). Sequencing was performed using the GS-FLX platform (Roche, Branford, CT, USA). A significant part of the mtDNA is comprised of non-coding sequences (~85%), the genes accounted for ~27% of the genome, 56.6% were represented by exons and the remainder by introns. For phylogenesis, the neighbor-joining method employing 23 conserved genes was used. As a result, *E. sativa* was found to be more closely related to the *Brassica* species and *Raphanus sativus* than to *Arabidopsis thaliana* (Fig. 3.1).

Very recently, de novo whole genome sequencing of three elite inbred lines has been obtained through short read illumina sequencing (Bell et al. 2020). This study represents the first attempt to investigate the *Eruca* genome. The genome size has been reported as ~851 Mb with 66.3% of transposable elements primarily represented by long terminal repeat (LTR) retrotransposons (37.3%). In total, 45,438 protein-coding genes were reported, which is higher as compared to those reported in *Arabidopsis thaliana*. The study highlighted several ortholog genes with other

Brassica species, in particular those responsible for glucosinolates and sulfur biosynthesis.

3.3.3 Genetic Resources Conservation

In the early 1990s, the International Plant Genetic Resources Institute (IPGRI) initiated, with support of the Italian Government, a project entitled Conservation and Use of Underutilized Mediterranean Species, aimed at promoting the conservation of neglected crops in the Mediterranean Region. As part of the project, a specific collaborative group named the Rocket Genetic Resources Network was established as a means to centralize and facilitate activities and strategies on conservation, management and uses of rocket resources (IPGRI 1999). This project led to an increase in germplasm collecting activities and expanded the knowledge of the resources stored in the different gene banks around the world (Pignone 1997).

Two decades later, the *Eruca* resources are still present in different gene banks worldwide. Among them, three institutions hold the greatest collections (> 100 accessions): the U.S. National Plant Germplasm System (USDA-GRIN, USA), the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) in Germany and the Banco de Germoplasma Vegetal-UPM César Gómez Campo (BGV-UPM) in Spain. The U.S. National Plant Germplasm System has up to 273 accessions of *E. vesicaria,* from which 247 accessions are still available. There are 236 described as the ssp. *sativa*, most of them collected in Pakistan, Italy, India and Iran (NPGS 2020). The IPK gene bank includes 130 accessions, all classified as *E. sativa* and with a great representation of Pakistani and Italian germplasm (GBIS/I 2020). Finally, the BGV-UPM includes 131 accessions, among them only 9 are specifically described as ssp. *sativa* (BGV-UPM 2020). Although the level of duplication among gene banks is not known with certainty, the transfer of materials in the past suggests that it might be high. In fact, Gómez-Campo (2007) reported that approximately 90% of the Brassicaceae collection could be duplicated in other banks according to the BGV-UPM records.

Genetic resources in the form seeds are conserved ex situ in other international gene banks such as the Centre for Genetic Resources (CGN; Wageningen, Netherlands) and the Royal Botanic Garden, Millennium Seed Bank (KEW, London). A listing of seed banks is provided in Appendix I.

As mentioned above, the germplasm resources are mainly conserved in gene banks; however, resources can be also found as wild populations in situ across a large number of regions where the plant grows spontaneously, without control of the evolution of these resources over time. The seeds of rocket salad are orthodox, thus facilitating their ex situ preservation. The BGV-UPM has successfully used cold preservation for more than 60 years for orthodox seeds, combining the storage in cold conditions (−5 to −10 °C) and low moisture content (~1.5–3% on a fresh weight basis) (Pérez-García et al. 2007). Similar conditions are commonly used in

other gene banks for seed conservation over the medium-long term, although the temperature can be slightly higher, above 0 °C.

Despite the simplicity of storing the seeds of rocket salad as *ex situ* resources in gene banks, it should be noted that the conservation and management of its germplasm have a number of critical points to be considered for gene banks and breeders. This issue was first described in the second meeting of the Rocket Genetic Resources Network, based on the experiences of the participating scientists (Pignone 1997). The main constraints are highlighted as follows:

(a) Dehiscence of the siliques. Siliques are easily opened at maturity, thus allowing the spread of seeds as a strategy for species survival. However, for conservation and breeding programs, this condition implies extra effort to collect the seed to minimize seed loss.
(b) Low germination rates. Collecting the immature siliques, in an attempt to reduce the seed losses, can result in a high percentage of immature seeds and, therefore, in low germination rates (Pignone 1997). However, germination studies in rocket salad have shown that high rates can be achieved under controlled environments, nearly 95% (Bakhshandeh et al. 2020; Barazani et al. 2012). Other aspects such as the genotype and/or the environment in which the mother plants were grown may affect germination as well, as the results of Barazani et al. (2012) suggest. A strategy that can be followed to increase the germination rate is soaking the seeds in gibberellic acid as a dormancy-breaking treatment; this is especially suggested for gene banks (González-Benito et al. 2011).
(c) Contamination with foreign pollen. Rocket salad is an allogamous species pollinated by insects. This increases the risk of cross-contamination with local pollen and/or pollen from other accessions if they are growing in the same or nearby fields. In order to reduce such risks, it is essential to isolate properly the materials of interest (e.g. cover with adequate mesh for preventing the pollen/insects to pass through).
(d) Self-incompatibility and inbreeding depression. The multiplication of allogamous species under isolated conditions for a long period can result in fertility problems for seedlings and inbreeding depression. In particular, rocket salad has been described as a species of high self-incompatibility (Sharma et al. 1985; Verma et al. 1977). Therefore, this is a trait to be considered in multiplication and breeding activities to determine the number of plants and the selection strategy.

With the consideration of these critical points, gene banks and breeders will be able to establish adequate strategies and experimental designs to achieve their goals.

3.4 Resistance to Pathogens in *Eruca* ssp.

Biotic stresses are responsible for significant crop losses each year; chemical control represents an additional cost for growers and is limited by several factors such as legislative restrictions, environmental concerns and loss of efficacy. The severe legislation on the use of pesticides adopted in the last few years in Europe, and other parts of the world, implies a need to develop new protection approaches which are more sustainable and less dependent on chemical control. The potential to identify and use sources of resistance to control biotic and abiotic stresses is a key component in future integrated pest management (IPM) strategies.

Baby-leaf crops are grown under high crop density in five to six cycles per year, with a lack of adequate crop rotation and a shortage of fungicides labelled for their control (Gullino et al. 2019). In the last years, the introduction of new pathogens and the intensification of existing problems in rocket production have arisen, as a consequence of the dynamism of the sector, massive diffusion of rocket species cultivation and specialization (Gilardi et al. 2013).

The susceptibility of rocket crop to several foliar and soil-borne pathogens constitutes a limiting factor for both the quality and the quantity of yield. This problem is more serious due to the intensive production systems used. Also, poor phytosanitary control in seed markets is making difficult the management of dangerous fungal and bacterial pathogens (Pane et al. 2017).

Downy mildew susceptibility observed in rocket varieties causes severe production losses and represents an increasing threat to crop production, especially in temperate climates (Caruso et al. 2018; Choi et al. 2010, 2018; Gilardi et al. 2013; Hladilova 2010; Koike, 1998; Larran et al. 2006; Minuto et al. 2004; Pimpini and Enzo, 1997; Romero and Zapata 2005; Sharma et al. 1991; Thines and Choi 2016).

Downy mildew is a polycyclic disease more severe under high humidity conditions and during mild, but cool (10–16 °C) seasons. Periods of high relative humidity, especially when leaf tissues are wet, favors rapid development of the disease and production can be lost in few days. Plants can be affected from the seedling stage to harvest, but the disease is more severe on young plants. Recently, Choi et al. (2018) classified the causal agent responsible for downy mildew on *Eruca sativa* as *Hyaloperonospora erucae* sp. nov., suggesting the distinction of this species from the one that also infects wild rocket, *Diplotaxis tenuifolia* (L.) DC., known as *Hyaloperonospora* sp.

The infection of the leaves with downy mildew occurs from the airborne conidia and typical disease symptoms are clearly observed on rocket plants a few days later. Highly susceptible tissues initially exhibit a whitish covering and dense conidiophores on the abaxial leaf surface, but both sides of the leaf are affected under favorable (to the mildew) conditions. Later, the leaves become completely chlorotic accompanied by dark lesions and rot in the case of severe attack. The quality of the leaves is severely affected, and diseased plants are destroyed (Choi et al. 2010; Romero and Zapata 2005). Even in the case of slight damage the product is considerably devalued (Pimpini and Enzo 1997).

To achieve downy mildew infection, only short periods of leaf surface wetness were required, combined with the easy airborne dispersion of spores in open fields and under protection, and the high susceptibility of rocket varieties; as a consequence there is a large incidence and severity of the disease. Fall et al. (2016) referred to temperature, relative humidity (RH), wind speed, solar radiation and leaf wetness duration, as the main environmental factors that determine the infection processes and the extent of production, dispersion and survival of the downy mildew *Bremia lactucae* conidia on lettuce crops.

The use of pesticides is limited by the short cultural cycle of rocket plants. Preliminary studies on rocket indicated the potential value of using varietal resistance to provide good control to downy mildew, which represents clear advantages in the reduction of environmental contamination problems and access to more secure food (Coelho et al. 2017).

Molecular phylogenetic analyses have provided solid evidence for a high degree of specialization within *Hyaloperonospora* species (Göker et al. 2009). Although the causal agent responsible for downy mildew in rocket belongs to the same *Hyaloperonospora* genus that attacks other *Brassica* crops, there exists a pathotype variation since there are no cross-infections between rocket and other *Brassica* species. Seedlings of *Eruca sativa* were resistant against the pathogen isolated from the wild rocket *Diplotaxis tenuifolia* host and other host plants and, similarly, rocket plants are not infected with isolates from other brassicas (swede, broccoli and cruciferous weeds, such as *Capsella bursa-pastoris* and *Thlaspi arvense*) (Hladilova 2010). An extended study about the virulence of pathogens would be useful, since the knowledge about the composition of field pathogen populations is important for a consistent conclusion about the behavior of the resistance genes existing in different *Brassica* genotypes.

Fungal attacks such as *Fusarium* spp., *Pythium* spp., *Phoma* spp., *Sclerotinia* spp., are also of concern among rocket producers (Pimpini and Enzo 1997). *Fusarium* wilts attributed to *F. oxysporum* (f. sp. *raphani* and *conglutinans*) causes serious losses on *Eruca sativa* in India (Chatterjee and Rai 1974) and in Italy (Garibaldi et al. 2003, 2006; Gilardi et al. 2007; Gullino et al. 2019). *Fusarium equiseti* is also identified in Italy (Garibaldi et al. 2011a). Diseased plants are stunted and chlorotic, with brown or black streaks in the vascular system. Differences in the host range of strains of *F. oxysporum* suggest the presence of different races of the pathogen. The causal agent of *Fusarium* wilt of rocket can be seed transmitted (Garibaldi et al. 2004).

White rust symptoms caused by the oomycete *Albugo candida* (Pers.) Kunze was observed in *Eruca sativa* plants (Latinović et al. 2019; Mangwende et al. 2015; Scheck and Koike 1999; Zapata et al. 2005). Symptoms appear as pale-yellow blotches on the upper leaf surface, which coincides with white sori emerging on the lower surface of the leaves. Numerous pustules are present on leaves and stems under the epidermis. Secondary infection of flower heads lead to staghead development.

The leaf spot disease caused by *Colletotrichum* spp. responsible for anthracnose has also been identified on rocket salad (Garibaldi et al. 2016; Patel et al. 2014).

Powdery mildew infection caused by *Erysiphe cruciferarum* was recorded on *Eruca sativa* and *E. vesicaria* (Gunasinghe et al. 2013). Also, *Alternaria* spp. pathogen attacks leaf blades, petioles and hypocotyls on *Eruca* spp. (Garibaldi et al. 2011b; Tidwell et al. 2014).

Clubroot caused by *Plasmodiophora brassicae* Wor. has been reported in Brazil (Paz Lima et al. 2004) on *Eruca sativa*. Diseased plants are severely affected with hypertrophic, malformed roots and root galls. Rocket is a susceptible host and should not be planted on *P. brassicae*-infested land.

Leaf spot diseases caused by bacteria were also reported on *Eruca* spp. The bacterium *Xanthomonas campestris* causing black rot was detected on *E. sativa* (Romero et al. 2008; Rosenthal et al. 2018). Plants presented V-shaped necrotic lesions on leaf margins and blackened veins with broad yellow halos, followed by leaf necrosis. The bacterial blight caused by *Pseudomonas cannabina* pv. *alisalensis* (formerly *P. syringae* pv. *alisalensis*) was reported on *E. vesicaria* (Bull and du Toit 2009; Bull et al. 2015). Initially, symptoms consist of small, angular, water-soaked spots that are visible on both sides of the leaf. The spots later enlarge, remain angular in shape and turn brown to tan. The bacterial wilt caused by *Ralstonia pseudosolanacearum* was reported on *E. vesicaria* ssp. *sativa* in Brazil (Albuquerque et al. 2016).

Eruca sativa has been identified as a natural *Tomato chlorosis virus* (ToCV) host plant, which has serious epidemiological implications and can become a major problem (Boiteux et al. 2016). In addition to these pathogens that attack leaves and roots, Soroka and Grenkow (2013) report that *E. sativa* was an excellent host of flea beetle (*Phyllotreta* spp.). The use of pesticide is the primary tool for controlling diseases, but foliar application on rocket is problematic. The very short interval between harvestings and the need to be applied prior to disease onset limits pesticides use (Caruso et al. 2018). At present, few products are available mainly in organic rocket production, and there is a risk of developing more virulent pathotypes resistant to fungicides.

3.5 Challenges for Abiotic Stresses

Rocket salad is also affected by abiotic stresses. The *Eruca* species has evolved as a fast-growing plant with an efficient root system capable of tolerating severe drought conditions (Garg and Sharma 2014). *Eruca sativa* can be cultivated in almost any type of soil in favorable climatic conditions in open fields and protected areas, whereas calcareous soils are preferable for growing wild rocket *Diplotaxis* spp. (Pimpini and Enzo 1997).

A relevant aspect in drought resistance is the germination rate of seeds. *Eruca sativa* germinates and establishes itself usually in 26–68 days depending on the season. Frequently, rocket salad is a winter crop of drier areas, suitable for marginal and poor lands, drought resistant, tolerant of biotic and abiotic stresses and has a fast-penetrating root system for moisture absorption from deeper soil profiles. In

India, during years of severe drought and late winter rains, is the only alternative crop which can thrive and bear fairly good yield with ensured returns (Bhandari and Chandel 1997). Although the crop is well adapted to dryness, proper irrigation is required to obtain tender leaves and high yields (Bianco and Boari 1997). Rocket is more sensitive to excessive watering than of drought; however, it is important to pay particular attention to drought, since similar to other types of stress, it may accelerate the flowering and endanger the good results of the entire cultivation (Pimpini and Enzo 1997). Plants exposed to excessive watering in conjunction with low temperatures (4–5 °C) tend to have reddish leaves, whereas, once exposed to higher temperatures, these turn yellow and are accompanied by reduced growth, loss of aroma and preservation qualities (Pimpini and Enzo 1997).

The increase of soil salinity causes a dramatic decrease in marketable yield of rocket salad. Furthermore, yield quality worsened with salinity increase due to evident leaf chlorosis and thickening (Bianco and Boari 1997). Salinity, drought, high temperature and nitrogen deficiency affect the phytochemical composition of plants, such as the glucosinolate accumulation (Martínez-Ballesta et al. 2015).

3.6 Qualitative and Nutraceutical Properties

Rocket salad's nutritional properties have stimulated a considerable amount of research in recent decades. As previously mentioned, it contains high quantities of bioactive compounds including vitamin C, glucosinolates, phenolic compounds, chlorophylls, carotenoids and fiber (Barillari et al. 2005; Tripodi et al. 2017). It is also considered a hyperaccumulator of nitrates (Santamaria 2006).

Nutritional studies in rocket salad have been mainly aimed at evaluating the levels of glucosinolates and phenolic compounds, while the characterization of other bioactive properties has been less considered. Baby-leaf products at a commercial stage represent the organs commonly evaluated, as will be summarized in this section. Moreover, the stability of different traits during the postharvest shelf life has been also considered as a key point in rocket salad research. Finally, the characterization of sprouts/microgreens and seeds should also be considered from a nutritional point of view, although such studies are scarce. Microgreens have gained increased attention since their appearance in the food industry in the early 1980s. Apart from the popularity as a decorative element, they also deserves attention as a potential food with high nutritional value (Xiao et al. 2016). Rocket salad was one of the first microgreens commercially available (Choe et al. 2018). Finally, seeds are also of interest for the production of oil (Barillari et al. 2005). Therefore, this section summarizes the main results obtained for the nutritional characterization of different rocket salad materials.

3.6.1 Glucosinolates and Hydrolysis Derived Compounds

Glucosinolates (GSLs) are characteristic of the order Brassicales (also known as Capparales), and includes the family Brassicaceae which is of high economic relevance (Romeo et al. 2018). Chemically, they have a carbon skeleton comprising a β-D-thioglucose moiety and a sulfonated oxime moiety, and a variable chain derived from a α-amino acid (Ishida et al. 2014). According to the α-amino acid precursor, GSLs can be subdivided into: 1) aliphatic, derived from methionine, valine, leucine or isoleucine; 2) aromatic, derived from phenylalanine or tyrosine and 3) indolic, derived from tryptophan (Agneta et al. 2014).

Glucosinolates play a part in the defense system of plants against generalist herbivores and pathogens (Angelino et al. 2015). On the contrary, these compounds are known, or considered, to have potential human health benefits (Bell and Wagstaff 2017). After tissue damage, e.g. by chewing, glucosinolates are hydrolyzed by GSL-specific enzymes known as myrosinases and a variety of compounds including isothiocyanates, thiocyanates, nitriles, epithionitriles and oxazolidines are released (Ahuja et al. 2010). The hydrolysis into different class compounds depends on the species, pH of the reaction and the presence of other specific molecules, among other factors (Angelino et al. 2015; Ciska et al. 2015; Hanschen and Schreiner 2017). Apart from their consideration as bioactive compounds, different hydrolysis products are also responsible for the characteristic bitter, pungent and sulfur flavor in the Brassicaceae (Bell and Wagstaff 2017).

Rocket salad materials present a large variability in total content of GSLs, as suggested by Bell et al. (2015). These authors determined contents of 3.1–11.6 mg g^{-1} DW in mature leaves for plants grown under controlled conditions. In a similar way, Villatoro-Pulido et al. (2013) found total concentrations of 6.12–12.33 mg g^{-1} DW among four accessions grown in the field. By contrast, a greater variability can be found according to Taranto et al. (2016), with a value range of 2.10–40.96 mg g^{-1} DW in the materials evaluated.

Apart from the genotype, other factors affect the concentration in total GSLs such the environment of cultivation. Bell et al. (2017b) found that growing rocket salad in open fields significantly reduced the values previously obtained under controlled conditions (Bell et al. 2015). In fact, values in that study were closer to those obtained by D'Antuono et al. (2008) also using an open-field growing system, obtained levels 0.35–2.68 mg g^{-1} DW. Rossetto et al. (2013) found that growing rocket salad under organic conditions led to an increase in the total GSLs content compared to conventional conditions. On the other hand, Di Gioia et al. (2018) obtained at increase of approximately 41% growing plants in a soilless system compared to soil cultivation. Kim and Ishii (2006) found that seeds accumulated approximately 11-fold the content determined in baby-leaves (125 vs. 11 μmol g^{-1} DW) for plants grown hydroponically. These authors also found that modifying the ammonium:nitrate ratio in the hydroponic nutrient solution has a significant effect on the accumulation of GSLs (Kim et al. 2006). The highest values were obtained for percent molar ammonium- nitrate-nitrogen rates of 50 and 75, while not

providing nitrate-nitrogen had a detrimental effect, probably due to ammonium toxicity. In another study, Chun et al. (2017) found that modifying the N-P-K concentrations in the fertilizer solution would lead to an increase of GSLs levels. The concentration of total GSLs can become a key point for consumer acceptance of rocket salad, as it would positively correlate to the bitter post-swallowing sensation as described by Bell et al. (2017a). Therefore, it can limit the breeding programs aimed at enhancing the nutritional value. Nevertheless, other factors like the sugar content can influence such perceptions as well.

The profile of GSLs in rocket salad has been well studied in a large number of investigations since the early 2000s. Table 3.2 summarizes the compounds commonly determined for the species. Glucoerucin, glucoraphanin and glucosativin/DMB (dimeric glucosativin) are the major GSLs in rocket salad, although great differences in terms of total and relative abundance are reported in the literature. Such differences could correspond to genetic differences but also to the phenological stage and the growing conditions, as in the total GSLs concentration. Seeds are especially rich in glucoerucin. Barillari et al. (2005) determined levels of 108 μmol g^{-1} DW, representing around 95% of the total fraction. The percentage was increased up to 98% in the work of Cataldi et al. (2007). On the contrary, sprouts reduce the levels in glucoerucin. Barillari et al. (2005) found a decrease to 79% due to the oxidation into glucoraphanin. A similar result was observed by

Table 3.2 Individual glucosinolates (GSL) commonly described for rocket salad and chemical structure

GSL	Chemical structure	References[a]
Aliphatic		
Diglucothiobeinin	4-(*β*-D-glucopyranosyldisulfanyl)butyl GSL	2, 4, 6
DMB	Dimeric-4-mercaptobutyl GSL	4, 6
Glucoalyssin	5-(methylsulfinyl)pentyl GSL	4, 6
Glucobrassicanapin	4-pentenyl GSL	3
Glucoerucin	4-(methylthio)butyl GSL	1, 2, 3, 4, 6
Glucoiberverin	3-(methylthio)propyl GSL	2, 3, 4
Gluconapin	3-butenyl GSL	3, 5
Glucoraphanin	4-(methylsulfinyl)butyl GSL	1, 2, 3, 4, 5, 6
Glucosativin	4-mercaptobutyl GSL	1, 2, 3, 4, 5, 6
Progoitrin	(R,S)-2-hydroxy-3-butenyl GSL	3, 6
Aromatic		
Gluconasturtiin	2-phenylethyl GSL	3, 5
Indolic		
4-hydroxyglucobrassicin	4-hydroxy-3-indolymethyl GSL	1, 3, 4
4-methoxyglucobrassicin	4-methoxy-3-indolymethyl GSL	1, 3, 5
Glucobrassicin	3-indolylmethyl GSL	3
Neoglucobrassicin	1-methoxyinfol-3-ylmethyl GSL	3

[a](1) Bennett et al. (2006); (2) Jin et al. (2009); (3) Villatoro-Pulido et al. (2013); (4) Bell et al. (2015); (5) Katsarou et al. (2016); (6) Taranto et al. (2016)

Bennett et al. (2006). For developed leaves, commonly harvested 20–40 days after sowing, the profile can vary significantly. In these tissues, glucosativin/DMB are major compounds (Bennett et al. 2002, 2006; Chun et al. 2013). These compounds derive from glucoerucin by means of S-demethylation (Kim et al. 2006), so it is related somehow to the presence of glucoerucin in seeds. Kim and Ishii (2006) found that 51.6% of the GSLs fraction was represented by DMB, while glucoerucin and glucoraphanin accounted for 29.8 and 11.4%, respectively. Villatoro-Pulido et al. (2013) found that glucosativin was the main compound in some genotypes, while others were also rich in glucoraphanin. In the work of Bell et al. (2015), the percentage of glucosativin/DMB reached to 91.3%. On the contrary, Di Gioia et al. (2018) found that the three compounds together accounted for around 69% of the total fraction. As previously described for the total GSLs content, the individual levels of glucosativin and glucoerucin significantly increased under a soilless system as compared to soil cultivation (around 60%).

Together with these major compounds, other GSLs can be found as well (Table 3.2). Kim et al. (2004) identified diglucothiobeinin in the GSLs fraction of rocket salad for the first time. This compound could be closely related to glucosativin (Kim et al. 2006); although Bennett et al. (2006) suggested that it may correspond to an artefact obtained during the extraction procedure. The work of D'Antuono et al. (2008) included 13 *Eruca sativa* accessions, and they found that materials were clustered according to the levels in total GSLs (low or high) and the profile.

On the other hand, GSL-derived compounds have been also studied in the species. According to Bell et al. (2017b), the hydrolysis mainly releases isothiocyanates (ITCs), especially sulforaphane and, in a lower quantity, sativin. Other minor compounds included erucin and sulforaphane nitriles, bis(4-isothiocyanatobutyl)-disulfide derived from sativin, and 4-isothiocyanato-1-butene that may be a breakdown product of other isothiocyanates. In a previous study, these authors also identified 4-mehtylpentyl ITC, hexyl ITC, 1-isothiocyanato-3-methylbutane, *n*-pentyl ITC and *n*-hexyl ITC (Bell et al. 2016). On the contrary, Raffo et al. (2018) obtained high levels of erucin (69.6 mg kg^{-1} FW) but no accumulation of sulforaphane and sativin due to their degradation into other compounds. In the case of sprouts, Fechner et al. (2018) found high accumulation of sativin (69%), erucin and sulforaphane (12% each) and bis(4-isothiocyanatobutyl) disulfide (6%), with a total content of 2.27 μmol g^{-1} FW.

Finally, some studies evaluating the stability of GSLs and their hydrolysis-derived compounds during shelf life have been conducted. The degradation of these compounds can translate into a loss of the bioactive added value in the commercial product, and also affect the organoleptic quality. Bell et al. (2017b) found that GSLs can increase during storage for a short time, which may be due to a stress response as a result of harvesting. The results of this work indicated a high stability of glucoerucin and glucoraphanin, while other GSLs like glucosativin were more affected. Moreover, the hydrolysis derived sulforaphane and sativin significantly increased after storage for 7 days. By contrast, Fechner et al. (2018) found that sativin was stable and even increased within 24 h at a plant pH of 5.2. Sulforaphane was also of

high stability, with a decrease of 31% within 24 h, while erucin decreased by 74% within 5 h. Jin et al. (2009) found that preharvest conditions affected the stability of compounds. Thus, plants growing under high light intensity did not decrease the levels of GSLs within 2 weeks of storage, while those growing under low intensity significantly decreased the levels by approximately 50% with storage.

3.6.2 Phenolic Compounds

Phenolic compounds represent the second major class of bioactive molecules studied in rocket salad. They are secondary metabolites occurring broadly in plants and can be grouped in different classes (phenolic acids, flavonoids, isoflavonoids, lignanes, stilbenes, coumarines, phenolic polymers) according to their chemical structure (Barba et al. 2014). Phenolic compounds are commonly conjugated to other molecules, and in this sense the term *aglycone* refers to the phenolic moiety.

The content in total phenolics can vary greatly according to genotype. The study by Pasini et al. (2012) showed a variation in the content of total phenolics, with a range of 9.99–30.33 g rutin eq. kg^{-1} DW. Taranto et al. (2016) determined a range of 0.82–10.16 mg g^{-1} DW with an average value of 8.13 mg g^{-1} DW. The levels determined by Bell et al. (2015) were lower, reaching up to only 3.8 g GAE (gallic acid equivalents) kg^{-1} DW and similar to the average values found by Barbieri et al. (2011). Moreover, they found that commercial varieties had higher contents as compared to gene bank accessions.

On the other hand, the growing conditions can affect the phenolic fraction as well. In the study by Barbieri et al. (2011), plants obtained during the spring cycle had higher levels than those from the summer. It was also observed that growing under different salt-stress conditions was translated into different levels of total phenolics. Jin et al. (2009) found that growing plants under high light intensity resulted in the highest levels of flavonoids. Moreover, cyanidin was also found in the plants grown under high light intensity, which could be linked to the anthocyanin production.

The main flavonoid aglycones found in rocket salad leaves are the flavonols kaempferol, quercetin and isorhamnetin, with marked differences with wild rocket (*Diplotaxis tenuifolia*) (Bell et al. 2015). Jin et al. (2009) identified kaempferol as the main aglycone flavonoid (up to 33.5 mg g^{-1} DW), followed by quercetin and isorhamnetin (9.53 and 3.23 mg g^{-1} DW, respectively). In a similar way, Pasini et al. (2012) found that the kaempferol derivatives accounted for 77–88% of total phenolics. Among the kaempferol derivatives, kaempferol-3,4′-di-glucoside stands out as the main flavonoid in rocket salad. In this sense, Martínez-Sánchez et al. (2007) found average levels of 97.8 mg 100 g^{-1} FW. The concentration determined by Taranto et al. (2016) was on average 5.54 mg g^{-1} DW. In the work of Pasini et al. (2012), the levels of this compound showed a range of 8.07–23.68 g kg^{-1} DW; isorhamnetin-3,4-diglucoside, the second major compound identified, was accumulated in less than 5 g kg^{-1} DW. According to Bell et al. (2015), this compound

represented 20.0–75.0% of the phenolic fraction in genebank accessions. Other flavonoids identified in rocket salad include myricetin, kaempferol-3-glucoside, kaempferol-3-di-glucoside-7-glucoside, kaempferol-3-(2-sinapoyl-glucoside)-4′-glucoside, kaempferol-3-sinapoyl-triglucoside-7-diglucoside, quercetin-3-glucoside, quercetin-3,3,4′-tri-glucoside, quercetin-3,4′-di-glucoside-3′-(6-caffeoyl-glucoside), quercetin-3,4′-di-glucoside-3′-(6-sinapoyl-glucoside), isorhamnetin-3-glucoside and isorhamnetin-3,4′-diglucoside (Bell et al. 2015; Pasini et al. 2012). In an different way, Bennett et al. (2006) found that seeds and sprouts of the materials evaluated mainly accumulated quercetin derivatives, and also Villatoro-Pulido et al. (2013) identified quercetin as the main aglycone in the materials tested.

In the addition to genotype and growing conditions, the application of postharvest treatments and the time of storage, can also affect the phenolic fraction. Thus, treating the harvested product with UV-C radiation prior to storage may increase the initial content in around 8% as observed by Gutiérrez et al. (2015), which could be related to the stress of the treatment. However, these authors observed that after 1 week of storage, the effect was reduced and levels remained similar to the control. By contrast, a second study showed that neither the UV-C radiation nor the ozone treatment significantly affected such content (Gutiérrez et al. 2018). Moreover, no decrease was found during the shelf life.

3.6.3 Vitamin C, Carotenoids and Chlorophylls

Together with glucosinolates and phenolic compounds, there are other bioactive compounds of interest in rocket salad, including vitamin C, carotenoids and chlorophylls. Vitamin C is a hydrosoluble molecule that can be present in reduced form or as ascorbic acid (AA), and an oxidized form or dehydroascorbic acid (DHAA). The latter can be regenerated in vivo into ascorbic acid, or irreversibly hydrolysed to diketogulonic acid (Adikwu and Deo 2013).

Vitamin C is an essential micronutrient but it is also a direct/indirect antioxidant when it is found in its reduced form (Adikwu and Deo 2013; Ashor et al. 2019). It can reach high levels in rocket salad, mainly in the form of ascorbic acid, although great variability can be found in the literature. Guijarro-Real et al. (2017) determined a percentage of DHAA below 6% of the total vitamin C. This percentage was significantly higher in the results of work by Gutiérrez et al. (2018), which revealed that DHAA represented up to 32% of the total vitamin C. Similar levels were found by Xiao et al. (2012) in microgreens, in which the percentage of DHAA represented approximately 28% of total vitamin C.

Most research studies focus on the study of AA since this is the form with antioxidant capacity. Levels are usually above 55 mg AA 100 g^{-1} FW (Acikgoz 2011; Guijarro-Real et al. 2017; Gutiérrez et al. 2018), while in the case of microgreens lower levels have been found (Xiao et al. 2012). The work of Colonna et al. (2016) showed significantly increased values, with an average content of 86.2 mg AA

100 g^{-1} FW. The accumulation of ascorbic acid can be affected by cultivation practices and also postharvest handling and storage. Barbieri et al. (2011) found very low contents for plants growing under hydroponic cultivation, as compared to the above cited works, with values of 15–20 mg 100 g^{-1} FW depending on the degree of salt stress applied. Nevertheless, those authors detected that leaves sprayed with 20 mM proline increased levels up to 30 mg 100 g^{-1} FW, which may correspond to the osmoprotective nature of this compound. On the contrary, cultivation under high or low light intensity, or in the field or greenhouse during spring, may not have a clear effect on the accumulation of AA (Colonna et al. 2016; Guijarro-Real et al. 2017). Regarding the postharvest conditions, Gutiérrez et al. (2018) found that both the UV-C radiation and the ozone treatments reduced the levels of AA. In addition, storage of both treated and control leaves decreased the levels up to 50% within 8 days. Finally, Barbieri et al. (2011) found that the decrease in the content in AA during storage may correspond to its oxidation to DHAA.

Chlorophylls and carotenoids are photosynthetic compounds with bioactive properties and can be found in high amounts in leafy vegetables, especially the former (Guzman et al. 2012). Chlorophylls can be estimated as a sum of chlorophyll *a* and chlorophyll *b*. Gutiérrez et al. (2015) measured total chlorophyll content in 210.5 mg 100 g^{-1} FW, from which around 70% corresponded to chlorophyll *a*. By contrast, those authors found that materials from other geographic areas had a lower content (on average, 98.4 mg 100 g^{-1} FW), while the percentage in chlorophyll *a* in these materials increased to 77% (Gutiérrez et al. 2017). These works showed that the treatment of the harvested product using UV-C irradiation reduced the levels up to 20% compared to untreated leaves. However, the treatment with gaseous ozone would not negatively affect the levels of chlorophylls (Gutiérrez et al. 2017). Apart from the treatments used, levels were further reduced during shelf life, although previous treatments may protect the chlorophylls, thus reducing the relative degradation during storage.

Other works evaluated instead the relative content in chlorophyll as SPAD units. Colonna et al. (2016) and Egea-Gilabert et al. (2009) obtained values of a 32.0–45.6 SPAD index. Such studies showed an inference of the light intensity on the levels measured, and also the presence of genetic variation. In a similar way, Jin et al. (2009) had found that growing plants under low light intensity was translated into a higher accumulation of chlorophylls, but those materials were more affected during shelf life with a faster senescence.

Carotenoids exhibited similar responses as chlorophylls in many cases. In rocket materials, this class of compounds mainly include lutein, zeaxanthin, violaxanthin and β-carotene. Xiao et al. (2012) evaluated microgreens of rocket salad. The levels obtained for β-carotene, lutein/zeaxanthin and violaxanthin were 7.5, 5.4 and 2.6 mg 100 g^{-1} FW, respectively. According to this study, microgreens may accumulate higher contents than mature leaves, although Gutiérrez et al. (2015, 2017) determined estimated values of 22.4–38.6 mg 100 g^{-1} FW on average.

Preharvest practices and growing conditions can affect the accumulation of carotenoids and may be used to increase their levels in rocket salad. According to

Esteban et al. (2014), moderate drought stress can significantly increase the content in total carotenoids. But the most effective treatment for such an increase is probably the exposure of plants to high light intensity, as they suggest. Thus, exposure for 4 days resulted in a significant increase within the next 14 days. Other practices for increasing such levels could include the use of osmoprotective compounds. Barbieri et al. (2011) found that spraying the plants with 20 mM proline increased the levels in total carotenoids by 8–18%.

As for the content in chlorophylls, carotenoids may be also affected by decontamination treatments. Gutiérrez et al. (2015) observed that the UV-C irradiation reduced initial levels by up to 15%. However, these results were not consistent in a second work (Gutiérrez et al. 2017), where the authors found that neither the treatment with irradiation nor gaseous ozone reduced the initial levels. Regarding the effect of storage, different results have been obtained. Barbieri et al. (2011) observed that levels in total carotenoids were higher for products that had been exposed daily to light conditions, compared to those ones stored in the dark. In any case, the prolonged storage reduced the initial contents. On the contrary, Jin et al. (2009) had described a great stability within 7 days of storage, with no differences for plants growing at different light regimes. Gutiérrez et al. (2015, 2017) showed that contents were reduced during shelf life, although the percentage of degradation could be reduced with an irradiation treatment prior to storage. Reduction within 12 days may reach up to 35% of the initial content.

3.6.4 Nitrates

Nitrates accumulate in plant tissue after root uptake, if the levels exceed the capacity of assimilation by the plant. Nitrates themselves are not toxic, although they can cause methemoglobinemia in infants and certain ethnic groups (Bondonno et al. 2018). By contrast, they may have the potential to derive carcinogenic *N*-nitrose compounds in the human body, although epidemiological studies are not clear in this regard (Quijano et al. 2017).

Considering that rocket salad is a hyperaccumulator (Santamaria 2006), the evaluation of the nitrate content has been addressed in numerous studies. The Commission Regulation (EU) No 1258/2011 (European Commission 2011) imposed maximum limits for the commercialization of rocket salad across Europe: 1) 6000 mg NO_3^- kg^{-1} for products harvested from 1 April to 30 September, and 2) 7000 mg NO_3^- kg^{-1} for harvests between 1 October and 31 March. The season affects the accumulation of nitrates, since low light intensity (commonly found in the autumn-winter season) reduces nitrate reductase activity thus increasing the levels in nitrates (Colonna et al. 2016). These authors found that plants harvested under low irradiance accumulated around 36.5% more nitrates than those obtained under high irradiance. Kyriacou et al. (2019) evaluated different commercial products acquired from markets during winter and summer cycles and found average values below 4000 mg kg^{-1} FW. However, these authors determined levels above the

maximum limits in both seasons, up to 6946 mg kg^{-1} FW in the summer, and 8279 mg kg^{-1} FW during winter. Colonna et al. (2016) also observed in their study an effect of the light intensity, although these authors grew plants that did not exceed limitations.

In addition, the use of different agronomic practices, such as the fertilizer management and the use of hydroponic cultivation, can also affect the nitrate accumulation. Different researchers have obtained adequate levels under hydroponic cultivation, as by Barbieri et al. (2011), Bozokalfa et al. (2009, 2011), Colonna et al. (2016), Egea-Gilabert et al. (2009); Fontana and Nicola (2009) and Vernieri et al. (2005). Fontana and Nicola (2009), however, observed that the hydroponic cultivation increased the levels up to 61% compared to the soil system. Regarding the use of fertilizer, Santamaria et al. (2002) determined that an increase in up to 52% can be obtained under different N solutions at a range of 1–8 mM. In a study by Kyriacou et al. (2019), the use of a basal-dressing N rate of 200 kg ha^{-1} produced an increase of 40–76.6% compared to control, for the winter and summer cycles, respectively. Kim et al. (2006) had previously determined that fertilizers combining NH_4^+/NO_3^- ions could be used to significantly reduce the levels in plants up to 50% when compared to the nitrate fertilization. However, the use of ammonium fertilizer would not be appropriate due to the toxicity of this ion to plants. Moreover, Vernieri et al. (2005) found that the use of biostimulants also had a positive effect on the accumulation of nitrates in rocket salad, so authors suggest its combined used as an alternative to reduce application of fertilizer in hydroponic cultivation.

3.7 Genetic Improvement Approaches

3.7.1 Genetic Improvement Objectives

Several are the constraints that affect rocket salad cultivation while few are the efforts toward genetic improvement. The overall increase of cultivated area and related consumption require a great effort to obtain new varieties suitable to meet the main challenges of the current agriculture such as climate change, emerging disease, and novel trends in consumption. The choice of appropriate varieties able to provide constant performance over time and space, and meeting market needs, is pivotal.

Breeding strategies for specific plant traits includes the improvement of pharmaceutical and nutritional properties, shelf life and increased resistance to biotic and abiotic factors like diseases, insects, drought, salinity and extreme temperatures expected under predicted global climate change. The use of partially-resistant cultivars is an interesting option, especially when associated with preventive cultivation measures (irrigation systems, ventilation, sowing density, use of certified seeds), as they reduce the multiplication of the inoculum and delay the progression of the infection. Also, partially-resistant responses are usually more durable and effective against different pathogen types.

Plant architecture and leaf morphology are the main agronomic traits to consider for varietal selection, having an impact on harvesting, yield and shelf life. Different typologies available in germplasm collection can be used as parent lines in breeding programs (Figs. 3.3 and 3.4).

Fig. 3.3 Example of morphological variability of *Eruca vesicaria* ssp. *sativa* accessions grown in open fields. (**a**) Marked leaf indentation, (**b**) Smooth leaves with an entire margin

Fig. 3.4 Variability of *Eruca vesicaria* ssp. *sativa* accessions grown in pots. (**a**) Leaf rosette with evident lobation, (**b**) Indented leaves with a remarkable spine, (**c**) Smooth leaves with evident apex lobation, (**d**) Leaves with entire margins, (**e**) Marked central leaf-spine, (**f-g**) Inflorescences with white petals, (**h**) Leaves with a pronounced central rib, (**i**) Leaf rosette with an erect bearing

3.7.2 Traditional Breeding

Unlike other crops, in rocket salad there is no possibility of obtaining fertile progenies deriving from crosses with wild relatives. In the case of wild rocket, it belongs to a different genus (*Diplotaxis*) and the related intergeneric crosses are unsuccessful in the transfer of genes of interest (Tripodi et al. 2017). Various intergenic hybrids have been obtained between *Brassica* species and *Eruca sativa* (Table 3.3) by the embryo rescue approach. However, crosses with *Brassica* species are not always possible due to incompatibility mechanisms (pollen-stigma interactions) (Sun et al. 2005). Intergeneric crosses have been applied to transfer cytoplasmic male sterility from *Brassica napus* or *B. oleracea* (Merete et al. 2008) to *E. sativa* plants and vice versa. Some *E. sativa* accessions are indeed a good source of sporophytic self-incompatibility genes and male sterility for *Brassica* species. By intergeneric crosses between *B. rapa* and *E. sativa,* a cytoplasmic male sterile line of *B. rapa* containing the cytoplasm genome of *E. sativa* was developed by Matsuzawa et al. (1999).

The breeding approaches used in this work required two main strategies:

(a) Embryo rescue after the first cross hybridization, subsequent chromosome doubling and backcrossing of the resulting hybrid *Brassica* x *Eruca* to *Eruca*.
(b) Using protoplast fusion from cytoplasmic male sterile *Brassica*. Somatic cell fusion allows overcoming the sexual incompatibility barriers between species. Subsequently, the regeneration of allogenic cells and crossing of the regenerated plant with pollen from *E. sativa* would be needed.

Embryo rescue consists of the manual excision of embryos after fecundation and their culture in a medium providing proper nutrients to support survival and growth. From embryo culture, a new individual can be generated. The method overcomes postzygotic barriers (endosperm abortion) occurring in distantly-related species.

Protoplast fusion (somatic fusion) consists of the removal of the cell wall through enzymatic methods to obtain protoplasts which are then fused using chemical or

Table 3.3 Intergeneric crosses between *Brassica* and *Eruca*

Type of cross	References
Brassica napus x *Eruca sativa*	Dai et al. (2004)
B. rapa x *E. sativa*	Agnihotri et al. (1990); Matsuzawa et al. (1999)
E. sativa x *B. juncea*	Sikdar et al. (1990)
B. oleracea x *E. sativa*	Merete et al. (2008)

physical (electroshock) treatment. The fusion allows the joining of cells and of their nuclei.

Both embryo rescue and protoplast fusion were obtained using different concentrations of auxin, cytokinin and naphthaleneacetic acid (Slater 2013).

Genetic improvement in *Eruca* mainly refers to crossbreeding and selection activities. Molecular approaches for gene mapping and QTL analysis as well as biotechnological strategies for genetic improvement are not yet reported. However, protocols for *Agrobacterium*-mediated transformation are available in *Eruca* (Slater 2013) and can be successfully applied.

3.7.3 Male Sterility and Double Haploids

As a minor vegetable crop, rocket salad has not gained much attention by the research and breeder communities and there are not many studies focused on its improvement. Nevertheless, some authors have attempted to facilitate the breeding opportunities for rocket salad, for example, by working on CMS-lines or doubled haploids that could be useful for the development of hybrid varieties.

Male sterility has been suggested as an alternative in breeding programs for the development of hybrid lines by several authors. Despite the potential interest noted, studies in this line are very scarce, while the use of *Eruca sativa* for improving *Brassica* crops has been more explored. In the case of rocket salad improvement, we should cite the work of Nothnagel et al. (2016). These authors developed male sterile lines of rocket salad by sexual hybridization with a cytoplasmic male sterile *Brassica oleracea* line. After five cycles of backcrossing with an *E. sativa* genotype, the genome of *B. oleracea* was considered as removed and tested by RAPD and GISH analyses, while the mtDNA and the cpDNA remained. The morphology and vigor of the developed lines did not differ from the *E. sativa* parent. This work could result in promising interest in the improvement of rocket salad, allowing for control of directional crosses in the breeding programs.

Also, the study of doubled-haploid production in rocket salad has been addressed. Leskovsek et al. (2008) published the first work in this line of research. The authors reported a successful protocol that allowed obtaining embryos from cultures of microspores, although a high variability in the results was reported due to the heterogeneity of the parental material. Despite the good response in the development of embryos, the percentage of conversion of plantlets from the embryos obtained was low for most of the treatments evaluated. In any case, the majority of the plantlets obtained (66%) were 2*n*, but whether they were diploid or double haploid remained uncertain. Despite the promising interest in doubled haploids for crop improvement, this research topic has not been further developed.

3.7.4 Somatic Embryogenesis

Plant regeneration through somatic embryogenesis has been obtained in cultivated rocket. Somatic embryogenesis is a process in which a plant is regenerated from a single somatic cell, which derives from tissue not normally involved in embryo development. Embryogenic culture is a suitable method for rapid plant regeneration of many plant species. The method, established by Chen et al. (2011), reports the successful production of embryogenic calli from over 90% of explants, mainly cotyledons. After preliminary sterilization with ethanol and hydrochloric acid, seeds were cultured on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) until germination. Cotyledon and hypocotyls were then cut and placed onto MS medium with different concentrations of hormones. Subsequently, the obtained calli with buds were propagated to produce new somatic embryos. In the various steps, Chen et al. (2011) optimized the protocols and concentrations of different chemicals and hormones to use for the development of somatic embryos (1.0 mg/l of 2,4-dichlorophenoxyacetic acid, 0.1 mg/l of kinetin, 6-benzylaminopurine, 0.1–0.5 mg/l of 1-naphthaleneacetic acid, 60–80 g/l sucrose).

The same research group identified three main proteins involved in the development of embryogenic callus, reporting the enolase highly expressed high transcription in embryogenic callus and not expressed completely in the nonembryogenic (Chen et al. 2012).

These studies are effective for breeding purposes and can be used in the application of further genome editing approaches.

3.8 Conclusions and Prospects

Despite global consumption of rocket salad has increased recently, few efforts has been made to develop new varieties by both private and public breeding programs. Although large genetic variability exists, breeding activities are mainly still carried on by traditional selection schemes such as single seed descent or mass selection. A lack of experimental mapping populations and QTL studies limits the exploitation of germplasm resources. Moreover, few genomic resources have been established.

New possibilities for the dissection of the genetic basis of complex traits and the development of molecular marker-assisted selection and breeding may be obtained by genome-wide association approaches. Furthermore, novel genotypes may be developed by tilling and mutagenesis. Genome editing is a promising approach for genetic improvement, however no studies are yet reported. Therefore, for *Eruca*, different approaches could be exploited in genetic research. Cultivars with increased amounts of nutraceutical compounds, resistance and other qualitative properties, will make the crop more attractive for consumers, growers and processing industries, respectively.

Appendix I: Research Institutes Relevant to Arugula or Rocket Salad (*Eruca vesicaria* ssp. *sativa*)

Institution name	Specialization	Address	Email and website
Centre for Genetic Resources (CGN)	Germplasm bank	Wageningen University, 6708PB Wageningen The Netherlands	https://www.wur.nl/en.htm
Leibniz-Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK)	Germplasm bank	Corrensstraße 3, 06466 Gatersleben, Germany	https://www.ipk-gatersleben.de/
Germplasm Resources Information Network (USDA-GRIN)	Germplasm bank	United States	https://www.ars-grin.gov/
Universidad Politécnica de Madrid (UPM)	Germplasm bank	28,040 Madrid, Spain	http://www.upm.es/
Millennium Seeds Bank KEW	Germplasm bank	United Kingdom	https://www.kew.org/
Research Centre for Vegetable and Ornamental Crops (CREA-OF)	Breeding, genetics, agronomy, pathology	Via Cavalleggeri 25, Pontecagnano-Faiano 84,098, Italy	e-mail: pasquale.tripodi@crea.gov.it, https://www.crea.gov.it/en/web/orticoltura-e-florovivaismo
Instituto Nacional de Investigação Agrária e Veterinária (IP)	Agronomy, breeding, genetics	Av. da República, Quinta do Marquês, 2784–505 Oeiras, Portugal	e-mail: paula.coelho@iniav.pt; http://www.iniav.pt/

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Chapter 4
Spring Onion (*Allium fistulosum* L.) Breeding Strategies

Fatimah Kayat, Arifullah Mohammed, and Ahmed Mahmood Ibrahim

Abstract Spring onion (*Allium fistulosum* L.) belongs to subgenus *Cepa,* genus *Allium* and family Liliaceae and popularly known as scallion, Welsh onion and Japanese bunching onion. Cultivation of spring onion dates back to 200 BC in China and reached Japan before 500 AD which later spread to Southeast Asia. Spring onion is grown worldwide, however the main area of cultivation remains in East Asia from Siberia to tropical Asia including China, Taiwan, Japan, the Philippines, Malaysia and Indonesia. The plant is a perennial herb, does not produce bulbs, and possesses hollow leaves and has traditionally been used in Chinese folk medicine to treat common cold, influenza, abdominal pain, headache and cardiovascular disease as well as having antifungal and antibacterial effects. Spring onion is known for its flavor and aroma and is a rich source of vitamin C, A and B_6, thiamine, folate, rhamnose, galactose, glucose, arabinose and xylose. Production of F_1 hybrids is considered as one of the main goals in crop breeding. The length of time taken is the main restriction in breeding programs as eight or more generations of inbreeding are needed to establish homozygous lines that can be applied in hybrid production. This process can be enhanced by using doubled haploid (DH) lines as components of hybrid cultivars. In this chapter, we give an overview of the origin, botanical classification, distribution, reported health benefits, genetic resource and conservation, crop cultivation practices and recent advances on biotechnology, and molecular biology and their application for crop improvement in connection with traditional breeding methods of spring onion. In this aspect, mutational breeding and somatic hybridization are the potential approaches for the development of new high-yielding and disease-resistant cultivars.

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Keywords *Allium fistulosum* · Japanese bunching onion · Scallions · Spring onion · Welsh onion

4.1 Introduction

Spring onion (*Allium fistulosum* L.) belongs to the Liliaceae family and in some countries is known as scallion, green onion, Welsh onion, salad onion, Japanese bunching onion, Spanish onion, two-bladed onion and green trail (Fig. 4.1). Other local names include *cong* in China; *ciboule*, *oignon de Strasburg* in France; *rohtenlauch*, winter *zwiebel* in Germany; *negi* in Japan; *pa* in Korea; *pijplook*, *bieslook* and *indischeprei* in The Netherlands, *cebolla*, *ceboletta* in Spain and *chung* in Taiwan (Inden and Asahira 1990; Sang et al. 2002). *Allium fistulosum* is a perennial, herbaceous plant which is usually grown for its edible tops, long leaf bases or young shoots. It does not develop bulbs, and possesses hollow leaves (*fistulosum* means *hollow*) which differ from leek, where their leaves are flat. A large number of *A. fistulosum* varieties resemble the leek, such as the Japanese *negi*, while smaller varieties resemble chives. Leaves of *A. fistulosum* are somewhat rounder in cross-section, not flattened adaxially.

Fig. 4.1 Spring onion sold at West End Market, Brisbane, Australia

4.1.1 Botanical Classification and Distribution

The inflorescence of spring onion lacks bracteoles and the size of the flower is about twice of *Allium cepa*. The scape is round, hollow, 40–75 cm tall, and does not have the typical bulge of onion scapes. Diameter of the umbel ranges from 3 to 7 cm and is not spherical. The order of opening of the pale yellow flowers begins at the top of the umbel and proceeds toward the base and becomes a distinguishing feature in contrast to most other alliums except chives (Rubatzky and Yamaguchi 1997). The filaments of the stamens are more protruded, they lack basal teeth and are not broadened at the base. This type of onion is harvested at an immature stage before the bulb has fully developed. Some useful characteristics in identifying different *Allium* vegetables are shown in Table 4.1. Their flavor and aroma much milder than red onion; they are eaten raw in salads and sandwiches. The green tops are used as a garnish or sliced in salads or stir-fries. It has been a common food to humans since the earliest times, along with garlic, leek, chive, bulb onion and shallot.

Spring onion belongs to subgenus *Cepa*, genus *Allium* and family Liliaceae; the basic chromosome number of *A. fistulosum* is 8; (2n = 2x = 16). The size of spring onion, *A. fistulosum* genome is estimated to be 11.7 pg/1C or 1.2×10^4 Mbp (Ricroch et al. 2005). It is self-compatible with a high degree of cross-pollination. Self-pollination may occur in some plants and show inbreeding depression and poor growth. Landraces and open pollinated varieties exhibit high levels of heterozygosity and heterogeneity (Tsukazaki et al. 2006; Wako 2016).

Table 4.1 Useful characteristic in identifying *Allium* vegetables

Allium	Chromosome number	Flower color	Bulb formation	Bulbils in inflorescence
Onion and shallot	16	White, green striped	Yes	Absent in most cultivars
Garlic	16	Lavender to pale green and white	Yes	Very common
Great headed garlic	48	White to purple	Yes	Usually not
Spring onion	16	Pale yellow to white	No	Absent in most cultivars
Chive	16, 24, 32	Purple or rose, rarely white	No	Rarely
Chinese chives	32	White	No	No
Rakkyo	16, 24, 32	Rose-purple	Yes	No
Leek and kurrat	32	White to purple	No	Sometimes

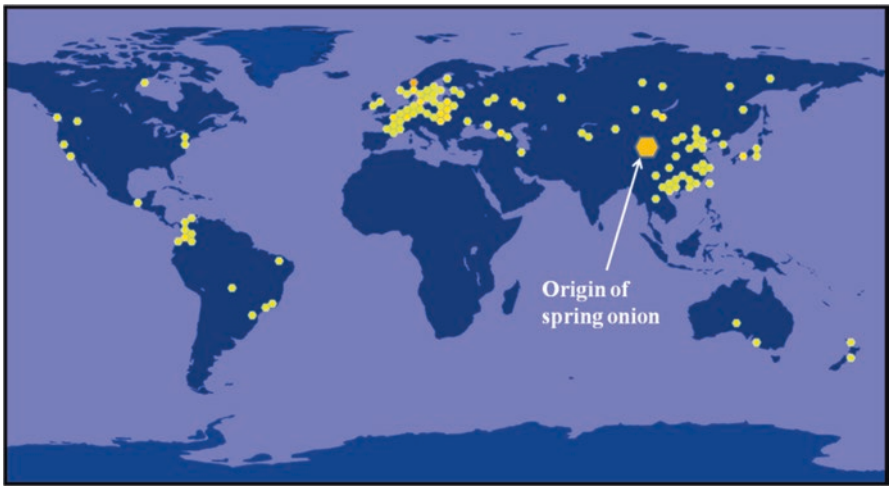

Fig. 4.2 Map of origin and distribution of spring onion. Yellow dots indicate production sites

4.1.2 Origin and Domestication

Traces of alliums have been found in the remains of Bronze Age settlements. Seven major edible *Allium* species from 500 to 650 varieties of *Allium* worldwide are among of the earliest domesticated crops for its flavor, medicinal and nutritional properties (Shigyo et al. 2018). Plants with the largest and quickest-growing bulbs were selected by early farmers which probably originated in northwestern China (Sang et al. 2002; Tsukazaki et al. 2010). Cultivation of spring onion dates back to 200 BC in China and reached Japan before 500 AD and later spread to Southeast Asia (Fig. 4.2). The earliest description of the crop and its cultivation is found in a Chinese book of 100 BC and mentioned the first time in Japanese literature in 720 AD (Inden and Asahira 1990).

4.2 Health and Economic Importance

4.2.1 Health Benefits

Allium fistulosum has traditionally been used in Chinese medicine to treat the common cold, influenza, abdominal pain, headache and cardiovascular disease. According to a dictionary of Chinese drugs, the bulbs and roots are used for treatment of febrile disease, headache, abdominal pain (Yamazaki et al. 2016), diarrhea, snakebite, ocular disorders, habitual abortion, as well as having antifungal and antibacterial effects (Sang et al. 2002; Ueda et al. 2013). Extracts from spring onion are a potential source of natural antioxidants (Aoyama and Yamamoto 2007; Wang

et al. 2006) and antimicrobial agents (Chang et al. 2013). It is also reputed to improve eyesight, help in digestion, perspiration, recovery from wounds and festering sores. It was traditionally served to sick people as a preservative against *evil spirits*. Kang et al. (2010) suggested that their fibrous roots have potential for as a hypoglycemic agent in controlling blood glucose.

Several studies indicate that *Allium fistulosum* is a rich source of vitamin C, A and B_6, thiamine, folate, potassium, copper, manganese, iron and chromium (Table 4.2). Leaf blades are rich in rhamnose, galactose, glucose, arabinose and xylose (Inden and Asahira 1990). The organoleptic quality of leaf-bunching onions improves under low temperature, which increases well-hydrated gels of cellulose, hemicellulose, protopection and water-soluble pectin, which embed free sugars

Table 4.2 Nutritional value of spring onion, *Allium fistulosum* per 100 g

Spring onion (raw), Nutritional value (per 100 g)		Daily values (%)
PROXIMATES		
Energy (kJ/Cal)	32 kJ (135 kcal)	2
Protein (g)	1.83	4
Fat (g)	0.19	n.a
Water (g)	89.83	n.a
Carbohydrate (g)	7.34	2
Sugars (g)	2.33	n.a
Dietary Fiber (g)	2.4	10
Ash (g)	0.81	n.a
VITAMINS		
Folate, Vit. B9 (µg)	64	16
Thiamine, Vit. B1(mg)	0.55	4
Riboflavin, Vit. B2 (mg)	0.08	5
Niacin, Vit. B3 (mg)	0.525	3
Vitamin B6 (mg)	0.061	3
Vitamin A (IU)	997	20
Vitamin C (mg)	18.8	31
Vitamin E (µg)	0.55	3
Vitamin K (µg)	207	259
MINERALS		
Calcium, Ca (mg)	72	7
Iron (mg)	1.48	8
Magnesium, Mg (mg)	20	5
Phosphorus, P (mg)	37	7
Potassium, K (mg)	276	8
Zinc, Zn (mg)	0.39	3
Sodium, Na (mg)	16	1
Copper, Cu (mg)	0.083	4
Manganese, Mn (mg)	0.16	8
Selenium, Se (µg)	0.6	1

Source: USDA National Nutrient Database for Standard Reference (2016)

carbohydrates storage in the leaf (Hang et al. 2004; Inden and Asahira 1990). Flavor is attributed to the enzyme allinase which acts on sulfur compounds when the tissues are disrupted. The strength of flavor increases with plant age (Peffley 1992). Spring onion is known to contain amino acids and peptides such as cysteine, and glutathione which act as a redox agent in dough, improving bread making properties (Seguchi and Abe 2003)

Flavonoids, carotenoids and some sulfur compounds are phytonutrients found in spring onion. They are packed with highly effective flavonoids known for their health-promoting effects. Aoyama and Yamamoto (2007) studied the effect of thermal treatment on antioxidant activity and flavonoid content, and observed an increase in antioxidant activity and decrease in flavonoids of green spring onion compared to three other vegetables (yellow and red onion varieties and white-sheath spring onion) during the boiling procedure. The study suggested that green Welsh onion, but not the white one, is a potent antioxidant food comparable to yellow onion, and is a good source of kaempferol. It possesses anti-platelet, antioxidative, anti-hypertensive and anti-hyperlipidemic properties (Sung et al. 2015), and may lower cholesterol level and decrease the risk of heart attack and stroke. Several studies indicate that spring onion has excellent antibacterial and antifungal properties. Sung et al. (2018) suggested that *Allium fistulosum* extracts could be used as functional food materials for weight control in obesity. The roots contain allicin and diallyl disulfide and have the potential to reduce the body fat mass.

4.2.2 Economic Importance

Spring onion is grown throughout the world, but the main area of cultivation remains in East Asia from Siberia to tropical Asia including China, Taiwan, Japan, the Philippines, Malaysia and Indonesia (Inden and Asahira 1990). It is an important ingredient in Asian cuisine particularly in China, Japan, Korea and Southeast Asia. In Japan, it is the second most important ingredient after *Allium cepa* but it stands first in China, where it is used in different dishes. Shredded green onions often make up the *green* part of the five colors in a traditional Korean and Japanese meal. It is used as flavoring marinade in *bulgogi* (Korean grilled beef) and to flavor kimchi. Negi, which is also known as Welsh or spring onion, is among the most important ingredient in Japanese cuisine. It is usually served with soba noodles and tofu and always found in miso soup, negimaki and widely used as a garnish in noodles or stir-fried. In Southeast Asia it is mainly grown for salads, or as an herb to flavor soups and other dishes. Dehydrated spring onion is used as an additive to pre-processed food such as instant noodles, potato chips and others. The young inflorescence is sometimes deep-fried and consumed as a snack. Fried *Allium* oil is widely used in traditional Chinese cooking and has recently grown in popularity in the food manufacturing industry (Zhang et al. 2019).

Statistical analysis spring onion production is often combined with other *Allium* spp. *Allium* vegetables gross production in 2014 were valued at USD 61,348 million

with 4% of it is contributed by green onions and shallots (Galmarini 2018). The main world production of this vegetable is in Japan, South Korea, China, and Taiwan ranking in their top ten vegetable crops. Bunching onion has the highest annual output of *Allium* crops in Japan in 2014 (Tsukazaki et al. 2017). The annual production in Japan is about 500,000 mt from 23,000 ha, whereas in South Korea, around 27,000 ha produces 723,000 mt, and in China, 20,754,000 mt from 545,000 ha. The main production areas are distributed in the southern parts of these countries (Galmarini 2018). Data on annual consumption per person shows their consumption is 6.6 kg/capita/year in Korea, 1.7 kg/capita/year in Japan and 5.1 kg/capita/year in China (Galmarini 2018). Meanwhile, the annual production in Indonesia in 1988 amounted to 163,000 mt from 24,500 ha. In the USA, they are grown mainly in Monterey, Riverside, and Ventura counties in California and in Arizona, Georgia, Idaho, New Mexico, Oregon, Texas and Washington. In South America, Colombia has a considerable production of Japanese bunching onion for domestic consumption (Galmarini 2018). It is the second largest vegetable crop produced in Colombia, with 327,000 mt from 17,000 ha.

Given the enormous area of cultivation, great adaptability to climate and varied uses, a very large number of cultivars exist. Each cultivar has a characteristic commercial value due to the proportion of green leaves and white sheathes (Yamazaki et al. 2016). The Kumazawa system, based on utilization and ecological characteristic, is widely accepted as a method to classify spring onion in Japan. The system divides the Japanese bunching onion into four groups. *Kaga* which show little tillering and is dormant in winter, has dark green thick leaves and is grown for the pseudostems. *Kujyo* which remains green during winter; its cold tolerance is generally low and it has tender, green leaves of excellent eating quality; they tiller profusely and are mostly grown for their green tops. *Senju* which continues to grow during winter, although at a reduced rate, is intermediate between the former two and is mainly grown for the pseudostem. Meanwhile, *Yaguranegi* produces numerous tillers in spring and summer but its growth stops in winter, produces no flowers but only bulbils; it is propagated by division of the basal cluster or bulbils (Inden and Asahira 1990).

Spring onion is among the 15 most important crops in China. It is widely cultivated in Shandong, Henan, Hebei and Shaanxi provinces (Wang et al. 2018). It is preferred compared to bulb onion as it has a stronger flavor to enhance Chinese cuisine. There are more than 200 cultivars of Chinese spring onion which are classified as long pseudostem type, LP (long pseudostem, soft leaves, low soluble solids concentration and mild flavor), fleshy root types, FR (fleshy roots, high soluble solids concentration, stronger pungency and larger sheath diameter) and short pseudostem type, SP (intermediate between the other two types) (Liu et al. 2009).

On the island of Java, Indonesia, the three types of spring onion planted are *bawang bakung* which is grown for the pseudostem; *bawang cina* for the leaves and *bawang daun* which is the intermediate between the two. Among the popular Indonesian cvs. are Plumpung, Mambo, Nyonya, Siih Kecil and Tosari. Mixed cropping with white cabbage, carrot and potato is common in the highlands (Sulistriarini et al. 2016).

The main varieties of spring onion grown in Australia are Straight Leaf, Dynasty Winter King and Summer King. The immature bulb of bulb onion (*Allium cepa*) varieties such as early Lockyer White, South Australian White, Savages White and Gladalan White are sold as spring onion if harvested early with leaves intact. It is estimated that about 3 million bunches, or 1000 mt, of spring onion are produced from the Swan Coastal Plain, mainly between Wanneroo and Baldivis, in Western Australia (Burt 2007).

4.3 Current Cultivation Practices

Spring onion is easy to grow. It maintains vegetative growth all year round except in winter. Propagation is generally by seed and/or transplanted as seedlings in early spring, summer or autumn (Zhu et al. 2019). The crop produces tillers and can be propagated by seed or division. Division is preferred by most growers as it ensures that they have the chosen clone, especially where winter hardiness is an important requirement. Although it grows throughout the year, the best quality and highest yields are produced in late spring. Żurawik et al. (2013) showed that the highest marketable yield obtained from seeds sown at the end of April compare to early April in cv. Sprint. In temperate areas, propagation is mainly by seed, sown directly in the field or in nurseries before being transplanted into the field. Seed requirements are 8–16 kg/ha for direct seeding while 2–4 kg/ha for transplanting. In nursery beds, seeds are either broadcast or sown in rows or in 5–6 cm wide bands. Seedlings are ready for transplanting at about 15 cm height when it has pencil-thick base.

In Southeast Asia the crop is propagated mainly using basal tillers and can be planted the whole year round. Plants are rarely raised from seed as imported cultivars are more difficult to sow under tropical conditions and more time-consuming. In Indonesia, spring onion is planted in uplands as well as on dry paddy fields. In Java, it grows well above 200 m elevation, but it is more common above 500 m. Tillers are transplanted into raised beds or ridges, which are alternated with furrows for irrigation and drainage. There are many local selections and commercial cultivars, reflecting the adaptation to a wide range of climatic conditions. Most spring onion cultivars are well adapted to variations in rainfall and more tolerant of heavy rainfall than other *Allium* spp.

In Poland, spring onion is grown on a small scale (Majkowska et al. 2014) and mostly cultivated as a perennial crop out of rotation for the period of 3–4 years. Cultivars with a short pseudostem, strong tendency to tillering, abundant foliage production and fast regrowth after harvest like Siedmiolatka Czerwona, Kroll, Vita or Flamenco, are usually recommended beside other high yielding cultivars including Parade, Sprintesa, Performer, Ishikura Long White and Totem (Adamczewska-Sowinska and Kolota 2014). In Brazil, spring onion is grown throughout the year in highland regions and during autumn-winter in lower regions. Cultivars grown

include Todo Ano, Nebuka, Evergreen, Natsu Hosonegui, Futonegui and Ibirité (Marcuzzo and Carvalho 2014).

The main advantage of growing from transplants is the possibility of obtaining earlier yields (Tendaj and Mysiak 2011). Seedlings are planted in furrows, and roots and bases are lightly covered by soil. The depth of the furrow is about 15 cm for pseudostem production and 5 cm for green top production. Distance between rows and within rows are 55–85 cm and 3–15 cm, respectively, depending upon tillering tendency (Warade and Kadam 1998). Single plants are grown in a square spacing 20 × 20 cm or 25 × 25 cm depending on the vigor of the clone. Seedlings are planted in groups of 3 or 4 approximately 20 cm each way when using seed (George 2011). Highest yield was obtained in cv. Sprint at a sowing rate of 8 kg/ha^{-1} and grown in rows 20 cm apart (Żurawik et al. 2013). Spring onion can be produced in most types of soil such as sandy loam, loam and clay loams with an optimum soil pH of 5.3–5.8. However, it is more successful at higher elevations (>1000 m) than lower. Ideally, it will grow efficiently in well-drained good fertile soil with good potential moisture retention as it has a sparse, shallow root system, and is easily rooted in waterlogged soil. Sprinklers are typically used, while drip irrigation is uncommon because of close row spacing. Typically, 25–38 cm of water is needed to meet evapotranspiration requirements (Smith et al. 2011).

Recommended rates of fertilizers in cultivation for branched pseudostems on soil with high organic matter is 200–300 kg of nitrogen, 100–200 kg of P_2O_5, and 150–200 kg of K_2O per ha (Kolota et al. 2013). In greenhouse studies spring onion performed best when nitrogen was supplied as nitrate, NO_3^-. Complete fertilizer is usually used during planting time followed by two or three additional applications of nitrogen fertilizer. The use of high N rates to increase large onion bulbs and excessive nutrient supply is common in intensive farms to maximize marketable yield. Due to spring onion's poor root system, this practice may cause the risk of nitrate leaching. Therefore, the application of N fertilizer at the optimum recommended rate is essential in spring onion production (Liu et al. 2009).

A power tiller and a fertilizer applicator-ditcher was developed to reduce labor and cultivation costs, as the task can be completed so much faster than by the conventional way (Katahira et al. 2006). Growth, yield, flavor intensity and nutritional value of spring onion can be highly influenced by genotype, sulfur nutrition, soil type, climatic conditions, edaphic factors and management practices (Abbey et al. 2015). Sulfur fertilization reduces the total soluble solid content by 30% in spring onion cvs. Sydney Bunching as compared to Paris Silverskin. It also reduced the bulb diameters of *Allium fistulosum* cvs. Fragrant and Sydney Bunching, while increasing the bulb diameters in *A. cepa* cvs. Winter White Bunching, Egyptian Bunching, Winter Over and Paris Silverskin (Abbey et al. 2002).

Kołota et al. (2012) found that similar yields can be produced by spring onion cv. Performer harvested in early June to September. Meanwhile, lower yields with a gradual decrease of dry matter, carotenoids, sugars, volatile oils and nitrates have been produced from those harvested in October. Delay in harvesting at 60–120 days after planting results in a substantial yield increment with a simultaneous depletion of vitamin C, carotenoids, chlorophyll a and b, sugars, volatile oils, nitrates, total N,

K and Ca content. Dry matter content in spring onion cv. Sprint yield depends significantly on the sowing date with seed sown in May having higher dry content than those sown in April (Żurawik et al. 2013). Tendaj and Mysiak (2011) showed that spring onion grown from transplants produce longer and wider diameter pseudostems compared to seeds sown directly in the field.

Allium fistulosum usually depends on arbuscular mycorrhizal (AM) fungi for P uptake, as the adventitious root system is shallow, especially in soils with a low to medium P level. Tawaraya et al. (1996) suggested that selection of suitable fungal species and optimal phosphate application is important for spring onion growth. High dependency on mycorrhizal colonization has been observed in cvs. Sydney Bunching, Winter Over, Vilr-Moscow, Ciboule 9379 and Kagoshimahanegi (Tawaraya et al. 2001). Perner et al. (2007) suggested that AM colonization may support the production of organosulfur compounds by compensating growth depression caused by changes in N-form ratios in field conditions.

Suggested field planting density is 400,000–500,000 plants per ha, depending on the production of green leaf or pseudostem, respectively. Green leaves can be harvested 2–3 month after transplanting. However, it will take about 6–9 months to harvest for blanching (Rubatzky and Yamaguchi 1997). Plants are ready to harvest in 8–10 weeks in summer and 12–14 weeks in winter (Burt 2007). Spring onion competes poorly with weeds as they initially grow slowly. Hand weeding is required but it is costly. Harvesting can be coupled with replanting, separating plantlets at intervals to ensure continuity of supply. The production requires a lot of manual labor as each onion must be lifted by hand, trimmed, cleaned and packed into bundles. Several efforts have been made by a number of researchers to develop customized machinery to enhance farm production. Mechanized lifters and trimmers have been developed to speed the harvesting process.

Spring onion varies according to the cultivar in both juvenile age and cold requirement. Su et al. (2007) studied genotypic differences in the interaction between low temperature and photoperiod of spring onion cvs. Chunwei, Changbao and Zhanqiu, and found that each suffered from stress at low temperatures during winter, but differed in their response; Chunwei is the most tolerant, followed by Changbao, while Zhangqiu is the least cold tolerant. Greenhouse culture and plug seedling transplanting of spring onion are common in Japan. It is usually cultivated continuously with other crops without rotation in Hokkaido. Seedlings are raised in the greenhouse for up to 2 months before being transplanted into open fields from late April to mid-June and being harvested 4 months later (August to October). Leaf sheaths of the plants are covered with soil three times by hilling every month to promote etiolated growth. Some farmers cultivate the plants in greenhouses from autumn to the following spring (Misawa et al. 2017).

Vernalization is important in spring onion in order to prevent bolting as it may lower the yield while seed producers prefer to induce flowering to speed up the seed production. Flower induction is controlled by temperature and day length. Low temperatures (generally less than 13 °C) and short days induce flowering when a seedling has more than 11 leaves or is more than 5–7 mm in pseudostem diameter. Specific seedling characteristics and sufficient time at low temperature are required

for bolting. The temperature and time for vernalization may vary with cultivar. Plants are not vernalized when they are grown at more than 20 °C (Inden and Asahira 1990) and under a photoperiod of 16 h. With certain Taiwanese cvs. like Pei Chung, 5 days at 5 °C or 20 days at 10 °C are sufficient for vernalization. Dong et al. (2013) showed that cultivar, sowing date, transplant location and their interactions could influence bolting in spring onion. Bolting-susceptible cultivars are sown in late September to avoid vernalization temperature during winter while bolting-resistant cultivars can be sown earlier. They suggested that sowing around October and transplantation into the open field could delay bolting in cv. Xia Hei, while sowing in late August and transplantation to a plastic tunnel could accelerate flower bud development for seed production. Two mid-season flowering cvs., Kincho and Asagi-kujo, exhibited similar responses to temperature in flower initiation and bolting. However, after flowering was initiated, the early stage of flower development is day-neutral while a long-day photoperiod promotes flower development after the floret formation stage, followed by elongation of the seed-stalk (Yamasaki et al. 2011).

4.4 Pest and Disease Management

Although spring onion is generally a healthy crop, there are several bacterial and fungal diseases common to most *Allium* crops. Several problems regarding yield have occurred due to purple blotch (*Alternaria porri*), which causes concentric spots on the leaves, and downy mildew (*Peronospora destructor*). Most commercial growers follow guidelines for sanitation, crop rotation, use of resistant varieties and frequent monitoring to avoid severe disease outbreaks.

Successive cropping results in white rot (*Sclerotium cepivorum*) infestation as the pathogen is very persistent in the soil. Poor nutrition and heavy rains also can stimulate the disease development. In Victoria, Australia, vegetable growers report that this disease has progressively increased over the years due to frequent use of spring onion monocultures in short rotations with other non-host crops (radish, endive, parsley). Drastic increases of pathogen (sclerotia) populations in the soil lead to high disease levels and considerable yield losses which limit spring onion production. Rot commonly range from 5% to 50%, but in some seasons when soil conditions are favorable for disease development over 80% of plants may be killed (Villalta 2005).

Onion yellow-dwarf virus (OYSV), cycas necrotic stunt virus (CNSV) and tomato spotted wilt virus (TSWV) are pathogenic to Japanese bunching onion (Inden and Asahira 1990). The most important virus disease caused by the onion yellow dwarf virus is transmitted by various aphid species causing mosaic-type symptoms, including chlorotic mottling, streaking and stunting, and distorted flattening of the leaves. The Kujyo group of cultivars was found to be tolerant to this disease. The most serious pests are beet army worm (*Spodoptera exigua*) and the American bollworm (*Heliothis armigera*). They are difficult to control due to the

waxy layer on the leaves and that the larvae hide inside the hollow leaves (Sulistriarini et al. 2016).

Onion thrips (*Thrips tabaci* L.) is one of the most important pests in spring onion causing both direct and indirect damage by feeding and ovipositing on leaves that may cause green onions (scallions) to be unmarketable and dry bulb onion size to be reduced. Rust *(Puccinia allii)* causes serious damage to the leaves. Purple blotch or alternaria leaf spot (*Alternaria porri*) causes heavy losses under moist conditions. Phytophthora blight (*Phytophthora nicotianae* var. *parasitica)* leaf spot *(Pleospora herbarum),* black spotted leaf blight *(Septoria alliacea),* botrytis leaf spot (*Botrytis cinerea)* and fusarium wilt (*Fusarium oxysporum*) are fungal diseases which may affect the crop. Although a parental line with rust resistance has been successfully developed (Wako et al. 2012; Yamashita et al. 2005), it shows only a moderate level of field resistance to rust and does not completely control the disease. Li et al. (2018) reported that cultivation of cucumber with Welsh onion as a companion plant reduces root knots and egg masses of root-knot nematodes (*Meloidogyne* spp.) by 77%.

4.5 Germplasm Resources

Crop wild relatives (CWR) provide breeders with useful traits for crop improvement in a wide range of crops. Collection of germplasm of landraces and wild relatives creates a broad pool of potential genetic resources for breeding and innovative research (Hajjar and Hodgkin 2007). Improvement in gene bank information systems has allowed for better storage and management of a large quantity of data. The genus *Allium* is widely distributed over different regions from the dry subtropics to the boreal zone. Evolution of the genus coevolved with ecological diversification (Fritsch and Friesen 2002). Major efforts to collect source material of spring onion for evaluation and selection, and several trials have resulted in the economically-valuable Premyera variety of Welsh onion in southwestern Siberia (Shishkina et al. 2019).

Spring onion has resistance to several diseases that affect common onion as well, and it exhibits broad adaptability to a wide range of climatic conditions, winter-hardiness and early flowering. Cultivated germplasm forms the primary gene pool of the crop. This clearly indicates the potential for conserving genetic variation in landraces of the species (Ford-Lloyd and Armstrong 1993). Collections of *Allium fistulosum* exist in The Netherlands, Japan, UK, USA, Germany and the former USSR. A total of 975 accessions of *A. fistulosum* are conserved in botanical gardens and gene banks from 18,539 accessions of cultivated *Allium* genetic resources held worldwide (Table 4.3). About 89% of the *Allium* genetic resources are cultivated species (Keller and Kik 2018) with many economically-important species belonging to subgenus *Cepa*, e.g. *A. cepa* (onion, shallot), *A. fistulosum* (bunching onion) and *A. schoenoprasum* (chives).

Table 4.3 Number of accessions of cultivated *Allium* species (sensu Fritsch and Friesen 2002) conserved per subgenus and in botanical gardens and gene banks

Subgenus/species	Number of accessions	Botanical garden	Gene bank
Allium			
ampeloprasum L.	2013	161	1852
sativum L.	4634	130	4504
macrostemon Bunge.	23	14	9
rotundum L.	127	30	97
Cepa			
fistulosum L.	975	105	870
altaicum Pall.	136	42	94
cepa L.	8660	215	8445
chinense G.	33	16	17
oschaninii O.	48	18	30
pskemense B.	66	38	28
schoenoprasum L.	607	256	351
proliferum Schard.	81	0	81
Amerallium			
canadense L.	49	38	11
hookeri Thwaites.	15	11	4
kunthii G.	12	7	5
neapolitanum Cyr	117	76	41
ursinum L.	177	103	74
wallichii Kunth.	30	26	4
Anguinum			
victorialis L.	150	104	46
Butomissa			
ramosum L.	103	45	58
tuberosum Rottl.	299	142	157
Polyprason			
obliquum L.	63	42	21
Rhizirideum			
nutans L.	121	60	61

Knowledge of genetic diversity aids in efficient management of germplasm and selection of parents for crossbreeding. Protein electrophoresis provides a source of marker genes for systematic studies within and between populations. Utilization of genes and their products such as isozymes and allozymes have been used to address issues dealing with local mating patterns, fine-scale structure within populations and broad-scale variation across species (Parker et al. 1998). Several studies using isozymes in *Allium fistulosum* are shown in Table 4.4. Most allozymes represent codominant Mendelian loci. However, their number is limited, and different taxonomic groups may exhibit variation and lack of polymorphism. It may also differ in metabolic function, and gene product could be exposed to selective processes in nature (Parker et al. 1998).

Table 4.4 Isozymes used in *Allium fistulosum*

Isozyme	Purpose	Country	References
Pgm-1 and *Adh-1*	Mapping of enzyme coding genes *Adh-1 and Pgm-1*	USA	Peffley and Currah (1988)
Got, *Idh*, *Pgi* and *Pgm*	Intraspecific differentiation and isozyme pattern in *A. wakegi*	Japan	Okubo and Fujieda (1989)
Adh-1	Introgression of *A. fistulosum* L. into *A.cepa* L.	USA	Peffley and Magnum (1990)
Idh-1, *Adh-1* and *Pgi-1*	Study of isozymes in progeny of (*A. fistulosum* × *A. cepa)* × (*A. cepa*)	USA	Cryder et al. (1991)
Got-1 and Got-2	Study of isozymes in progeny of (*A. fistulosum* × *A. cepa)* × (*A. cepa*)	Netherlands	van der Valk et al. (1991)
Acp-1, *Acp-3, Est-3*, *Got-1, Got-2* and *Lap-1*	Study of isozyme genes in *A. fistulosum*	Japan	Haishima and Ikehashi (1992)
Pgm-1, Adh-1, Acp-1, Acp-3, Lap-1, Got-1 and Got-2	Study of isozymes in *A. fistulosum*	Japan	Haishima et al. (1993)
Adh-1, 6-*Pgdh-1*, 6-*Pgdh-2, Pgm-1* and *Skdh-1*	Study of isozymes inheritance in *A. fistulosum*	USA	Magnum and Peffley (1994)
Got-1 and Got-2	Mapping of glutamate oxaloacetate gene loci	Japan	Shigyo et al. (1994)
Pgi-1	Genetic analysis of *Pgi* isozymes in *Allium* subgenus *Cepa*	Japan	Shigyo et al. (1996)
Lap-1, Got-1, Got-2 6-Pgdh-2, Idh-1, Pgi-1, Adh-1 and Gdh-1	Identification of monosomic addition lines from *A. cepa* in *A. fistulosum*	Japan	Shigyo et al. (1996)
Est-1	Tacking the introgression between *A. fistulosum* and *A. cepa*	USA	Hou et al. (2001)
Gdh-1	Study of alien chromosomes transmission in self progenies of *A. fistulosum* × A. *cepa*	Japan	Shigyo et al. (2003)
Got-2	Characterization of alien chromosome addition in shallot from *A. fistulosum*	Japan	Hang et al. (2004)
Lap-1, Got-1, Got-2, Pgi-1 and *Gdh-1*	Identification of alien chromosome addition in shallot from *A. fistulosum*	Japan	Yaguchi et al. (2009)
AAT, CAR, Est, MDH, ME, SOD	Isozyme variation in genus *Allium*	India, Bangladesh	Mukherjee et al. (2013)
Lap-1, Got-1, Got-2	Study of rust resistance using alien chromosome addition lines from *A. cepa* into *A. fistulosum*	Japan	Wako et al. (2015)
Lap-1, 6-Pgdh-1, Pgi-1, Got-1 and Gdh-1	Characterization of alien chromosome addition in *A. fistulosum* from *A. roylei*	Japan, Vietnam, Russia	Ariyanti et al. (2015)

Notes: *Pgm* phosphoglucomutase, *Adh* alcohol dehydrogenase, *Pgi* phosphoglucoisomerase, *Acp* acid phosphatase, *Est* esterase, *Got* glutamate oxaloacetate transaminase, *Lap* leucine aminopeptidase, *Gdh* glutamate dehydrogenase, *6-Pgdh* 6-phosphogluconate dehydrogenase, *AAT* aspartate aminotransferase, *CAR* carbonic anhydrase, *MDH* malate dehydrogenase, *ME* malic enzyme, *SOD* superoxide, *Skdh* shikimate dehydrogenase, *Idh* isocitrate dehydrogenase

Phylogenetic relationships and evolution within *Allium* species have been investigated by several researchers. Molecular marker technology is been widely used for diversity analysis, varietal identification and facilitates selection of certain agronomic traits for crop improvement such as their polymorphic nature, natural behavior, easy and fast assay preparation, high reproducibility and easy exchange of data between laboratories (Chinnappareddy et al. 2013). Restriction fragment length polymorphism (RFLP), random-amplified polymorphic DNA (RAPD), sequence characterized amplified region (SCAR), simple sequence repeat (SSR) and inter-simple sequence repeat (ISSR) markers are widely used for diversity analysis, varietal identification and study of DNA transfer within species in *Allium* (Table 4.5). Wilkie et al. (1993) reported the use of RAPD for diversity analysis of seven cultivars of onion and a single cultivar each of spring onion, chive, leek and *A. roylei* (a wild relative of onion). Application and impact of molecular markers on evolutionary and diversity studies in the genus *Allium* was reported by Klaas (1998) and Klaas and Friesen (2002).

Chloroplast and mitochondrial DNA are useful for the study of hybridization and introgression as they are inherited in a non-Mendelian, cytoplasmic fashion which usually relates to maternal transmission (Parker et al. 1998). Bark et al. (1994) demonstrated the use of RFLP in the chloroplast and nuclear genomes to assess DNA transfer from spring onion to bulb onion. Bradeen and Havey (1995) reported maternal phylogenetic relationship for *Allium altaicum, A. fistulosum, A. cepa* and *A. vavilovii* using RFLP of the chloroplast DNA. Use of mitochondrial DNA to distinguish cytoplasm of cultivated and wild *Allium* in subgenus *Cepa* was reported by Yamashita et al. (2000). RFLP analysis of mtDNA and cpDNA of two cultivated species of *A. fistulosum* and *A. cepa* along with four wild species in subgenus *Cepa* by Yamashita et al. (2001) demonstrated that phylogenetic relationships among the species closely correspond to the crossing ability test reported by Van Raamsdonk and Vries (1992). Yusupov et al. (2019) published a complete chloroplast of *A. fistulosum* consisting of 82,237 bp long single copy and 17,907 bp small single copy regions separated by 26,510 bp inverted repeat regions which could later be used for population genomic studies, phylogenetic analysis and genetic engineering studies of the genus *Allium*.

Nuclear DNA contains both unique single copy regions which generally code for a gene product and nonunique repetitive regions which consist of core sequences repeated in varying degree. Phylogenetic reconstruction based on nuclear DNA of *Allium* in subgenus *Cepa* was presented by Van Raamsdonk et al. (1997). A study using RAPD and RFLP by Friesen et al. (1999) showed that *A. fistulosum* originates from an *A. altaicum* progenitor. This result is contradictory to results published by Bradeen and Havey (1995) suggesting that phylogenetic hypotheses partly depend on the marker systems used. Development of RAPD marker for phylogenetic study in *Allium* subgenus *Cepa* has been reported by Shigyo et al. (2002). A study by Ricroch et al. (2005) revealed a significant interspecific variation in the amount of nuclear DNA and GC content of different onions and their wild allies. A phylogeny was constructed using internal transcribe spacer (ITS) sequences of 43 accessions representing 30 *Allium* species belonging to 3 major subgenera and 14 sections

Table 4.5 Molecular markers used for diversity analysis and fingerprinting in *A. fistulosum*

Marker type	Purpose	Country	References
Random-amplified polymorphic DNA (RAPD)	Genetic analysis in *Allium*	UK	Wilkie et al. (1993)
Restriction fragment length polymorphism (RFLP)	Analysis of *A. fistulosum* × *A. cepa* hybrid progeny	USA	Bark et al. (1994)
Restriction fragment length polymorphism (RFLP)	Phylogenetic relationship between *Allium* species	USA, Japan	Bradeen and Havey (1995) and Yamashita et al. (2001)
Random-amplified polymorphic DNA (RAPD)	DNA-based phylogenies in *Allium* subgenus *Cepa*	Netherlands	Van Raamsdonk et al. (1997)
Random-amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP)	Evolutionary and diversity studies of *Allium* species	Germany	Klaas (1998) and Klaas and Friesen (2002)
Random-amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP)	Phylogenetic analysis of *A. altaicum* and *A. fistulosum*	Germany	Friesen et al. (1999)
Restriction fragment length polymorphism (RFLP)	Study of mitochondrial DNA in *Allium* subgenus *Cepa*	Japan	Yamashita et al. (2000) and Yamashita et al. (2001)
Random-amplified polymorphic DNA (RAPD)	Development of RAPD in *Allium* subgenus *Cepa*	Japan	Shigyo et al. (2002)
Internal transcribe spacer (ITS) region	Phylogenetic and diversity studies of *Allium* species	Germany, France	Ricroch et al. (2005), Friesen et al. (2006), and Gurushidze et al. (2007)
Simple sequence repeat (SSR)	Intraspecific F_1 hybrid and interspecific taxonomic analysis in *Allium* subgenus *Cepa*	Japan	Araki et al. (2009)
Inter-simple sequence repeats (ISSR)	Species relationship study among *Allium* species	Korea	Son et al. (2012)
Amplified fragment length polymorphism (AFLP)	Development of a comparative genomic database for *Allium*	New Zealand	McCallum et al. (2012)
Simple sequence repeat (SSR)	Identification of cytogenetic marker for monitoring of alien genetic material	Russia, Belgium	Kirov et al. (2017)
Simple sequence repeat (SSR)	Study of the chloroplast genome of *A. fistulosum*	China, Uzbekistan	Yusupov et al. (2019)

showed a tendency towards a decrease in genome size within the genus. Friesen et al. (2006) divide *Allium* into 15 subgenera and 56 sections based on internal transcribed spacer region (ITS) of nuclear ribosomal DNA with an estimation of 780 *Allium* species currently being recognized. Phylogenic analysis of *Allium* subgenus *Cepa* by Gurushidze et al. (2007) using sequences of the nuclear ribosomal DNA

internal transcribed spacer (ITS) region showed that the subgenus is monophyletic with three species group consisting (a) *A. altaicum*/*A. fistulosum*, (b) *A. farctum*/*A. roylei*/*A. asarense*/*A. cepa*/*A. vavilovii* and (c) *A. galanthum*/*A. oschaninii*/*A. praemixtum*/*A. pskemense.*

Tandem repeats are usually associated with chromosomal landmarks such as centromeres, telomeres, subtelomeric and other heterochromatic regions can be used for chromosome identification and to study plant chromosome evolution in many crops. Son et al. (2012) used ISSR analysis to study the relationships among *Allium* species. A comparative genomic database for genetic mapping and marker data from *Allium* species and population reported by McCallum et al. (2012) provide a valuable resource for genetic and genomic studies of *Allium* genome including *A. fistulosum*. Kirov et al. (2017) reported two cytogenetic markers, *HAT58* and *Cat36* for identification of individual chromosomes in *A. fistulosum*. Development of SSR markers for identifying intraspecific F_1 hybrids and interspecific taxonomic analysis in *Allium* subgenus *Cepa* are reported by Araki et al. (2009).

4.6 Breeding Strategies

4.6.1 Traditional Breeding

Most cultivated alliums including spring onion are seed propagated (Havey 2002). Traditionally farmers raise their own seed or planting material. Breeders aim to improve cultivar homogeneity and adaptation to specific ecological conditions. High consumer and farmer preferences of low pungency, high sugar content, disease resistance, high yield, delayed bolting and suitability for mechanization are among the main important trait in spring onion breeding (Tsukazaki et al. 2017). Miyagi et al. (2011) showed that general consumers prefer strong flavor and sweetness of fresh and heated (boiled or grilled), besides soft texture of grilled bunching onion. Preferences on pungency, stickiness and texture for fresh, boiled and grilled spring onion vary depending on the consumer. Japanese food restaurants tend to value taste and flavor, while other types of restaurants tend to consider price and spring onion size.

Interspecies hybridization plays an important role in onion breeding as it allows introgression of valuable traits with improved characteristics. Among the interspecific crosses in *Allium*, those between *A. fistulosum* and *A. cepa* have been studied extensively because the former represents a rich source of several agronomic traits including resistance to diseases and pests (Budylin et al. 2014; Kik 2002) lacking in bulb onion. Breeders have used introgression of resistant genes from *A. fistulosum* into *A. cepa* for decades with many crosses between these species achieved (Table 4.6). Some commercial interspecific hybrids obtained are Beltsville Bunching which is an amphidiploid species of *A. cepa* and *A. fistulosum*, Delta Giant (backcross of *A. cepa* var. *ascalonicum* with an amphidiploid of shallot × *A. fistulosum*),

Table 4.6 Interspecific crosses involving *A. fistulosum*

Cross	References
A. cepa × *A. fistulosum*	Levan (2010), Saini and David (1967), El-Gadi and Elkington (1975), Dolezel et al. (1980), Peffley and Mangum (1990), Ulloa et al. (1994), and Kudryavtseva et al. (2019)
A. fistulosum × *A. ascalonicum*	Cochran (1950) and Arifin and Okudo (1996)
A. fistulosum × *A.cepa*	Dolezel et al. (1980), Peters et al. (1984), Corgan and Peffley (1986), Song et al. (1997), and Kudryavtseva et al. (2019)
A. fistulosum × *A. galanthum*	El-Gadi and Elkington (1975)
A. fistulosum × *A. royeli*	McCollum (1982)
A. fistulosum × *A. schoenoprasum*	Umehara et al. (2006a)

Top Onion which is a diploid interspecific between *A. cepa* × *A. fistulosum* and Wakegi Onion which is a diploid interspecific between shallot × *A. fistulosum* (Kik 2002).

Studies on interspecific hybridization between *Allium cepa* and *A. fistulosum*, and research has continued over the years (Chuda and Adamus 2012). However, low fertility of the interspecific F_1 hybrid between these two species has made progress rather slow. Van der Valk et al. (1991) suggested that strong pre-fertilization and post-fertilization barriers limit the recombination between the chromosomes of bulb and bunching onions. Ulloa et al. (1994), showed that the number of bridges and fragments varied between the F_1 hybrid of *A. fistulosum* × *A. cepa* and BC_1 progenies (*A. fistulosum* × *A. cepa*) × (*A. cepa*). The F_1 hybrid and all BC_1 progenies were either sterile or very low seed set. Development of addition lines (Peffley et al. 1985) and the use of *A. roylei* as a bridging species (Khrustaleva and Kik 1998) was proposed to increase the possibility of genetic introgression from *A. fistulosum* into *A. cepa* (Martinez et al. 2005).

Since the 1980s, F_1 hybrids cultivars have been released in Japan with 80% new cultivars released per year. There are over 120 registered cultivars of spring onion in Japan with some developed using male sterility. Shigyo et al. (1996) established a series of alien addition lines, representing the 8 different chromosomes of shallot (*Allium cepa* Aggregatum group) in an *A. fistulosum* background. Yamashita et al. (1999) suggested that a male sterile line of *A. fistulosum* developed by continuous backcrossing with *A. galanthum* could be useful for spring onion breeding to eliminate the emasculation process and to produce large numbers of F_1 seeds. In Japanese bunching onion, male sterility is controlled by the interaction between a cytoplasmic factor *S*, and two nuclear genes Ms_1 and Ms_2 (Moue and Uehara 1985) with male sterility occurring when it is homozygous recessive (Havey 2002). The genotype of the male sterile plants and their maintainer are $Sms_1ms_1ms_2ms_2$ and $Nms_1ms_1ms_2ms_2$, respectively (Inden and Asahira 1990). Cytoplasmic male sterility (CMS) is

important in spring onion breeding as it allows easy propagation of male sterile plants using an appropriate maintainer line (Havey 2004; Yamashita et al. 2009). However, the CMS expression depends on the genetic relationship between the cytoplasm donor species and bunching onion. Yamashita et al. (2009) reported that the frequency of male sterile plants in 8 of 135 spring onion accessions from Japan, China, Mongolia, Korea and Taiwan varied from 1.7% to 24.5%.

Kudryavtseva et al. (2019) revealed that spontaneous chromosome duplication produced allotetraploids from interspecific hybrids between *Allium cepa* and *A. fistulosum* by using GISH analyses. Interspecific hybrids between spring onion (*A. fistulosum*) and chive (*A. schoenoprasum)* by reciprocal crossing through ovary culture producing vigorous growth and higher edible parts than their parents when using *A. fistulosum* as the seed plant (Umehara et al. 2006b). Yamasaki et al. (2011) reported that 24 h photoperiod treatment increased plant height and leaf number in both Kincho and Choetsu cvs. However, 16 h photoperiod only increased the plant height and leaf sheath diameter of cv. Choetsu but did affect cv. Kincho. Meanwhile, 16% of treated plants bolted under 24 h, 54% under 16 h and 77% under 8 h in cv. Kincho for 30 days of treatment. More than 30 days of treatment increased the percentage of bolting plants for 16 h and 24 h photoperiods. However, no differences in percentage of bolting were observed in the 60-day treatment for the 3 photoperiods (8, 16, 24 h) tested. Long-day photoperiod delayed bolting and increased the number of leaves in both cultivars. They suggested that spring onion requires facultative short-day during flower initiation process and that the inhibition of flower initiation by a long photoperiod can be overcome at low temperature.

4.6.2 Molecular Breeding

Spring onion is second most important species in the genus *Allium* because of its disease resistance, ecological adaptability and close relationship to *A. cepa*. Alien chromosome addition lines from *A. fistulosum* could enhance the possibility of breeding for selective chemical components in *A. cepa* (Yaguchi et al. 2009). Breeding of spring onion is time-consuming and requires a large area. It takes many years, usually 1–2 years per generation, because of slow plant growth. Development of molecular markers is important in facilitating the establishment of a genetic basis for plant breeding. Quantitative trait loci (QTL) analysis based on genetic linkage maps is required to reveal the mode of inheritance of selective agronomic traits targeted for crop improvement. Several studies on the use of molecular markers for the development of linkage map and QTL analysis are reported (Table 4.7). Yamashita et al. (1999) published the use of isozymes and RAPD analysis to develop genetic markers linked to the fertility restoring gene (*Rf*) for cytoplasmic male sterility (CMS) in backcross progenies between *A. galanthum* and *A. fistulosum*. Sequence characterized amplified region (SCAR) markers linked to fertility restoring gene for CMS were reported by Yamashita et al. (2002) and could be applied for marker associated selection (MAS).

Table 4.7 Molecular markers used for the development of linkage map and QTL analysis in *Allium fistulosum*

Marker type	Purpose	Country	References
Random-amplified polymorphic DNA (RAPD)	Study of gene for CMS of *A. fistulosum*	Japan	Yamashita et al. (1999)
Random-amplified polymorphic DNA (RAPD) and sequence characterized amplified region (SCAR)	Study of fertility restoring gene for CMS in *A. fistulosum*	Japan	Yamashita et al. (2002)
Simple sequence repeat (SSR)	Organization of SSR in heterochromation of *A. fistulosum*	Russia	Fesenko et al. (2002)
Simple sequence repeat (SSR)	Development of microsatellite marker in *A. fistulosum*	Japan, China	Song et al. (2004), Tsukazaki et al. (2007), and Yang et al. (2015)
Amplified fragment length polymorphism (AFLP)	Relationship between heterosis and genetic distance in *A. fistulosum*	Japan	Ohara et al. (2005b)
Amplified fragment length polymorphism (AFLP), cleaved amplified polymorphic sequence (CAPS) and simple sequence repeat (SSR)	Genetic linkage map of *A. fistulosum*	Japan, Korea	Ohara et al. (2005a)
Random-amplified polymorphic DNA (RAPD), cleaved amplified polymorphic sequence (CAPS)	To confirm the hybridity between *A. fistulosum* × *A. schoenoprasum*	Japan	Umehara et al. (2006b)
Simple sequence repeat (SSR)	Construction of SSR-based chromosome map in *A. fistulosum*	Japan	Tsukazaki et al. (2008)
Simple sequence repeat (SSR) and cleaved amplified polymorphic sequence (CAPS)	Mapping QTL controlling seedling growth in *A. fistulosum*	Japan	Ohara et al. (2009)
Simple sequence repeat (SSR)	Identification of alien chromosome additions from *A. fistulosom* in shallot	Japan & Vietnam	Yaguchi et al. (2009)
Random-amplified polymorphic DNA (RAPD) and sequence characterized amplified region (SCAR)	Cytoplasmic male sterility (CMS) analysis in *Allium*	China	Gai and Meng (2010) and Gao et al. (2013, 2015)
Simple sequence repeat (SSR)	Classification of *A. fistulosum* varieties	Japan	Tsukazaki et al. (2010)
Expressed sequence Tag (EST)- simple sequence repeat (SSR), simple sequence repeat (SSR), single nucleotide polymorphisms (SNP)	Comparative genomics resource for *Allium*	Japan, New Zealand, Netherlands	McCallum et al. (2012)

(continued)

Table 4.7 (continued)

Marker type	Purpose	Country	References
Random-amplified polymorphic DNA (RAPD), simple sequence repeat (SSR), sequence-tagged site (STS), expressed sequence tag (EST)	QTL analysis for pseudostem pungency in *A. fistulosum*	Japan	Tsukazaki et al. (2012)
Amplified fragment length polymorphism (AFLP) and sequence characterized amplified region (SCAR)	Development of SCAR marker for CMS	China	Wang et al. (2013)
Simple sequence repeat (SSR) and ribonucleic acid sequencing (RNAseq)	Study of cuticular wax-related genes in *A. fistulosum*	China	Liu et al. (2014)
Simple sequence repeat (SSR) and single nucleotide polymorphisms (SNP)	Genetic linkage map of *A. fistulosum*	Japan	Tsukazaki et al. (2015)
Single nucleotide polymorphisms (SNP)	SNP markers for introgression breeding in onion	Netherlands	Scholten et al. (2016)
Simple sequence repeat (SSR) and expressed sequence tag (EST)	QTL for bolting time in *A. fistulosum*	Japan	Wako (2016)
Simple sequence repeat (SSR)	Association of tandem repeat to major chromosomal landmarks in *A. fistulosum*	Russia, Belgium	Kirov et al. (2017)
Single nucleotide polymorphisms (SNP)	Development of KASP marker for CMS	China	Gao et al. (2018)
Single nucleotide polymorphisms (SNP)	Varietal identification of open-pollinated onion cultivars	Korea	Lee et al. (2018)
Mitochondrial genome sequences	Development of molecular markers distinguishing between *A. cepa* and *A. fistulosum*	Korea	Kim and Kim (2019)
SSR and ISSR	Genetic diversity of *A. fistulosum*	Nigeria	Abutu et al. (2020)

Organization of a 378-bp satellite repeat in terminal heterochromatin of *Allium fistulosum* was reported by Fesenko et al. (2002). Isolation and development of SSR markers in spring onion as a primary basis for breeding was described by Song et al. (2004) and Yang et al. (2015). A total of 1796 SSR clones from SSR-enriched DNA libraries of spring onion were identified by (Tsukazaki et al. 2007) which are applicable for phylogenic analysis and construction of genetic linkage maps. Ohara et al. (2005b) used AFLP markers to study the relationship between heterosis and genetic distance in intervarietal F_1 hybrids of *A. fistulosum*. Tsukazaki et al. (2008) isolated 1940 SSR clones from size-fractionated genomic DNA libraries with potential use

for cultivar identification and hybrid purity identification. A genetic map based on single nucleotide polymorphisms (SNPs) markers was assembled by using interspecific F_1 hybrid between *A. roylei* × *A. fistulosum* (Scholten et al. 2017).

The first linkage map of spring onion with AFLPs was constructed by Ohara et al. (2005a) using reciprocally backcrossed progenies of two inbred lines of Japanese bunching onion (*A. fistulosum*) based on 149 AFLP markers, 1 cleaved amplified polymorphic sequence (CAPS) and 13 SSRs using reciprocally backcrossed progenies. Tsukazaki et al. (2008) constructed a detailed linkage map consisting of 17 linkage groups with 212 bunching onion SSR markers and 42 bulb onion (*A. cepa*) SSR, InDel, CAPS or dCAPS markers, covering 2069 cM. Ohara et al. (2009) added 24 SSRs and 1 CAPS to the previous map developed by Ohara et al. (2005a) on reciprocally backcrossed progenies resulting into 16 linkage group for the J map and 15 linkage groups for the D map. QTL analyses conducted showed that the seedling growth is controlled by many QTLs that exhibit various modes of gene actions, additive, dominant and overdominant.

Classification and identification of 30 bunching onion varieties based on simple sequence repeat (SSR) markers was reported by (Tsukazaki et al. 2010). Numerous DNA markers based on SSRs, SNPs and InDels were developed (Tsukazaki et al. 2015). Identification of restorer genes involved in the restoration of fertility in CMS is essential for the establishment of molecular breeding system. RAPD and SCAR marker distinguishing between *N* and *S* cytoplasm in several spring onion cultivars were reported by Gai and Meng (2010) and Gao et al. (2013, 2015). SCAR marker developed from AFLP marker distinguishing between CMS and fertile lines could be used by breeder to extract maintainer lines from open-pollinated spring onion (Wang et al. 2013). Gao et al. (2018) reported a competitive allele-specific PCR (KASP) marker developed from SNP detected in the *atp6* gene could discriminate between male-sterile and normal cytoplasmic types.

Sequencing a huge genome by the high-throughput method has been initiated, as well as the development of molecular markers and linkage mapping. Allium Map for cultivated *Allium* vegetable including spring onion was developed by McCallum et al. (2012) and provides a genetic map and marker data from multiple *Allium* species. High throughput SNP genotyping, functional genomics using RNAi and transcriptome mapping can be exploited to understand the function of genes in the genome. Transcriptome sequencing utilizing next-generation sequencing (NGS) has become an effective tool to discover novel genes in several crops. A total of 798 genes, representing 1.86% of total putative unigenes, were differentially expressed between waxy spring onion and non-waxy mutant spring onion varieties (Liu et al. 2014). More than 50,000 unigenes were obtained from transcriptome shotgun assembly (TSA) of next-generation sequencing (NGS) data.

Genomic SSRs from bunching onion and EST-SSRs from bulb onion were used by Wako (2016) to study the genetic basis of bolting time in bunching onion. They reported that 2 QTLs associated with bolting time were detected on the linkage groups of chromosomes 1 (Chr. 1a) and chromosome 2 (Chr. 2a). However, the QTL on Chr. 1a was not detected in the KiC population (inbred line from ever-flowering, Kitanegi and late-bolting, Cho-etsu) grown in a heated greenhouse under

unvernalized conditions. A single QTL with major effect was identified exclusively on the linkage group Chr. 2a in the SaT03 population (cross between early-bolting line, Chuukanbohon Nou 1 and late-bolting, Fuyuwarabe) were evaluated under field conditions. Tsukazaki et al. (2017) reported 27 QTLs for the 6 morphological traits (plant height, leaf length, pseudostem length, leaf width, pseudostem width, number of leaf sheath) in 16 regions of 11 linkage groups, with a major QTL for the number of leaf sheaths repeatedly detected on Chr. 8. They discovered 2 QTLs associated with pseudostem pigmentation on linkage groups Chr. 4a and Chr. 5a-2. Tsukazaki et al. (2012) revealed a major QTL for pseudostem pungency located within a 24.2 cM interval on Chr. 2a of spring onion.

A SSR-tagged breeding scheme to enhance the rapidity, ease and accuracy of variety identification and F_1 purity test was suggested by Tsukazaki et al. (2006). Tsukazaki et al. (2009) later proposed to protect breeders' rights and confer traceability in allogamous crops. The scheme consists of three steps (Fig. 4.3):

(a) Selection of a small number of highly polymorphic SSR loci that are not tightly linked to each other. Selection of prevailing allele at each locus, since the parental lines of the F_1 hybrids must carry different alleles at each selected locus.
(b) Selection of plants in a foundation seed field that are homozygous for the prevailing allele at all the SSR loci selected.
(c) Harvest of foundation seed from the plants selected. For F_1 breeding, one parental line should be homozygous at each selected SSR locus and the other should be homozygous for another allele.

Production of stock seed normally will be followed by marketing of seed. Open-pollinated varieties should be homozygous and uniform at the selected SSR loci while the F_1 varieties should be uniformly heterozygous. Open-pollinated varieties and the parental lines of the F_1 varieties should not exhibit inbreeding depression since most of the loci can maintain their original heterogeneity.

4.6.3 Tissue Culture Applications

Micropropagation of *Allium fistulosum* can avoid the maintenance of male sterile lines by a non-restorer fertility line. A large number of identical offspring from a limited amount of parent material could be obtained to increase the number of female plants for the production of F_1 hybrid seed. Song and Peffley (1994) reported on in vitro culture of *A. fistulosum* and *A. cepa* interspecific derivatives (Table 4.8). Furthermore, the high proliferation of adventitious shoots were obtained from shoot tip culture of spring onion using MS medium (Murashige and Skoog 1962) supplemented with 2 mg/l 6-furfurylaminopurine (kinetin) and 0.5 mg/l 1-naphthylacetic acid (NAA) at 20 °C. Fujieda et al. (1977) found that in the presence of cytokine it stimulated the multiplication of adventitious shoots, but completely inhibited adventitious roots.

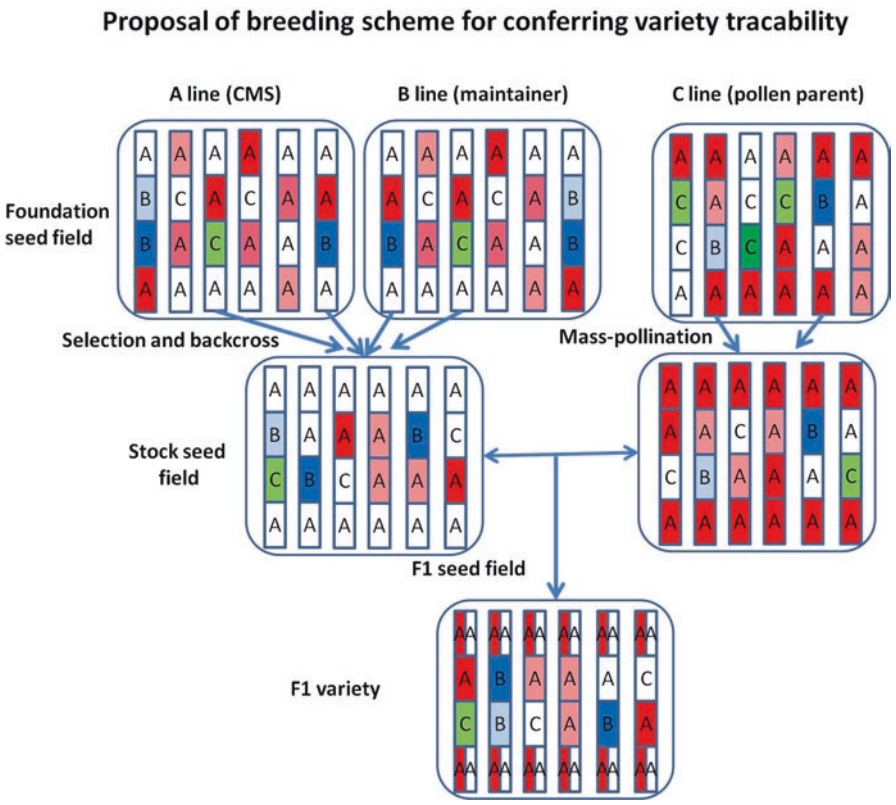

Fig. 4.3 The SSR-tagged breeding scheme for conferring variety traceability

Organ culture such as embryo and ovary can facilitate the recovery of progeny from high sterile interspecific F_1 hybrids and backcross progeny to overcome incompatibility in interspecific hybrids. Regeneration into haploid plantlets has been achieved in spring onion using flower and ovary culture (Ibrahim et al. 2016). Hybrid plants were obtained from embryo cultured on B5 medium (Gamborg et al. 1968) without growth regulators (Peffley 1992). Gonzales and Ford-Lloyd (1987) demonstrated successful embryo rescue of hybrids from crosses between *Allium cepa* and *A. fistulosum* using B5 medium. Umehara et al. (2006b) reported the production of interspecific hybrids between *A. fistulosom* × *A. macrostemon* through ovary culture using B5 medium modified by Dunstan and Short (BDS) containing 30 g l^{-1} sucrose. Hybrids produce from germinated embryos transferred to BDS medium containing 1 mg l^{-1} 6-benzylaminopurine and 15 g l^{-1} sucrose followed by subculturing on phytohormone-free B5 medium showed intermediate traits of both parents.

Somatic embryogenesis can provide regeneration of a large number of individuals from a single progenitor. However, the occurrence of somaclonal variation can

Table 4.8 Summary of tissue culture studies on *Allium fistulosum*, A. fistulosum × A. cepa and A. cepa × A. fistulosum.

Species	Type of tissue culture	References
A. fistulosum	Callus	Lin and Cui (1982), Tashiro et al (1985), and Kim and Soh (1996)
	Callus and embryo	Van der Valk et al (1992) and Phillips and Hubstenberger (1987)
	Ovary and ovule	Ibrahim et al. (2016)
A. fistulosum × *A. cepa*	Callus	Peffiey (1992) and Phillips and Hubstensberger (1987)
	Embryo	Lu et al. (1989) and Van der Valk et al. (1991)
	Somatic hybrid	Shimonaka et al. (2002)
A. cepa × *A. fistulosum*	Callus	Shahin and Kaneko (1986)
	Embryo	Gonzalez and Ford-Lloyd (1987)
	Micropropagation	Mar'yakhina et al. (1983)
	Ovary and ovule	Guan and Peffley (1989)
A. fistulosum × *A. macrostemon*	Ovary	Umehara et al. (2006b)

be an issue in determining desired clones. Mutations and polyploidization are quite frequent in callus culture and thus enables their possible use in breeding program using somaclonal variation (Inden and Asahira 1990). Plant regeneration from callus culture is well established in *Allium fistulosum* and interspecific hybrids between alliums. Phillips and Hubstenberger (1987) developed procedures for micropropagation and plant regeneration from callus for *A. fistulosum, A. altaicum, A. galanthum, A. roylei* and selected progeny of interspecific crosses of *A. cepa* × *A. fistulosum, A. cepa* × *A. galanthum* and *A. cepa* × *A. oschaninii*. Lu et al. (1989) reported high frequency somatic embryo production from callus culture using BDS media supplemented with moderate to high auxin level and 2.5 g l^{-1} proline and Shahin's vitamins. A reproducible protoplast culture system using BDS basal medium supplemented with 5mM potassium nitrate, 2 μM 2,4,dichlorophenoxyacetic acid (2,4,-D) and 0.2 or 1 μM 6-benzylaminopurine (BAP) and a combination of 0.2 M sucrose and 0.2 M glucose has been established by Shimonaka et al. (2001). Plantlet regeneration was achieved 2–3 months after inoculating protoplast-derived calli to a half N MS medium. This finding enables the introduction of targeted genes such as disease resistance or male sterile genes from *Allium* species into spring onion using cell fusion or electroporation. Interspecific somatic hybrids between *A. fistulosum* and *A. cepa* are reported by Shimonaka et al. (2002) using protoplast electrofusion. Several amphidiploids and alloplasmic plants which are valuable for spring onion breeding have been produced.

The term *haploid* refers to a plant which possesses the gametophytic number of chromosomes in their sporophytes. Monoploids contain a single genome from

diploid species while polyhaploids containing two or more genomes are derived from polyploid species. Haploid plants will become doubled-haploids (DHs) following chromosome doubling. This doubled-haploid methodology offers several advantages to plant improvement programs as it provide a rapid approach to achieve homozygosity. Since haploid plants carry only one set of alleles at each locus, upon doubling, homozygous and homogeneous lines are available. This allows identification of superior parental combinations, evaluation of environment × genotype interactions, avoids masking of recessive genes, and evaluation of qualitative and quantitative traits (Snape 1988).

Ibrahim et al. (2016) developed frequency of embryogenesis in spring onion using flower and ovary culture using cultured in BDS medium supplemented with 2 mg l^{-1} 2,4-D and 2 mg l^{-1} BAP fortified with 100 g/l sucrose, 200 mg l^{-1} proline and 500 mg l^{-1} myo-inisitol. Calli were recorded around 90 days after ovary inoculation and shoot induction was observed after 60 days of callus induction in BDS media (Dunstan and Short 1977). The cultures were green in color during the first 3 months and gradually turned yellowish as the culture progressed. Calli from the ovule were easily recognized with ovary burst after 4–5 months of inoculation (Fig. 4.4).

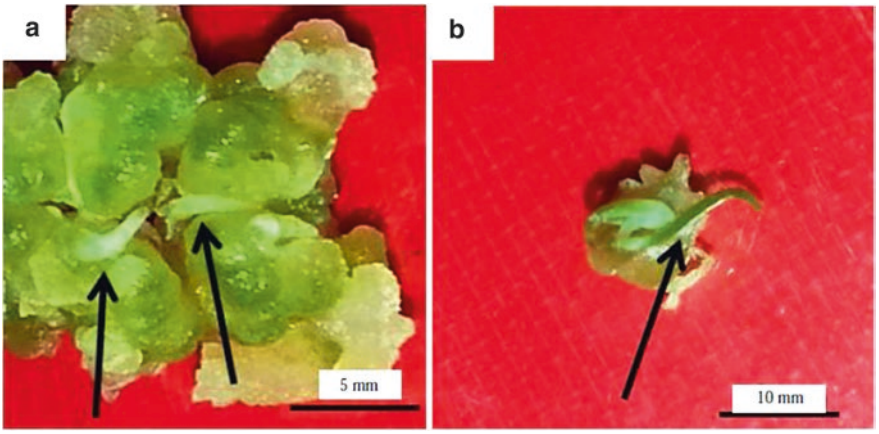

Fig. 4.4 (**a**) Shoot formation has been observed from callus inoculated from the ovary culture in BDS media, (**b**) Shoot regeneration has been observed after 150 days of culture. (Source: Ibrahim et al. 2016)

4.7 Conclusions and Prospects

There are seven major edible species among the hundreds of *Allium* varieties available worldwide. Great adaptability to varied climatic conditions has created many different varieties of the crop. Each cultivar has a commercial characteristic value due to usage including in food and Chinese medicine. Different studies show spring onion has excellent antibacterial and antifungal properties. Some species also purportedly show anti-platelet, antioxidative, anti-hypertensive, and anti-hyperlipidemic properties, and reduction of cholesterol level which may decrease the risk of heart attack. The plants are rich source of vitamin C, A and B_6, thiamine, folate and minerals.

Spring onion can be grown in most types of soil but in recent years several countries have reported different diseases on spring onion due to continuous cultivation. Japan and Taiwan are good examples for great adaptability of the crop, where intense cultivation is combined with selection, breeding and the development of a good marketing network which has led to greatly increased production; this indicates that there is great scope for the development of better cultivars and increased commercialization and intensification of production in Southeast Asia.

Several new methods developed to achieve high yields and disease resistance in spring onion; organ culture and somatic embryogenesis will help to retain the F_1 progeny and produce large single progenitor respectively. Recent efforts in developing high yielding cultivars with specific and broad adaptability, precocity, resistance to various pest and diseases, and good marketing quality have been initiated, and there is a great need to use biotechnological approaches for the development of new cultivars with high yield and better disease resistant characteristics.

Appendix I: Research Institutes Relevant to Spring Onion

Institution name	Specialization and research activities	Address	Contact information and website
Institute for Horticultural Development, Agriculture Victoria	Study on growth and dry matter production of spring onion, white rot disease management	Private Bag 15, Ferntree Gully Delivery Centre, Victoria 3156, Australia	daryl.joyce@nrevic.gov.au; https://www.ausvegvic.com.au/pdf/r%26d_VG01096_white_rot_onion_integrated_control_strategy_booklet.pdf
University of Queensland	Disease forecasting system in spring onion	Queensland 4343, Australia	v.galea@uq.edu.au https://researchers.uq.edu.au/researcher/200
Department of Genetics and Plant Breeding, Bangladesh Agricultural University	SNP markers, molecular breeding	Mymensingh 2202, Bangladesh	sathisbioinfo@gmail.com https://www.mdpi.com/2073-4395/8/9/179
Center of Medical Genetics, Ghent University	Cytogenetics, molecular markers, spring onion breeding	Ghent, Belgium	kirovez@gmail.com https://pubmed.ncbi.nlm.nih.gov/28150039/
Instituto Federal Catarinense	Crop improvement	C. Postal 441, 89163-356 Rio do Sul-SC, Brazil	marcuzzo@ifc-riodosul.edu.br; https://www.scielo.br/scielo.php?script=sci_arttext&pid=S0102-05362014000300363
University of Saskatchewan	Phylogenetics and taxonomy of *Allium*	Saskatoon, Canada	hugo.cota@usask.ca https://www.researchgate.net/publication/263500245_A_taxonomic_revision_of_Allium_Alliaceae_in_the_Canadian_prairie_provinces
Kunming Institute of Botany, Chinese Academy of Sciences	Study of chloroplast genome of *A. fistulosum*	Kunming, China	sunhang@mail.kib.ac.cn; https://www.tandfonline.com/doi/pdf/10.1080/23802359.2018.1545532

Beijing Technology and Business University (BTBU)	Nutritional value of *A. fistulosum*	Beijing 100048, China	chenht@th.btbu.edu.cn https://europepmc.org/article/med/31108752
Beijing Academy of Agriculture and Forestry Science (North China)	Genetics and breeding of spring onion, transcriptome sequencing	Beijing 100097, China	wangyongqin@nercv.org; https://web.b.ebscohost.com/abstract?direct=true&profile=ehost&scope=site&authtype=crawler&jrnl=16616596&AN=117067465&h=rpJH2%2fnRPYhGXzYAIQ5jM6HOBvVg%2fFbcqJHYXlaSayCx34ik0ZiFQYjpy2GM%2fi%2bbH0nL%2fuL%2bpCRml%2bFebRMIQA%3d%3d&crl=c&resultNs=AdminWebAuth&resultLocal=ErrCrlNotAuth&crlhashurl=login.aspx%3fdirect%3dtrue%26profile%3dehost%26scope%3dsite%26authtype%3dcrawler%26jrnl%3d16616596%26AN%3d117067465
University of Chinese Academy of Science	Genomic study of *A. fistulosum*	Beijing, China	sunhang@mail.kib.ac.cn https://search.proquest.com/openview/03b13ee11d15e8b54850a2f6dd17e8b8/1?pq-origsite=gscholar&cbl=3933403
College of Horticulture and Gardening, Yangtze University	SSR markers, RNA sequencing, molecular markers	Jingzhou, China	lchliu18@yangtzeu.edu.cn https://pdfs.semanticscholar.org/b581/67fcada93d4f194e6f5a0d8a834632f038ff.pdf
College of Horticulture, Northwest A&F University	Physiology of *A. fistulosum*	Yangling, Shaanxi 712100, People's Republic of China	chengzh@nwsuaf.edu.cn https://pubmed.ncbi.nlm.nih.gov/24199907/
College of Resources and Environmental Sciences, China Agricultural University	Effect of mycorrhizal colonization on pungency	Beijing, China 100094	junlingz@cau.edu.cn https://pureadmin.qub.ac.uk/ws/files/452805/GuoT%20et%20al%202007.pdf
College of Horticulture, Shandong Agricultural University	SSR markers, transcriptome, molecular markers, molecular breeding, cytoplasmic male sterility	Tai'an 271018, P.R. China	sqliu@sdu.edu.cn https://www.mdpi.com/journal/ijms/special_issues/plant-molecular-biology?view=abstract&listby=type

(continued)

Institution name	Specialization and research activities	Address	Contact information and website
Institute of Medical Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College	Physiology of spring onion	Beijing 100193, China	qianwangcau@126.com https://www.cabdirect.org/cabdirect/abstract/20143400467
Yantai Agricultural Science Academy of Shandong Province	Effect of spring onion root against root knot nematodes	Yantai, Shandong, P.R.China	ytnkyscs@163.com https://journals.plos.org/plosone/article/authors?id=10.1371/journal.pone.0201471
The First Hospital of China Medical University	Medicinal value of *Allium*	Shenyang 110001, China	zli@cmu.edu.cn; https://onlinelibrary.wiley.com/doi/abs/10.1111/ajco.13133
Shanghai Jiao Tong University	Supply and demand of spring onion	Shanghai, People's Republic of China	gengna@sjtu.edu.cn;
Dept of Food Science and Technology, Chia Nan University of Pharmacy and Science	Nutritional and medicinal value of spring onion	60 Erh-Jen Road, Section 1, Pao-An, Jen-te Hsiang, Tainan Hsien, Taiwan	ipdduh@mail.chna.edu.tw
Key Laboratory of Biology and Genetic Improvement of Horticultural Crops (North China), Ministry of Agriculture	SSR markers, RNA sequencing, molecular breeding	Beijing 100097, China	wangyongqin@nercv.org
Beijing Academy of Agriculture and Forestry Science	Effect on nitrogen and sulfur on the growth of spring onion	Beijing, China	wangyongqin@nercv.org https://www.researchgate.net/publication/269356745_Effect_of_Nitrogen_and_Sulphur_on_the_Growth_and_Qualities_of_Bunching_Onion

Yantai Agricultural Science Academy of Shandong Province	Nematodes control in spring onion	Yantai, Shandong, P.R.China	ytnkyscs@163.com https://www.researchgate.net/publication/326703074_Inhibitory_effects_of_components_from_root_exudates_of_Welsh_onion_against_root_knot_nematodes
Shanghai Jiao Tong University	Seed demand forecasting of spring onion	China	gengna@sjtu.edu.cn; https://journals.plos.org/plosone/article/authors?id=10.1371/journal.pone.0219889
Colombian Corporation for Agricultural Research (Corpoica)	Agronomic Evaluation of Bunching Onion in the Colombian Cundiboyacense High Plateau	CI Palmira, Colombia and CI Tibaitatá, Mosquera, Colombia	galeanomendoza@gmail.com https://core.ac.uk/download/pdf/205389639.pdf
Department of Food Science, Yuanpei University	Nutritional and medicinal value of *Allium*	Hsinchu, Chinese Taipei	hungder@mail.ypu.edu.tw, https://www.scirp.org/html/22-2700684_35298.htm
Department of Plant Medicine, National Chiayi University	Molecular breeding, crop improvement	60004, Taiwan	chienjui.huang@mail.ncyu.edu.tw; https://web.b.ebscohost.com/abstract?direct=true&profile=ehost&scope=site&authtype=crawler&jrnl=11254653&AN=130550885&h=v9HLDeIM%2fFJLxFvToiyvNQAzGyQbJykSwJ%2btnfTxbaKX7SScpNpWq0Un7BNWHLaetSGxWGqKfHD5CtN2Utoy6A%3d%3d&crl=c&resultNs=AdminWebAuth&resultLocal=ErrCrlNotAuth&crlhashurl=login.aspx%3fdirect%3dtrue%26profile%3dehost%26scope%3dsite%26authtype%3dcrawler%26jrnl%3d11254653%26AN%3d130550885
Université Paris-Sud	*Allium* genomic study	CNRS UMR 8079, Bâtiment 360, 91405 Orsay, France	agnes.richroch@esv.u-psud.fr https://www.sciencedirect.com/science/article/abs/pii/S0378111997003958

(continued)

Institution name	Specialization and research activities	Address	Contact information and website
Botanical Garden of the University of Osnabrück	Phylogenetics, molecular markers	Albrechtstr 29, 49076 Osnabrück, Germany	nfriesen@uni-osnabrueck.de https://pubmed.ncbi.nlm.nih.gov/26639102/
Leibniz Institute of Vegetable and Ornamental Crops	Physiology of spring onion	Theodor-Echtermeyer-Weg 1, 14979 Großbeeren, Germany	perner@igzev.de https://pubmed.ncbi.nlm.nih.gov/18457399/
Institute of Plant Genetics and Crop Plant Research (IPK)	RAPD and non-coding chloroplast DNA study, cytogenetics, molecular markers	Correnssstr 3, 06466 Gatersleben, Germany	rutten@ipk-gatersleben.de
Dept. of Horticulture, University of Georgia	Physiology of onion	1111 Miller Plant Science Bldg., Athens, GA 30602	gboyhan@uga.edu
Indian Institute of Horticultural Research	Molecular markers, *Allium* genome and breeding	Bangalore, India	dclreddy@gmail.com
University of Bari 'Aldo Moro'	Physiology of spring onion	Valenzano, Bari, Italy	mariano.fracchiolla@uniba.it
National Institute of Vegetable and Tea Science (NIVTS), National Agriculture and Food Research Organization (NARO)	Microsatellite, SSR, AFLP and molecular markers, genetic mapping, QTL study, molecular breeding	360 Ano-Kusawa, Tsu, Mie 514-2392, Japan	tsuka@affrc.go.jp
National Agricultural Research Center for Tohoku Region (NARCT)	SSR markers, molecular breeding, crop improvement	4 Akahira, Shimo-kuriyagawa, Morioka, Iwate 020-0198, Japan	amhonjo@affrc.go.jp
Institute of the Society for Techno-innovation of Agriculture, Forestry and Fisheries (STAFF)	SSR markers, molecular breeding	446-1 Ippaizuka, Kamiyokoba, Tsukuba, Ibaraki 305-0854, Japan	kan@staff.or.jp

Tohoku Agricultural Research Center, National Agriculture and Food Research Organization (NARO)	QTL study, molecular breeding	92 Nabeyashiki, Shimo-kugiyagawa, Morioka, Iwate 020-0123, Japan	tsuka@affrc.go.jp
National Research Institute of Vegetables, Ornamental Plants and Tea	Physiology of *A. fistulosum*, molecular markers, QTL mapping, molecular breeding	Kurume, Fukuoka 839-8503, Japan	yamasaki@narc.affrc.go.jp
Western Region Agricultural Center NARO	QTL analysis, molecular breeding	1-3-1 Senyu-cho, Zentsuji, Kagawa 765-8508, Japan	kenyamas@affrc.go.jp
Osaka City University	Study on antioxidant activity and flavonoid content	Sugimoto 3-3-138, Sumiyoshi-ku, Osaka 558-8585, Japan	yamamoto@life.osaka-cu.ac.jp
Institute of Plant Genetics and Crop Plant Research (IPK)	Phylogenetics, molecular markers, molecular breeding	Corrensstr 3, 06466 Gatersleben, Germany	blattner@ipk-gatersleben.de
Botanical Garden of the University of Osnabrück	Phylogenetics, molecular markers	Albrechtstr 29, 49076 Osnabrück, Germany	nfriesen@uni-osnabrueck.de
Plant Nutrition, Institute of Crop Science, Humboldt University Berlin	Effect of fertilizer on yield of spring onion	nvalidenstr. 42, 10115 Berlin, Germany	george@igzev.de
Leibniz Institute of Vegetable and Ornamental Crops	Physiology of spring onion	Theodor-Echtermeyer-Weg 1, 14979 Großbeeren, Germany	perner@igzev.de
Institute of Plant Genetics and Crop Plant Research (IPK)	RAPD and non-coding chloroplast DNA study, cytogenetics, molecular markers	Corrensstr 3, 06466 Gatersleben, Germany	rutten@ipk-gatersleben.de
Dept. of Horticulture, University of Georgia	Physiology of onion	1111 Miller Plant Science Bldg., Athens, GA 30602	gboyhan@uga.edu

(continued)

Institution name	Specialization and research activities	Address	Contact information and website
Indian Institute of Horticultural Research	Molecular markers, *Allium* genome and breeding	Bangalore, India	dclreddy@gmail.com
University of Bari 'Aldo Moro'	Physiology of spring onion	Valenzano, Bari, Italy	mariano.fracchiolla@uniba.it
National Agricultural Research Center for Tohoku Region (NARCT)	SSR markers, molecular breeding, crop improvement	4 Akahira, Shimo-kuriyagawa, Morioka, Iwate 020-0198, Japan	amhonjo@affrc.go.jp
Institute of the Society for Techno-innovation of Agriculture, Forestry and Fisheries (STAFF)	SSR markers, molecular breeding	446-1 Ippaizuka, Kamiyokoba, Tsukuba, Ibaraki 305-0854, Japan	kan@staff.or.jp; i-kono@staff.or.jp
Tohoku Agricultural Research Center, National Agriculture and Food Research Organization (NARO)	QTL study, molecular breeding	92 Nabeyashiki, Shimo-kugiyagawa, Morioka, Iwate 020-0123, Japan	tsuka@affrc.go.jp
National Research Institute of Vegetables, Ornamental Plants and Tea	Physiology of *A. fistulosum*, molecular markers, QTL mapping, molecular breeding	Kurume, Fukuoka 839-8503, Japan	yamasaki@narc.affrc.go.jp
Western Region Agricultural Center, NARO	QTL analysis, molecular breeding	1-3-1 Senyu-cho, Zentsuji, Kagawa 765-8508, Japan	kenyamas@affrc.go.jp
Osaka City University	Study on antioxidant activity and flavonoid content	Sugimoto 3-3-138, Sumiyoshi-ku, Osaka 558-8585, Japan	yamamoto@life.osaka-cu.ac.jp
Tohuku University	Biochemical characterization in *A. fistulosum*	Katahira 2-1-1, Aoba-ku, Sendai 980-8577, Japan	koji.muramoto.d5@tohoku.ac.jp

Faculty of Agriculture, Tottori University	Micropropagation of spring onion, cytogenetics, microsatellite markers in *Allium*	4-101 Koyama-Minami, Tottori 680-8553, Japan	itai@muses.tottori-u.ac.jp
Faculty of Agriculture, Saga University	Isozymes markers, molecular markers in *Allium,* onion breeding	Saga 840-8502, Japan	tashiroy@cc.saga-u.ac.jp
Faculty of Agriculture, Yamagata University	Mycorrhizal colonization in *A. fistulosum*	Tsuruoka 997-8555, Japan	tawaraya@tds1.tr.yamagata-u.ac.jp
Faculty of Agriculture, Hokkaido University	Mycorrhizal colonization in *A. fistulosum*	Sapporo, 060-8589, Japan	tatsu@agr.nagoya-u.ac.jp
Faculty of Agriculture, Kyushu University	Biological control, pest management	46-01, Fukuoka 812, Japan	ueno@grt.kyushu-u.ac.jp
Faculty of Agriculture, Yamaguchi University	Molecular markers in *Allium,* QTL mapping, *Allium* genome study, molecular breeding	1677-1 Yoshida, Yamaguchi 753-8515, Japan	shigyo@yamaguchi-u.ac.jp
Faculty of Agriculture, Kyoto Prefectural University,	Biodiversity of *Allium*	Shimogamo, Sakyo, Kyoto, Japan 606-8522	yfujime@rio.odn.ne.jp.
Faculty of Life and Environmental Science, Shimane University	Micropropagation, physiology of spring onion	Matsue, Shimane 690-8504	yano@life.shimane-u.ac.jp
University of Toyama	Medicinal value of *A. fistulosum*	2630 Sugitani, Toyama, 930-0194, Japan	lee@pha.u-toyama.ac.jp
Faculty of Pharmaceutical Sciences, Sojo University	Medicinal value of *A. fistulosum*	22-1, 4-Chome, Nishi-ku, Ikeda, Kumamoto, 860-0082, Japan	none@ph.sojo-u.ac.jp.
Kansai University of Welfare Sciences	Molecular cytogenetic, molecular breeding	Kashiwara, Osaka 582-0026, Japan	myamamoto@tamateyama.ac.jp

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Institution name	Specialization and research activities	Address	Contact information and website
Department of Biotechnology, Maebashi Institute of Technology	Micropropagation of spring onion	Maebashi 371-0816, Japan	ihonda@maebashi-it.ac.jp
Kobe Women's University	Food science	Suma-Ku, Kobe City, Japan 654-8585	seguchi@kobe-wu.ac.jp
Gakushuin Women's College	Food Science	Tokyo, Japan 162-8650.	
Department of Pharmacognosy, Kyoto Pharmaceutical University	Food chemistry of *A. fistulosum*	Misasagi, Yamashina-ku, Kyoto 607-8412, Japan	matsuda@mb.kyoto-phu.ac.jp
Department of Pharmacognosy, Kyoto Pharmaceutical University	Nutritional and medicinal value of spring onion	Misasagi, Yamashina-ku, Kyoto 607-8412, Japan	matsuda@mb.kyoto-phu.ac.jp
Kazusa DNA Research Institute	RNA sequencing, biochemical analysis and molecular breeding	2-6-7 Kazusa-kamatari, Kisarazu, Chiba 292-0818, Japan	hh@kazusa.or.jp
Tottori Horticultural Experiment Station	Micropropagation and physiology of spring onion, rust resistance	Kurakoshi, Tottori 682-0948	shiraiwah@pref.tottori.jp
Fukuoka Agricultural Research Center	Molecular marker, ovary culture	Yoshiki 587, Chikushino, Fukuoka 818-8549, Japan	umehara@farc.pref.fukuoka.jp
Mibyeong Research Center, Korea Institute of Oriental Medicine	Nutritional and medicinal value of spring onion	1672 Yuseong-daero, Yuseong-gu, Daejeon 305-811, Korea	hkkim@kiom.re.kr
Suchon National University	SNP markers, molecular breeding	Suncheon 57922, Korea	my-656@hanmail.net; nis@sunchon.ac.kr
Sungkyunkwan University, Suwon	Development of spring onion harvester	Kyeonggi, Republic of Korea	seung@skku.edu

Institute of Traditional Medicine and Bioscience, Daejeon University	Nutritional value of *A. fistulosum*	Daejeon 300-716, Republic of Korea	sksh518@dju.kr
Institute for Food Sciences, Inje University	Nutritional and medicinal value of *A. fistulosum*	607 Obang-dong, Gimhae, 621-749 Korea	fdsnkiji@inje.ac.kr
Hankyong National University, Anseong	Nutritional and medicinal value of *A. fistulosum*	Kyeonggi, Republic of Korea	wypark@hknu.ac.kr
National Academy of Agricultural Science, Rural Development Administration, Suwon	Nutritional value of *A. fistulosum*	Suwon 441-707, Korea	wgkim@rda.go.kr
Mibyeong Research Center, Korea Institute of Oriental Medicine	Nutritional value of *A. fistulosum*	1672 Yuseong-daero, Yuseong-gu, Daejeon 305-811, Korea	dskim@kiom.re.kr; yysung@kiom.re.kr
National Academy of Agricultural Science, Rural Development Administration, Wanju	Nutritional value of *A. fistulosum*	Wanju, Korea	hgjaz@korea.kr
Center for Horticultural Seed Development of GSP	SNP markers, molecular breeding	Jeonnam, Suncheon 57922, Korea	jihee0830@cnu.ac.kr
Biotechnology Institute, Nongwoo Bio Co. Ltd	Molecular markers, linkage map, molecular breeding	Yeoju, South Korea	bk54@snu.ac.kr
Institute of JinAn Red Ginseng, Jinan-Eup	Nutritional and medicinal value of spring onion	Jinan-Gun, Chonbuk 567-801, Republic of Korea.	e-yoo0612@hanmail.net
Mokpo Experiment Station, National Institute of Crop Science	Mapping of AFLP markers	293-5 Cheongcheon, Cheonggye, Muan, Jeonnam 534-833, Korea	yssong25@rda.go.kr
Wageningen University and Research Centre	*Allium* genomic study, molecular marker	Postbus 16, 6700 AA, Wageningen Netherlands	sjaak.vanheusden@wur.nl

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Institution name	Specialization and research activities	Address	Contact information and website
Wageningen UR Plant Breeding, Wageningen University and Research Centre	SNP markers, molecular markers, molecular breeding	The Netherlands	olga.scholten@wur.nl
Laboratory of Genetics, Wageningen University	Spring onion breeding	Wageningen (The Netherlands)	hans.dejong@wur.nl
DLO-Centre for Plant Breeding and Reproduction Research CPRO-DLO, Department of Vegetable and Fruit Crops	Cytogenetics, DNA-based phylogenies in *Allium* subgenus *Cepa*, spring onion breeding	PO Box 16, 6700 AA Wageningen, The Netherlands	c.kik@cpro.dlo.nl
The New Zealand Institute for Plant & Food Research Ltd	*Allium* genomic study, molecular marker	Private Bag 4704, Christchurch, New Zealand	john.mccallum@plantandfood.co.nz
Faculty of Environment, Society and Design, Lincoln University	*Allium* genomic study, molecular marker	PO Box 84, Lincoln 7647, New Zealand.	yanbo.deng@gmail.com
University of Agriculture in Cracow	GISH study, molecular markers, molecular breeding	29 Listopada 54, 31-425 Cracow, Poland	a.chuda@org.ur.krakow.pl
Department of Horticulture, Wroclaw University of Environmental and Life Sciences	Physiology of spring onion	PI, Grunwaldzki 24a, 50-363 Wrolaw, Poland	katarzyna.a-sowinska@up.wroc.pl
Department of Vegetable Crops and Medicinal Plants University of Life Sciences in Lublin	Physiology of spring onion	Leszczyńskiego 58, 20-068 Lublin, Poland	maria.tendaj@up.lublin.pl

West Pomeranian University of Technology	Agronomy of spring onion	P.Pawla VI 1, 71-459 Szczecin, Poland	azurawik@zut.edu.pl
Department of Plant Cytology and Embryology, Institute of Botany, Jagiellonian University	Molecular markers, micropropagation, cytogenetics of spring onion	Gronostajowa 9, 30-387 Cracow, Poland	patryk.mizia@uj.edu.pl; a.joachimiak@uj.edu.pl
University of Warmia and Mazury in Olsztyn	Physiology of spring onion	Olsztyn, Poland	majkowska-gadomska@uwm.edu.pl
Center of Molecular Biotechnology, Russian State Agrarian University-Moscow Timiryazev Agricultural Academy (RGAU-MTAA)	GISH study, cytogenetics, spring onion breeding	49, Timiryazevskaya Str., 127550 Moscow, Russia;	khrustaleva@timacad.ru
All-Russia Research Institute of Vegetable Breeding and Seed Production	GISH study, cytogenetics, spring onion breeding	Moscow oblast, p/o Lesnoy Gorodok, 143080 Russia	mail@vniissok.ru
NakhonPhanom Agricultural Research & Development Center	Integrated cultivation of spring onion with other vegetables	Thailand	niyom_sp@hotmail.co.th
Institute of Biocience and Technology, Cranfield University	Study on growth and dry matter production of spring onion, water deficit stress and soil type	Silsoe, Bedfordshire MK45 4DT, UK	l.abbey.s99@cranfield ac.uk
University of Warwick	Physiology of spring onion	Coventry, UK	dezer@turing.ac.uk
Horticulture Research International	Study on growth and dry matter production of spring onion	Wellesbourne, Warwick CV35 9EF, UK	l.abbey.s99@cranfield ac.uk

(continued)

Institution name	Specialization and research activities	Address	Contact information and website
University of New Hampshire	Physiology of *Allium fistulosum*	38 Academic Way, Durham, NH 03824	becky.sideman@unh.edu; http://www.colsa.unh.edu/aes/
US Department of Agriculture, Agricultural Research Service, South Central Agricultural Research laboratory	Physiology of *Allium fistulosum*	911 Highway 3 W, Lane, OK 74555, USA	russo_vincent@hotmail.com
Department of Plant Science, Rutgers University	Food chemistry, antifungal constituents in *A. fistulosum*	65 Dudley Road, New Brunswick, New Jersey 08901-8520	ssang@rci.rutgers.edu
Department of Plant and Soil Science, Texas Tech University	Somatic embryogenesis and micropropagation and cytogenetics of *A. fistulosum*, spring onion breeding, isozyme markers	Lubbock TX 79409-4169,USA794	epeffley@ttacs.ttu.edu
Plant Sciences Department, The University of Tennessee	Physiology of spring onion	252 Ellington Plant Sciences, 2431 Joa Johnson Drive, Knoxville, TN 37996-4561	dkopsell@utk.edu
USDA-ARS and Department of Horticulture, University of Wisconsin	Molecular markers, molecular breeding	Madison, WI 53706, USA	michael.havey@ars.usda.gov
Seminis Vegetable Seeds, DeForest	Cytogenetics, spring onion breeding	WI 53532, USA	llblacktw@netscape.net
Institute of Botany, Academy Sciences of Uzbekistan	Genomic study of *A. fistulosum*	Tashkent, Uzbekistan	ktojibaev@mail.ru

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Friesen N, Pollner S, Bachmann K, Blattner FR (1999) RAPDs and noncoding chloroplast DNA reveal a single origin of the cultivated *Allium fistulosum* from *A. altaicum* (Alliaceae). Am J Bot 86(4):554–562. https://doi.org/10.2307/2656817

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Chapter 5
Water Spinach (*Ipomoea aquatica* Forsk.) Breeding

Moumita Gangopadhyay, Anup Kumar Das, Subhendu Bandyopadhyay, and Samanwita Das

Abstract *Ipomoea aquatica* Forsk. (Convolvulaceae) is a commonly grown vegetable in the Americas, Africa and especially Southeast Asia, including India. Due to the presence of numerous secondary metabolites, this plant has considerable therapeutic as well nutraceutical value and is categorized among highly prioritized but neglected leafy vegetables. Proper identification of the higher quality genotypes of *I. aquatica* will help scientists explore major genes to develop future high-quality varieties. Therefore, an integrated approach combining traditional and molecular plant breeding should be carried out to strengthen future breeding programs. Identification of traits controlling genes by extensive database searching with bioinformatics, followed by genomics and transgenic approaches, opens a new possibility to use these beneficial vegetables as potent nutraceuticals, especially in developing countries where malnutrition is a matter of concern. Application of plant cell culture technique can be an attractive field of research for this plant species. In this context micropropagation is the best choice for producing year around pilot-scale production within a short time span. In vitro plantlets can also be conserved as artificial seed to maintain elite plant lines with augmented secondary metabolites. Screening by the use of hairy root culture under photoautotrophic condition to detect contaminants and pollution can assure cultivars are safe to consume. This chapter presents an overview of the origin, distribution, botanical classification, breeding through classical and molecular approaches, tissue-culture practices like rapid micropropagation for high frequency regeneration, use of elite clones and conservation by alginate entrapment, prospects of using hairy root culture, recent developments and future scope of biotechnology and molecular biology using bio-

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informatics and transgenic approaches and their application for improvement of *I. aquatica*.

Keywords Biotechnology · Breeding · Cultivars · *Ipomoea* · Molecular diversity · Secondary metabolites · Vegetables

5.1 Introduction

Ipomoea aquatica Forsk. is an aquatic plant possessing long and hollow stems containing large numbers of air passages and often with rooting present at the nodes. Leaves of the plant are elliptical in shape; white or pale purple flowers are funnel- or cone-shaped; the fruit is in the form of a capsule (Anonymous 1959; Edie and Ho 1969; Gamble 1921; Payne 1956; Synder et al. 1981).

Ipomoea aquatica is believed to have originated in China (Umar et al. 2007; Edie and Ho 1969). It is found throughout Tropical Asia, India, Sri Lanka, Africa and Australia, as shown in Fig. 5.1 (Kirtkar and Basu 1952). The plant grows as a weed in India and the USA (Anonymous 1959; Reed 1977) while the plant is grown commercially in Southeast Asia (Candlish et al. 1987; Chen et al. 1991).

The plant contains vitamins such as A, B_1, B_2, B_6, B_{12}, C, E and K (Igwenyi et al. 2011) in addition to S-methylmethionine, a reputed treatment for diseases of the gastrointestinal tract (GIT) (Roi 1955). It also contains secondary metabolites such as flavonoids, amino acids, alkaloids, lipids, steroids, saponins, phenols, reducing sugars, tannins, β-carotene, glycosides and minerals (Bergman et al. 2001; Pandjaitan et al. 2005).

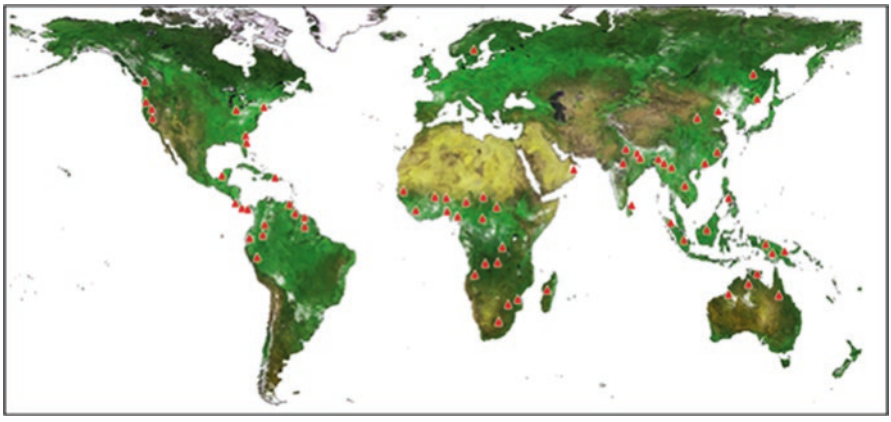

Fig. 5.1 World distribution of *Ipomoea aquatic*. Red triangle indicates the area of occurrence. (Source: Austin 2007)

In traditional medicine, it is used to treat constipation, migraine and sleep-related disorders (Burkill 1966). It is also recommended for the treatment of liver-related disorders (Badruzzaman and Husain 1992), diabetes, mental illness and intestinal problems (Samuelsson et al. 1992). It is a treatment for nosebleeds and high blood pressure (Duke and Ayensu 1985; Perry and Metzger 1980), as an anthelmintic (Nadkarni 1954), antiepileptic and hypolipidemic agent (Dhanasekaran and Muralidaran 2010), and antimicrobial and anti-inflammatory agent (Dhanasekaran and Muralidaran 2010).

The plant is also said to inhibit prostaglandin generation (Tseng et al. 1992). The plant extract is purported to be effective against arsenic poisoning. Poultices of plant extract are also reputed to be effective against itching (Khare 2007).

The above facts illustrate that it is a nutritious comestible plant with a high potential medicinal significance, but is usually neglected due to the lack of scientific knowledge. Therefore, this book chapter provides a complete overview of water spinach taxonomic description, chemistry, cultivation and restoration preservation methodologies and socioeconomic benefits of this vegetable so that people become more aware of its attributes, include it in their diet and begin to promote its cultivation on a larger scale.

5.1.1 Classification

Kingdom: Plantae
Family: Convolvulaceae
Genus: *Ipomoea* Lour.
Species: *Ipomoea aquatica* Forssk.
Synonyms:

- *Ipomoea clappertonii* R. Br.
- *Ipomoea incurve* G. Don
- *Ipomoea natans* Dinter & Süsseng.
- *Ipomoea repens* Roth
- *Ipomoea reptans* Poir.
- *Ipomoea sagittifolia* Hook. & Arn.

Local names: Swamp morning glory, water spinach, water convolvulus and swamp spinach.

5.1.2 Botanical Aspects

Ipomoea aquatica is a sedentary plant that can grow beyond 1 year. Water spinach plants have a rooting system, which can spread in all directions and can penetrate the soil to a depth of 60–100 cm, and expand horizontally to a radius of 150 cm or more. Roots are small to medium sized having a woody or soft core. Branching of roots is minimal.

Water spinach is an annual or perennial, aquatic herb whose stem is hollow, spongy and 2–3 m long. It trails or floats on water and is glabrous, or hairy at the nodes. The stem also contains a milky sap. The surfaces of the leaves are even and arrowhead-shaped. The blades extend up to 5–15 cm and are 2–6 cm broad, whereas the petiole is about 3–14 cm in length. The leaves generally float on water. The fruits are oval-shaped and enclose 1–4 seeds. Seeds may differ in color ranging from gray, brown to black. The plants accumulate heavy metals when cultivated near polluted water, thus posing a risk of biomagnifications. The flowers are bisexual, funnel-shaped and are white to light purple in color. They are found either singly or in clusters between the petiole and stem; the different plant parts are shown in Fig. 5.2.

5.1.3 Habitat Description

Ipomoea aquatica generally grows on moist soils along stagnant streams, fresh water bodies or near wet rice fields. It occurs in both wild and cultivated forms and can be easily propagated through stem cuttings. Within few weeks of planting, it grows rapidly and produces a dense mass of foliage. Water spinach is considered to

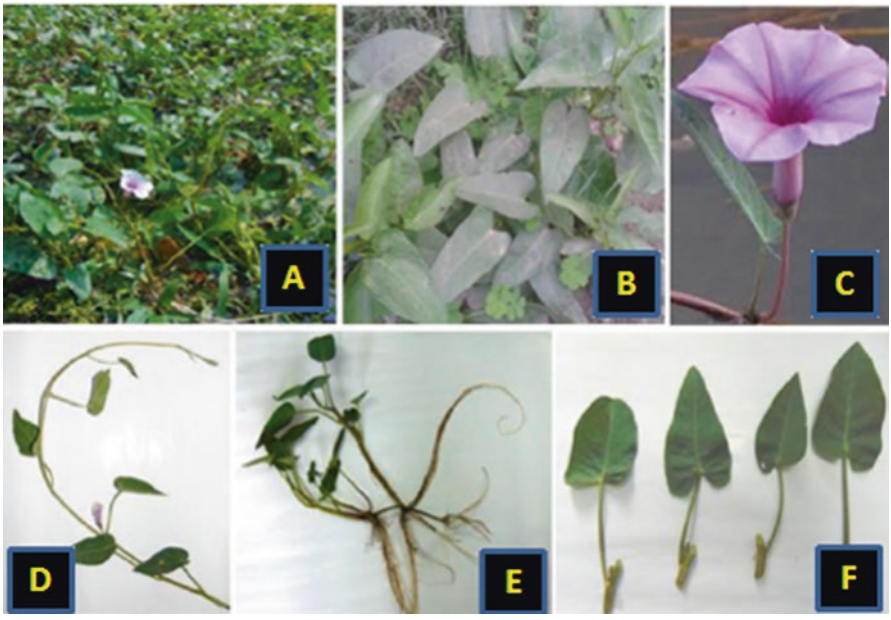

Fig. 5.2 Different plant parts of water spinach, *Ipomoea aquatica.* The morphology of I. aquatica Forssk. (**a**) The plant floating on water, (**b**) The plant creeping on moist soil; (**c**) The funnel form flower of I. aquatica, (**d**) flowering twig with simple and alternate leaves, (**e**) rooting at nodes, (**f**) leaves arise from nodes. (Source: Dua et al. 2015; https://translational-medicine.biomedcentral.com/articles/10.1186/s12967-015-0430-3)

be among the most accepted leafy vegetable (Kala and Prakash 2004; Anonymous 1959; Candlish et al. 1987; Chen et al. 1991; Edie and Ho 1969; Payne 1956; Synder et al. 1981; Wills et al. 1984).

5.1.4 Origin and Distribution

The scientific literature reveals that *Ipomoea aquatica* may have originated in China (Edie and Ho 1969; Umar et al. 2007). Its distribution is mapped and presented in Fig. 5.1. Water spinach is found throughout Tropical Asia, Africa and Australia (Kirtkar and Basu 1952). In India and the USA it grows wild as a weed (Anonymous 1959; Reed 1977) while it is cultivated commercially in Southeast Asian countries (Candlish et al. 1987; Chen et al. 1991).

5.1.5 Nutritional Components

The leaves of water spinach are known to contain proteins, carotenoids, amino acids, vitamins and polyphenols (Chen and Chen 1992; Chitsa et al. 2014; Chu et al. 2000; Daniel 1989; Miean and Mohamed 2001; Ngamsaeng et al. 2004; Rao and Vijay 2002; Umar et al. 2007; Wills et al. 1984). Water spinach has higher antioxidant activity than land spinach with high phenol/flavonoid content (Mariani et al. 2019). Vitamin C, starch, dietary fiber and minerals such as sodium, potassium, calcium, iron, magnesium and zinc contents for *Ipomoea aquatica* were studied by Kala and Prakash (2004) and Wills et al. (1984). Candlish et al. (1987) studied the dietary fiber and starch content in *I. aquatica*. The tocopherol content of *I. aquatica* was also compared and analyzed by Candlish (1983). Imb and Pham (1995) detected lipids, fatty acid and triglycerides content of water spinach. Vitamin C and iron content were investigated by Duc et al. (1999). Munger (1999) compared the nutritional value of *I. aquatica* with rice, sugarcane and maize. It can be argued that investment in water spinach may be an appropriate and an effective way to supplement human nutritional deficiency in developing countries. Irrespective of human nutritional aspect, this leafy vegetable also act as a protein source for indigenous pigs in Vietnam.

5.2 Cultivation

Ipomoea aquatica is a tropical plant native to China, but widely cultivated in East Asia and the Indian subcontinent (Naskar 1990), as well as in California, Texas and the U.S. Virgin Islands (Daniel 2007). As a tropical plant, water spinach produces its highest yield under conditions of high temperature and full sunshine. Below

500 m elevation and 25 °C, plant growth is very slow. This plant is adapted in variable soil condition but organic soils with pH 5.3–6.0 have shown best results (Westphal 1993). Three types of cultivation practices are used for water spinach:

(a) Plants are grown in small lakes or ponds, floating on the water surface.
(b) Wetland cultivation is practiced on hydric soil or saturated ground where seeds or stem cuttings are planted. This cultivation is popular in Southeast Asia and some parts of Africa, but is declining because upland cultivation is more productive. Planting is done by cutting or transplanting from nursery beds. A water level of 15–20 cm is suitable for cultivation and plants are cut 5–10 cm above ground level. The major problem of this form of cultivation is serious losses due to diseases, pests or weeds.
(c) Plants are cultivated on dry soil like normal land plants. However, here special attention should be taken by providing additional fertilizers (mostly N fertilizer) and excess irrigation which is very expensive.
(d) First harvesting is generally done 1 month after planting and thereafter harvesting of leaves can be done at weekly intervals; additional top dressing is recommended after each cutting. Apical dominance is broken by removal of the apical part of the shoot; due to profuse lateral branching of the plant, it forms a bushy architecture (Kaur et al. 2016). Once the plants are established in a particular area, cuttings of can be used as secondary source for cultivation (Edie and Ho 1969).

White rust (*Albugo ipomoeae-panduratae)* is the most common disease of water spinach. Damping off (*Pythium* sp.), root-knot nematodes (*Meloidogyne* spp.) are also reported. Caterpillars like *Spodoptera litura* and *Diacrisia strigatula,* and aphids cause serious threats to this plant.

5.3 Traditional Breeding

The presence of high variability in chromosome numbers and structural ecogeographic variations are observed factors that can be exploited in the implementation of a water spinach breeding program. Although this leafy vegetable grows well under tropical condition, due to its high nutritive value, strategies are needed to develop it for cultivation as well in temperate climatic zones. Up to now, cultivation in temperate regions is only possible under controlled condition using aeroponics or hydroponics (Hoang and Böhme 2001; Pinker et al. 2004) which are expensive and require skilled technicians. Westphal (1993) reported the selection of seeds from Southeast Asia landraces with low quality, low germination rate and high variability, but not for cultivation in temperate regions. Therefore, a holistic approach for developing new varieties by searching and incorporating genes from locally-adapted high elevation varieties into commercial varieties is of utmost importance for water spinach breeders. Researching genetic variability of locally-adapted biotypes began worldwide in the 1980s and 1990s (Synder et al. 1981). Rich genetic variability was

observed among biotypes in Southeast Asia, the Americas and also in Africa, by several earlier researchers. Westphal (1993) reported two main wild biotypes in Southeast Asia on the basis of color variation. One is called Red with green/purple stems, dark green leaves with sometimes purple petioles or veins; another called White characterized by green/white stems, green leaves and green/white petioles. In the USA (Florida), two floating biotypes grown in fresh water or ponds were identified (Van and Madeira 1998) by color differentiation. One is a Red type with red flowers and another White type with white flowers. An Upland cv. was also identified and cultivated commercially in Florida. All of these morphological variations followed by cytogenetic differentiation can be use in breeding programs, especially using genes from nutritionally-rich floating cultivars to upland cultivars to develop nutritionally-enhanced cultivars to grow on a large scale under organized field cultivation.

As water spinach is a highly nutritious and low-cost leafy vegetable, breeding of new spinach varieties with high nutritional value as well as low accumulation of health hazardous compounds at their edible part is the main challenge for genetic improvement programs of this plant species. The work of Chauhan et al. (2017) has shown that various quantitative characters specially related to leaf characteristics like foliage color, yield, leaf length, leaf width, dry matter, chlorophyll content exhibited a strong genotype dependent variation in terms of phenotypic (PCV) and genotypic coefficient of variance (GCV) for selection by separating out environmental influence from total variability. This may be used by plant breeders for the improvement of leaf characteristics (since leaf is the economic part) through selection. This study also revealed that the degree of association in terms of heritability coupled with high genetic advances for genotype-specific leaf traits is due to additive gene effects that may successfully be used in breeding programs towards the improvement of green foliage yield in water spinach. Using D^2 analysis of 25 water spinach genotypes for foliage characteristics, Chauhan and Singh (2018) clustered genotypes into 5 groups suggesting that crossing between genotypes of distant clusters resulted in better recombinants with wider spectrum genetic variability is the promising choice for breeding program for better heterosis in successive generations.

In Africa, there have also been observed different ecotypes with morphological and cytological differences (Ogunwenmo and Oyelana 2009). Two perennial savanna-restricted ecotypes exhibit slightly larger cotyledons and germinated within 2 weeks, while a sporadic annual forest type with smaller cotyledons usually germinates in soil after 6–8 weeks. In normal cases the chromosome number of this species is 2n = 30 (Fedorov 1969), but perennial and annual biotypes showed chromosome number (2n) 30 and 28, respectively; the chromosome size ranged from 1–2.5 μm and 1.5–3 μm, respectively.

In *Ipomoea aquatic*, two cytotypes (https://shodhganga.inflibnet.ac.in/bitstream/10603/15812/7/07_chapter%202) with different morphological traits exist. Broad-leaved aquatic Variant I bears large flowers with a bitter tasting leaves, while a narrow-leaved terrestrial Variant II has small flowers and a sweet taste. Both variants possess significantly different karyotypes and idiograms in their somatic

chromosome. Variant I showed 18 metacentric, 12 submetacentric and 2 pairs of satellite chromosome ($m_{18} + sm_{12} + st_o + t_o = 2n = 30$) whereas Variant II possessed 16 metacentric, 14 submetacentric with 4 pairs of satellite chromosome ($m_{16} + sm_{1}{}_{4} + st_o + t_o = 2n = 30$).

Singh et al. (2016) analyzed 10 genotypes of water spinach to evaluate nutritional and anti-oxidant properties in terms for protein, sugar, chlorophyll, carotenoid, phenol, proline, flavonoids and ascorbic acid contents. This work clearly demonstrated that biochemical traits related to nutritional and anti-oxidant potentialities are largely monitored by additive genes and less influenced by the environment. The high heritability along with high genetic advances indicate genotypic-dependent expression which can be utilized for genetic improvement program through convention plant breeding.

From an integrated metabolomics approach a correlation with phenotypic and metabolic, as well antioxidants level, were observed among different biotypes in terms of nutritional quality (Lawal et al. 2017). Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy combined with multivariate data analysis revealed that Special Pointed Leaf (K-11) cv. had high phenolic content and was most active due to the presence of epicatechin, 4,5-dicaffeoylquinic, protocatechuic acid and rutin as compared to two other cultivars namely Broad Leaf (K-25) and Bamboo Leaf (K-88). To the contrary, K-88 had higher sugars and some amino acids while K-25 possessed a higher content of organic acids. Water spinach breeders need to develop nutritionally enhanced cultivars combining all desirable traits by gene pyramiding, aimed at enhancing trait performance (nutritional quality) using hybridization-based breeding programs.

Today's consumers have specific preferences with regard to the quality of the product, e.g. plant structure, nutritional quality, plantation schedule. East-West Seed Company in Thailand has developed a few popular cultivars for commercial uses. A few examples are for single harvest such as cv. Yangtze with broad leaves and grown year-round, in contrast to cv. Chinwin, suitable for multiple harvesting, cv. Salween with small bamboo-like leaves and suitable for the hot rainy season and cv. Liao with bamboo-like leaves for the dry season (http://www.eastwestseed.com).

Concerning the gene pool resources with morpho-cyto and chemical polymorphism conservation of indigenous resources, or different ecotypes, these are important criteria for strategic implementation in future breeding programs. In this context in situ or field conservation is an essential approach for assembly and preservation of various indigenous land races with genetic diversity. AVRDC (World Vegetable Centre in Taiwan) has already begun this venture, not only for germplasm conservation but also to support research and cultivation (http://203.64.245.173/avgris/).

5.3.1 Breeding for Pollution Safe Cultivars

Water spinach is a valuable, low-cost, leafy vegetable useful as a powerful model plant for phytoremediation by accumulating heavy metals from contaminated water (Li et al. 2016; Zhang et al. 2014) or soil (Ng et al. 2016a, b). Although from the

nutraceutical point of view, higher accumulation of heavy toxic metals (Rai and Sinha 2001) becomes hazardous to human health via bioaccumulation and biomagnifications along the food chain. Therefore, developing pollution safe cultivars (PSCs) to ensure food safety with higher nutritional qualities and adaptabilities is the biggest challenge of plant breeders. PSCs are safe for consumption due to the lower accumulation of specific pollutants in their edible parts which is a practical method of minimizing the concentrations of heavy metals in crops (Grant et al. 2008). The PSC strategy is based on the fact that genotypic variation of edible parts accumulating pollutants is large enough at the cultivar level.

Cadmium (Cd) is one of the most toxic and non-degradable health hazardous heavy metals that accumulate in soil and water used in agriculture and transmit through food chain (Lane et al. 2015). Cultivars of water spinach vary widely in Cd concentration in the cellular vacuoles of the edible part, mainly the shoot portion (Xin et al. 2010). Xiao et al. (2015) and Huang et al. (2016) reported that Cd detoxification, i.e. inhibition in translocation from root to shoot in water spinach, is highly genotype dependent. Henceforth breeding strategies should focus on screening and development of cultivars with lower accumulation of Cd in edible part to assure safety for human consumption. After investigating 38 water spinach genotypes for low Cd accumulation in edible part, Tang et al. (2018) selected 4 genotypes, i.e. *JXDY*, *GZQL*, *XGDB*, and *B888* as pollution safe cultivars without health risk. A Low Shoot Cd cv. (QLQ 56) and a High Shoot Cd cv. (Taiwan 308) of water spinach were documented by Wang et al. (2009) and Xin et al. (2010), validating the molecular mechanism of the differentially-expressed genotypes for cadmium accumulation. Cd-induced gene expression differences of the two water spinach cultivars (QLQ 56, Taiwan 308) have been investigated using suppression subtractive hybridization (SSH) (Huang et al. 2009a, b). Almost 13.3% of the cultivars were found to be vulnerable to Cd contamination in soils (non-Cd-PSC), including cvs. Taiwan 308, Xianggangdaye, Sannongbaigeng, and Jieyangbaigeng, while 6 cvs., Daxingbaigu, Huifengqing, Qiangkunbaigu, Qiangkunqinggu, Shenniuliuye and Xingtianqinggu, were treated as typical Cd-PSCs (Grant et al. 2008). The work of Lian et al. (2010) revealed that the discrepancy in Cd accumulation among cultivars is genotype-dependent rather than from edaphic factors. It is hypothesized that genotyping dependent Cd accumulation of water spinach is mainly controlled by root processing mechanisms (Xin et al. 2013a). Moreover, Xin et al. (2013b) clearly indicated that this is due to the presence of cultivar specific thicker phloem and outer cortex cell walls in the roots which restricts root to shoot translocation. Although there are some studies (Milner et al. 2014; Papoyan and Kochian 2004) focusing on the molecular mechanisms of root processing regarding Cd hyperaccumulation in several species, but the molecular mechanisms of cultivar dependent difference in Cd accumulation of crops are still not adequately studied (Yamaguchi et al. 2010). Huang et al. (2018) reported the role of *metallothioneins* for differential cultivar-dependent Cd accumulation and the mechanism of detoxification. The work of Li et al. (2015) has revealed that unlike Cd, other heavy metals such as Pb and Zn also share similar transport uptake mechanism.

Just like in Cd, differential abilities of lead (Pb) accumulation by different cultivars were observed (Lian et al. 2010). Genotype dependence Pb accumulation at the

cultivar level (Xin et al. 2010, 2011) as well root-specific uptake has been reported (Huang et al. 2009a, b). It is assumed that high variability among the cultivars led to the explanation of intraspecific species (Folorunso et al. 2013) being involved in the mechanism of evolution and variability. However, relevant information regarding hereditary patterns of heavy metal accumulation is quite limited.

5.4 Molecular Diversity

The genus *Ipomoea* belongs to the family Convolvulaceae, consisting of 650 species distributed throughout different parts of the world and comprising shrubs, herbs and small trees, with the majority of species being twining, climbing plants (Mabberley 1997; Miller et al. 2004). Austin (2007) found a close alignment of *Ipomoea aquatica* with other species like *I. cairica, I. ochracea* and *I. obscura* in section *Leiocalyx* (Van Oostroom 1940) due to the polyphyletic nature of section *Erpipomoea*. Ogunwenmo (2003) reported a study based on morphometric and qualitative characters of mature cotyledons in 18 taxa of *Ipomoea*; they identified these species at seedling stages with different morphological traits. In another study, Das and Mukherjee (1997) investigated 12 species of *Ipomoea* and traced their homology and linkage through isozyme markers such as esterase and peroxidase. They performed a comparison between isozyme data and morphological data and successfully determined their interrelationships.

Ipomoea aquatica is an important member of this genus, often consumed as a vegetable. It has also several medicinal properties due to the presence of vitamins, minerals, flavonoids, alkaloids and many secondary metabolites (Malakar and NathChoudhury 2015). Therefore, genetic diversity analysis in different populations of *I. aquatica* it is very important to assess their nutritional as well as medicinal potential for proper utilization.

Genetic diversity analysis with morphological and molecular markers has not been done in great detail for *Ipomoea aquatic*; there are only few reports available for the species. Van and Madeira (1998) conducted a study on three biotypes of *I. aquatic* from Florida using RAPD markers and differentiated all three biotypes, although no clear cut distinction was made for wild types and cultivated biotypes (Fig. 5.3). ITS region and waxy gene sequence based on phylogenetic analysis in *Ipomoea* genus revealed that *I. aquatica* and *I. diamantinensis* are a sister group to *I. quamoclit*; *I. Aquatic* has also been found to be placed consistently in section *Erpipomoea* and is native to the Old-World tropics (Miller et al. 1999). Pollen and flower morphology-based analysis of some *Ipomoea* species in Nigeria classified all the species successfully and two important traits emerged for classification (Jayeola and Oladunjoye 2012).

In another interspecies study conducted by Das (2011), based on morphological and RAPD markers within 12 species of *Ipomoea* using 12 different sets of RAPD primers among which only 4 primers gave amplification for all the examined accessions. The data obtained with the primer OPA 1(5'CCGGCCCTTC3') is shown here in Fig. 5.4 with 11 different lanes corresponding to different *Ipomoea* species

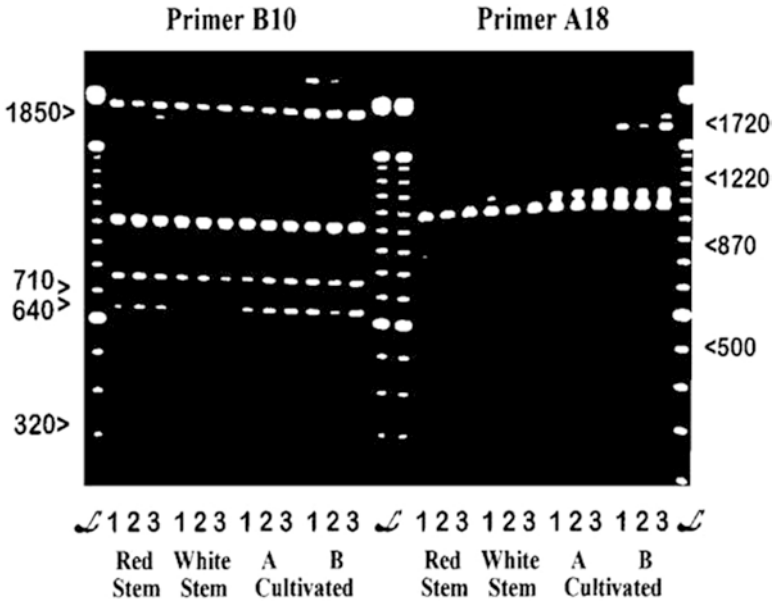

Fig. 5.3 A study on three biotypes of *Ipomoea aquatic* of Florida using RAPD. Lane 6 – *Ipomoea aquatica* RAPD profile. All the other lanes correspond to different *Ipomoea* species. (Source: Van and Madeira (1998))

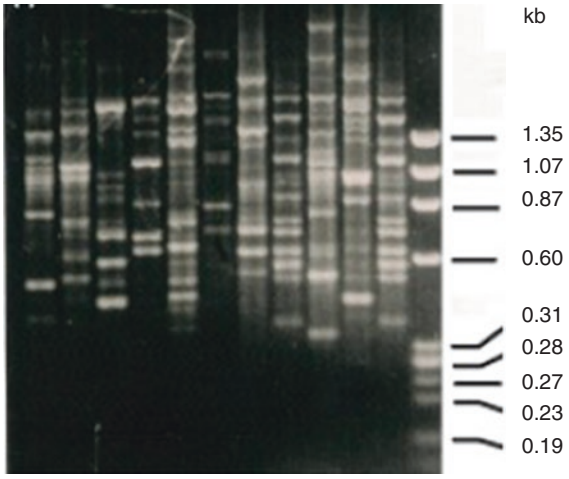

Fig. 5.4 RAPD fragment profile of different *Ipomoea* species with OPA 01 primer. Lane 1-11, 1. *I. quamoclit*, 2. *I. hederifolia*, 3.*I. pes-caprae*, 4. *I. fistulosa*, 5. I. *aquatica*, 6. *I. sepiria*, 7.*I.nil*, 8. *I. obscura*, 9. *I. chryseides*, 10.*I. pes-tigridis*, 11.*I. triloba*, M-DNA marker**.** (Source: Das (2011))

including *I. aquatica*. Moreover, the species *I. hispida* produced least number of bands. It has also been found that the maximum number of amplified fragments were obtained for *I. aquatica* and a sharp congruence was obtained between morphological and molecular data. Nahar and Alam (2016) in their study Anthelmintic based on karyotyping and RAPD markers estimated the effect of industrial effluents on the chromosomes of different samples of *I. aquatica*. They opined that industrial wastes contributed to significant DNA damage in the species. Chauhan et al. (2017) collected 25 genotypes of *I. aquatica* plants from different sites in the district of Chattisgarh, India and studied 15 parameters related to heritability and genetic advance to select a better genotype. Among the 15 parameters, researchers found that traits like leaf length, leaf width and green foliage yield could be successfully for future improvement of the green foliage yield in water spinach.

Here a short account of molecular diversity reports has been provided for related *Ipomoea* species, *I. batata* L. (Sweet potato) which is a species from the same genus and is considered as least risky vegetables to grow due to its rapid growth rate. It is also highly nutritive due to high starch level and elevated content of vitamins and minerals (Moulin et al. 2012). In one study, estimation of genetic variation of different germplasms collected from the local market of Rio de Janeiro, Brazil has been conducted using RAPD and ISSR markers. The results of this study showed that traditional farmers maintain sweet potato genotypes with good genetic diversity (Moulin et al. 2012). Another study reported the genetic diversity analysis between 18 different cultivars each from both South Africa and Papua New Guinea. Using RAPD markers, the study demonstrated the low genetic variation in Papua New Guinea cultivars (Zhang et al. 1998). Molecular diversity report for another species named *Ipomoea triloba*, an annual plant and a close relative to *I. aquatica* (used as a vegetable in West Africa and several other countries) have been investigated by Rane and Patel (2015) using RAPD. The study clearly showed similarity between different species of *Ipomoea* by constructing a dendrogram with UPGMA method based on RAPD data. Molecular evaluation of another species *Ipomoea lacunose* L. generally a weedy species and a close wild relative of *I. batata*, has been done with 14 ISSR primers and their evolutionary diversification was assessed in mid-south of USA (Burgos et al. 2011). Besides, a study has been done using RAPD markers for generating DNA fingerprints of 12 *Ipomoea* species to the morphologically classified sections *Pharbitis, Quamoclit* and *Batatas*. The greatest genetic distance found to be between section *Batatas* and the two ornamental sections (*Pharbitis* and *Quamoclit*). The section *Quamoclit* showed a rather high heterogeneity of the comprised species (Ardelean et al. 2004).

In view of the food value of the species, more molecular marker-based inter-population studies are required to properly distinguish genotypes with higher food value. Exact identification of the higher quality genotypes will also help scientists to explore major nutrition responsive genes to develop high-quality varieties in future. Therefore, association analysis for morphological and nutraceutical traits in *Ipomoea aquatica* should be done to strengthen future breeding programs.

5.5 Water Spinach Tissue Culture

5.5.1 Problems in Conventional Agriculture

Water spinach, being a highly nutritive and low-cost plant with the presence of most of the important essential amino acids, has attracted the attention of researchers for use as an ideal dietary protein and food supplement in place of soybean or eggs (Faruq et al. 2002; Gupta et al. 2005; Hongfei et al. 2011; Kala and Prakash 2004; Ogle et al. 2001) especially for the underprivileged. This neglected vegetable reproduces by sexual and asexual means. Asexual reproduction through vegetative fragmentation is slow due to the dependency on some carrier for their separation of a propagule from the main body and its migration to a different location for establishment (Patnaik 1976). The major problems in sexual reproduction in water are poor seed germination rates and slow initial seedling development (Edie and Ho 1969; Palada and Crossman 1999). Conventional cultivation seems unpromising due to problems of seasonal availability and the endemic nature of cultivars (South Asia, India, South China). In this context the in vitro micropropagation technique is well suited to meet ever growing market demand to produce year around pilot-scale production within a short time span for commercial exploitation.

5.5.2 Application of Plant Tissue Culture

Carotenoids and other antioxidants are present in *Ipomoea aquatic*. Prasad et al. (2005) isolated and characterized a compound 7-o-β-Dglucopyranosyl-dihydroquercitin-3-o-α-D-gluco-pyrannoside having potential antioxidant properties. Prasad et al. (2006) also reported callus-mediated tissue culture with high antioxidant potential. Kiradmanee et al. (2006) and Cha-um et al. (2007) utilized in vitro culture systems as a means to study how the various environmental conditions of photoautotrophic cultivation affect the growth of water spinach under temperature and salt stress, so as to select stress-tolerant clones. Tissue-culture raised transgenic plants developed through nodal co-cultivation with *Agrobacterium tumefaciens* bearing a gene of interest, was reported by Masanori et al. (1997). Normally salinity adversely affects germination process as well normal growth development by hampering physiological processes in plants. However, researchers using in vitro system as testing model with *Ipomea aquatic* Forssk. stated that salt tolerance of water spinach increased as the response towards increasing salinity (Ibrahim et al. 2019) which impart possibility of growing this plant in saline prone ecosystem. Plant regeneration through in vitro tissue culture remains necessary for rapid multiplication, in vitro conservation as well genetic manipulation and source for the isolation of bioactive compounds. Therefore, development of specific protocols especially for the identification of optimized culture media recipe for tissue culture establishment is very important. Stephen and Bopaiah (2014) demonstrated formulation of

an ideal culture medium for the in vitro propagation of *Ipomoea palmata* Forssk. (Synonym: *Ipomoea cairica.* L. Sweet).

5.5.3 Photoautotrophic Hairy Root Culture

Due to the potential of active and rapid propagation, and a high metabolic rate induced by *Agrobacterium rhizogenes*, hairy root culture can be used as an effective tool to develop high yielding cell lines. Several researchers established hairy root culture for the production of antioxidative enzymes like peroxidase (Taya et al. 1989) and superoxide dismutase (Kino-oka et al. 1991). Several researchers reported that higher illumination elicits the accumulation of more chlorophyll and develops greener (Fig. 5.5) transformed root clones (Masahiro et al. 1996; Nagatome et al. 2000; Taya et al. 1994). Hirofumi et al. (2000) reported the development of a phototropic cell line with augmented chlorophyll and activities of 5-ribulose-biphosphate carboxylase content. Kiuo-oka et al. (1996) presented a kinetic model regarding hairy root growth and chlorophyll formation in a photoautotrophic condition under continuous illumination. Ninomiya et al. (2001) investigated differential elongating behavior in photoautotrophic hairy roots and reported changes in chlorophyll content under a photoautotrophic condition (Ninomiya et al. 2002). From his work on green hairy root of pak bung (water spinach), a correlation between growth pattern and oxygen uptake (Ninomiya et al. 2003) was established.

Currently, phytoremediation has become an important sustainable technique for the removal of environmental pollutants (Agostini et al. 2013). Since hairy roots have become a tool for screening the accumulation potentialities of pollutants in different cultivars to tolerate or accumulate, pak bung (*Ipomea aquatica*) hairy roots were used as a model system for assessing pollutant absorption capacity**.** Since herbicides are potent environmental pollutants, green pak bung hairy roots showed

Fig. 5.5 Photoautotrophic and heterotrophic hairy roots of *Ipomea aquatica*. (**a**) Green hairy root under illumination, (**b**) Hairy roots under dark condition. (Source: Taya et al. 1994)

differential herbicidal response in comparison to heterotrophic hairy roots when grown under herbicidal stimuli using image analysis (Ninomiya et al. 2002, 2003).

5.5.4 In Vitro Conservation Through Synthetic Seed

A major downside to commercialization of conventional tissue culture is the need for continuous subculturing, which often produces undesirable results and the plant material is not easily exchangeable (West et al. 2006). Artificial seed or synthetic seed technology offers an efficient technique for exchange of plant germplasm, short- or long-term storage, and direct transfer of in vitro material to ex vitro conditions (Ara et al. 2000; Germana et al. 2011; Rai et al. 2009; Standardi and Piccioni 1998). By virtue of this method, artificially encapsulated micropropagules can be used as seeds and directly planted in the nursery and grown into a plant under in vitro or in vivo conditions. Nodes with axillary buds from *Ipomoea aquatica* have been successfully encapsulated and were well established when planted in the field (Tang et al. 1994). As this plant possesses high nutritive values, the alginate entrapment process can also be used as a potential tool for enhancing plant secondary metabolites with health benefits like other plants where immobilized cells showed a higher accumulation of secondary metabolites (Hall et al. 1989; Hussain et al. 2012). Table 5.1 provides a list of plants showing high levels of secondary metabolite accumulations using alginate entrapment.

5.6 Transgenic Approaches for Phytoremediation Studies

Phytoremediation is the removal of toxic compounds from polluted soil or water bodies by plants. Environmental pollution is a present-day threat to human lives in ever-greater proportion. In this context identification of plants with phytoremedial potential is of high concern and scientists are continuing work on it. *Ipomoea aquatica* is such a plant with immense potential as a metal chelator, which has already been proved in several scientific reports.

In view of the phytoremedial qualities of water spinach, and its use as a vegetable, gene transfer studies are very important as they will help in the development of better quality genotypes with higher food qualities or better pollutant removal qualities. In this context it is important to note that very few genetic engineering studies in *Ipomoea aquatica* have been done to date. A genetic transformation protocol was standardized by Khamwan et al. (2003), using cut cotyledons to infect with *Agrobacterium* harboring the *GUS* gene. The resulting *I. aquatica* plants showed stable *GUS* expression, indicating a successful transformation system. Sakulkoo et al. (2005) developed transgenic *I. aquatica* plants capable of hyperassimilation of sulfate and showing tolerance to toxic levels of sulfide and cadmium. This study demonstrated introduction of the *Arabidopsis* adenosine *phosphate sulfate*

Table 5.1 Some plants producing various secondary metabolites using alginate entrapment

Common and scientific names of plants	Secondary metabolites	References
Madagascar periwinkle (*Catharanthus roseus*)	Ajmalicine	Brodelius et al. (1979)
Pepper (*Capsicum annuum*)	Capsaicin	Johnson et al. (1991)
Opium poppy (*Papaver somniferum)*	Codeine	Furuya et al. (1984)
Foxglove (*Digitalis lanata*)	Digitoxin	Alfermann and Reinhard (1980)
Velvet bean (*Muczma pruriens*)	L-DOPA	Wichers et al. (1983)
Egyptian henbane (*Hyoscyamus muticus*)	Solavetivone	Ramakrishna et al. (1993)
Kalmegh (*Andrographis paniculata*)	Andrographolide	Chauhan et al. (2019)
Vinca (*Catharantus roseus*)	Ajmalicine	Akimoto et al. (1999) and Lee-Parsons and Shuler (2005)
Vinca (*Catharantus roseus*)	Indole alkaloid	Zhao et al. (2001)
Datura (*Datura innoxia*)	Tropane alkaloids	Gontier et al. (1994)
California poppy (*Eschscholzia californica*)	Benzophenanthridine alkaloid	Villegas et al. (2000)
Buckthorn (*Frangula alnus*)	Anthraquinones	Sajc et al. (1995)
Egyptian henbane (*Hyoscyamus muticus*)	Sesquiterpenes	Curtis et al. (1995)
Flax (*Linum usitatissimum*)	Neolignan	Attoumbré et al. (2006)
Tobacco (*Nicotiana tabacum*)	Scopolin	Gilleta et al. (2000)
Tobacco (*Nicotiana tabacum*)	Phenolics	Shibasaki-Kitakawa et al. (2001)
Parthenocissus tricuspidata	methyl(2R,3S)-2- benzamido-methyl-3- hydroxybutanoate	Shimoda et al. (2009)

transferase gene of a sulfur assimilation pathway that strengthened the sulfur assimilation capabilities of the plant. Meerak et al. (2006) reported the development of transgenic *I. aquatica* plants coexpressing the *Arabidopsisserine acyltransferase* gene and a rice cysteine synthase gene which conferred them higher sulfur assimilation qualities. Furthermore, Moontongchoon et al. (2008), reported that two transgenic lines of *I. aquatica* plants coexpressing *Arabidopsis* serine acyltransferase gene and rice cysteine synthase gene effectively mitigated detrimental effects of cadmium toxicity by efficiently developing and storing sulfur compounds.

Ipomoea aquatica is known as an accumulator of different environmental pollutants like heavy metals, sulfates and phosphates (Ng et al. 2016a, b; Rai and Sinha 2001). Hence, for better exploration of the species in relation to phytoremediation, two *I. aquatica* cultivars have been compared at the transcriptome level to understand the molecular mechanisms underlying the property of cadmium accumulation (Huang et al. 2016). The Low Shoot Cd (QLQ) cv. showed higher expression for cell wall biosynthesis genes, such as *GAUT* and *laccase*, and three Cd efflux genes (*Nramp5*, *MATE9*, *YSL7*). The High Shoot Cd cv. (T308) highly expressed sulfur and glutathione metabolism pathway genes like sulfate transporter and cysteine synthase. Exploration of metallothioneins (MTs) in a Low Shoot Cd (QLQ) cv. and a High Shoot Cd (T308) cv. has been done by Huang et al. (2016). Results showed that three *IaMT* genes, *IaMT1, IaMT2* and *IaMT3*, have been variably expressed in response to Cd stress in different cultivars. All three genes have been cloned and characterized in bacterial system where their roles have been properly elucidated. *IaMT1* was found to be unresponsive towards Cd stress whereas *IaMT2* and *IaMT3* increased Cd accumulation in *Escherichia coli*. Chuang Shen et al. (2017) demonstrated the role of miRNAs in cadmium accumulation and translocation in a Low Shoot Cd (QLQ) cv. and a High Shoot Cd (T308) cv. They reported that five different miRNAs have exclusively been regulated in the QLQ cultivar among them miRNA395 was shown to be upregulated, hypothesized to enhance the Cd retention and detoxification. Apart from miRNA395, several others, miRNA5139, miRNA1511 and miRNA8155, showed altered expression during Cd stress and were found to be involved in attenuation of Cd translocation into the shoot of QLQ. In the T308 cultivar, complex responses of miRNAs were revealed; miRNA397 regulation was found to be related to Cd influx, whereas miRNA3627 was involved in the efflux of Cd. These studies provided a new standard for molecular-assisted breeding of Low Cd cultivars for leafy vegetables.

In an updated and very recent review by Prasad (2019), phytoremedial prospects of high biomass producing aquatic plants have been discussed including transgenic approaches. It has been suggested that industrial-waste water which contains several toxic metal ions can be made free from these toxic elements with the help of aquatic plants like *I. aquatica*. Molecular biological studies can be performed for overexpression of genes for accumulation and detoxification of toxic metals and metalloids in these plants for phytoremediation purposes. In this respect, several important genes (like metallothionins, phytochelatins and several other uptake and transport related genes) can be used for making transgenic *I. aquatica* plants so that these modified plants can be used for phytoremediation purposes in future.

5.7 Bioinformatics

Bioinformatics is the study of biological samples using computer science and information technology. In the current genomic era, sequence information obtained from different scientific projects related to genome sequencing has generated huge

quantities of data that need proper handling and interpretation. In this context bioinformatics databases and tools provide wide-ranging opportunities to analyze and discover new biological insights. In crop development, bioinformatics has played a very significant role. The genomes of staple crops like rice, wheat and maize have all been sequenced, as well as important vegetables and legumes like tomato (Sato et al. 2012), potato (Xu et al. 2011), soybean (Schmutz et al. 2010) and chickpea (Varshney et al. 2013). Sequencing of these genomes has provided vast information that will provide important information regarding future improvement of these crop plants, which in turn will benefit humankind.

The full genome of *Ipomoea aquatic* has not yet been sequenced; a literature search revealed one in silico study of antibacterial and antioxidant potential of the plant and another such analysis of compounds blocking bacterial life cycle receptors. Gas chromatography based-MS analysis demonstrated the presence of five major compounds subjected to in silico analysis with bacterial receptors such as LuxS (1JVI), FtsZ (1S1J), FtsZ (3VOB) and LsrB (1TM2) from *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus* and *Salmonella typhii*, respectively, by using autodock 4.2 and Cygwin. Results from the in silico study supported the fact that compounds from *I. aquatica* exhibited antibacterial properties (Chandra and Shamli 2015).

The nucleotide database of National Centre for Biotechnology Information (NCBI) contains 219 entries for *Ipomoea aquatica* (Table 5.2). Among them, several full-length genes are present (MG471389, *Ipomoea aquatic* metallothionein MT3 (MT3) mRNA;MG471388, *Ipomoea aquatic* metallothionein MT2 (MT2) mRNA; MG471387, *Ipomoea aquatic* metallothionein MT1 (MT1), mRNA), several partial gene sequences are also available (MH792116.1, *Ipomoea aquatica* voucher DMB 8 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence). Given the importance of the species the information available in the database was found to be inadequate; hence, there is ample scope for more work in this field.

5.8 Conclusions and Prospects

Ipomoea aquatica is a low cost vegetable increasingly gaining attention around the world because of its high nutritional potential. Scientific findings on this plant reveal a treasure trove of several bioactive compounds with nutraceutical as well pharmaceutical importance. Phytochemical screening of the plant as well as investigation on its hyperaccumulation qualities has been done in great detail, but molecular data for the plant are available in a much smaller amount. There is a broad scope of work to be done regarding molecular-marker based identification of different genotypes, biotypes and cultivars through which we can pinpoint high value genotypes. An organized and targeted specific breeding program or gene pyramiding effort is needed, as well as marker-assisted selection using locally-adapted cultivars

Table 5.2 Different genes expressing various proteins in different accessions *of Ipomoea aquatic*

Accession No.	Gene/Protein name	Features	Source
GU135247.1	Ribulose1,5-bisphophate carboxylase/oxygenase large subunit (rbcL); partial sequence	567 bp linear DNA	Abbott JR et al. Submitted (27-OCT-2009) Florida Museum of Natural History, University of Florida, P.O. Box 117800, Gainesville, FL 32611-7800, USA
GU135418.2	Psba gene; partial sequence	468 bp linear DNA	
GU135084.1	matK gene; partial sequence	827 bp linear DNA	
MK309395.1	Ribulose1,5-bisphophate carboxylase/oxygenase large subunit (rbcL); partial sequence	567 bp linear DNA	Sasikumar, K. and Anuradha, C. Submitted (18-DEC-2018) Department of Botany, Periyar Evr College, Khajamalai, Thiruchirapalli, Tamil Nadu 620023, India
MK347242.1	matK gene; partial sequence	655 bp linear DNA	
MH189790.1	Ribulose1,5-bisphophate carboxylase/oxygenase large subunit (rbcL); partial sequence	545 bp linear DNA	Zhang W and Zhao H Submitted (10-APR-2018) Marine College, Shandong University (Weihai), 180 Wenhua Xilu, Weihai, Shandong 264209, China
MN153541.1	SKK-004 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	534 bp linear DNA	Kadam S Submitted (09-JUL-2019) Department of Biochemistry, Shivaji University, Shivaji university, Kolhapur, Maharashtra 416004, India
MH796546.1	*Ipomoea aquatica* voucher DMB 8 ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, chloroplast partial sequence	1249 bp linear DNA	Ranathunga APDT et al. Submitted (24-AUG-2018) Department of Molecular Biology, University of Peradeniya, Peradeniya, Kandy, Central 20000, Sri Lanka
MH796544.1	*Ipomoea aquatica* voucher DMB 6 ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, chloroplast partial sequence	1249 bp linear DNA	
MG471389.1	*Ipomoea aquatica* metallothionein MT3 (MT3) mRNA, complete sequence	518 bp linear mRNA	Huang Y-Y Submitted (11-NOV-2017) School of Life Sciences, Sun Yat-Sen University, Xingang Xi Road 135, Guangzhou, Guangdong 510275, China
MG471388.1	*Ipomoea aquatica* metallothionein MT2 (MT2) mRNA, complete sequence	596 bp linear mRNA	
MG471387.1	*Ipomoea aquatica* metallothionein MT1 (MT1) mRNA, complete sequence	656 bp linear mRNA	

(continued)

Table 5.2 (continued)

Accession No.	Gene/Protein name	Features	Source
MH792116.1	*Ipomoea aquatica* voucher DMB 8 small subunit ribosomal RNA gene,partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	633 bp linear DNA	Ranathunga AP et al. Submitted (24-AUG-2018) Molecular Biology and Biotechnology, University of Peradeniya, Peradeniya, Kandy, Central 20000, Sri Lanka
MH792115.1	*Ipomoea aquatica* voucher DMB 7 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	633 bp linear DNA	
MH792114.1	*Ipomoea aquatica* voucher DMB 6 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.	633 bp linear DNA	
AY100958.1	*Ipomoea aquatica* ribulose-1,5-bisphosphate carboxylase/oxygenase, large subunit (rbcL) gene, partial cds; chloroplast gene for chloroplast product	1372 bp linear DNA	Stefanovic S, Krueger L and Olmstead,R.G. Submitted (30-APR-2002) Botany, University of Washington, Box 355325, Seattle, WA 98195-5325, USA
AY100856.1	*Ipomoea aquatica* PsbE (psbE) gene, partial cds; PsbF (psbF) and PsbL (psbL) genes, complete cds; and PsbJ (psbJ) gene, partial cds; chloroplast genes for chloroplast products	758 bp linear DNA	
AY100749.1	*Ipomoea aquatic*a ATP synthase beta subunit (atpB) gene, complete cds; chloroplast gene for chloroplast product	1473 bp linear DNA	
AF111127.1	*Ipomoea aquatica* granule-bound starch synthase (waxy) gene, partial sequence	589 bp linear DNA	Miller RE, Rausher MD and Manos PS Submitted (07-DEC-1998) Botany, Duke University, Durham, NC 27708, USA

(continued)

Table 5.2 (continued)

Accession No.	Gene/Protein name	Features	Source
HQ142108.1	*Ipomoea aquatica* UDP glucose flavonoid 3-glucosyltransferase (UF3GT) mRNA, partial sequence	806 bp linear mRNA	Toleno DM, Durbin ML, Lundy KE and Clegg MT Plant Species Biol 25 (1): 30–42 (2010)
HQ142087.1	*Ipomoea aquatica* MADS (MADS) mRNA, partial sequence	402 bp linear mRNA	
HQ142068	*Ipomoea aquatica* alpha isopropylmalate synthase (IPMS) mRNA, partial sequence	822 bp linear mRNA	
HQ142048.1	*Ipomoea aquatica* isopropylmalate dehydrogenase (IMDH) mRNA, partial sequence	672 bp linear mRNA	
HQ142029.1	*Ipomoea aquatica* isolate 1 DFRB (DFRB) mRNA, partial sequence	453 bp linear mRNA	
HQ142028.1	*Ipomoea aquatica* DFRB (DFRB) mRNA, partial sequence	486 bp linear mRNA	
HQ142017.1	*Ipomoea aquatica* chalcone synthase (CHS-E) mRNA, partial sequence	808 bp linear mRNA	
HQ141992.1	*Ipomoea aquatica* chalcone synthase (CHS-D) mRNA, partial sequence	854 bp linear mRNA	
HQ141973.1	*Ipomoea aquatica* anthocyanidin synthase (ANS) mRNA, partial sequence	873 bp linear mRNA	
HQ141953.1	*Ipomoea aquatica* acetolactate synthase (ALS) mRNA, partial sequence	738 bp linear mRNA	
FJ795794.1	*Ipomoea aquatica* maturase K (matK) gene, chloroplast partial sequence	819 bp linear DNA	Tugume A K et al. Submitted (25-FEB-2009) Department of Applied Biology, University of Helsinki, P.O. Box 27 (Latokartanonkaari 7), Helsinki FIN-00014, Finland
KU182875.1	*Ipomoea aquatica* internal transcribed spacer 2, partial sequence	222 bp linear DNA	Li YB and Wu B Submitted (23-NOV-2015) Research Center of Natural Resources of Chinese Medicinal Materials and Ethnic Medicine, Jiangxi University of Chinese Medicine, Xingwan Road of Wanli District, Nanchang,Jiangxi 330000, China.

(continued)

Table 5.2 (continued)

Accession No.	Gene/Protein name	Features	Source
KP261915.1	Ipomoea aquatica voucher FRI 70037 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	530 bp linear DNA	Simoes AR, Culham A and Carine M Bot J Linn Soc 179 (3):374–387 (2015)
KR025049.1	Ipomoea aquatica voucher FRI 70037 trnK-rps16 intergenic spacer, partial sequence; and ribosomal protein S16 (rps16) gene, partial cds; chloroplast	674 bp linear DNA	
KR024912.1	Ipomoea aquatica voucher FRI 70037 maturase K (matK) gene, chloroplast partial sequence	749 bp linear DNA	
AY101067.1	Ipomoea aquatica tRNA-Leu (trnL) gene, partial sequence; trnL-trnF intergenic spacer, complete sequence; and tRNA-Phe (trnF) gene, partial sequence; chloroplast genes for chloroplast products	809 bp linear DNA	Stefanovic S, Krueger L and Olmstead RG Am J Bot 89 (9):1510–1522 (2002)
HM367065.1	Ipomoea aquatica clone Q5 metallothionein type 3-like protein mRNA, complete sequence	201 bp linear mRNA	Huang B et al. J Agri Food Chem 57 (19):8950–8962 (2009)
AF110919.1	Ipomoea aquatica internal transcribed spacer 1, 5.8S ribosomal RNAgene, and internal transcribed spacer 2, complete sequence	598 bp linear DNA	Miller RE, Rausher MD and Manos PS Submitted (04-DEC-1998) Department of Botany, Duke University, Box90338, Durham, NC 27708, USA

Source: Sources of the sequences are mentioned properly in the source column. All the sequences have been taken from National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/) with proper citation of authors names and affiliation. The majority of the data have been directly submitted in the database and those sequence data presented in journals are listed in the references section

specifically for creating pollution-safe, nutritionally-rich genotypes. Differential gene expression analysis based on high throughput RNA sequencing can be done for different cultivars of *Ipomoea aquatica* (showing differences in pollutant accumulating or sequestering properties) for identification of novel genes and transcription factors which can be a step toward making a transgenic elite plant in future for the betterment of humankind. Although this plant is normally cultivated under tropical condition, due to its huge potential as an important food supplement around the world, developing new strategies for cultivation under temperate conditions represents a promising future research area.

Appendices

Appendix I: Research Institutes Relevant to Water Spinach

Institution	Specialization and research activities	Contact information and website
State Key Laboratory for Biocontrol and School of Life Sciences	Pollution-safe cultivar through traditional breeding, transcriptomics	Sun Yat-sen University, Guangzhou,510275, China Website: http://www.sysu.edu.cn
Research and Instructional Farm of Horticulture, Department of Vegetable Science	Traditional and molecular breeding, agronomy	Indira Gandhi Krishi Vishwavidyalaya, Raipur, Chhattisgarh, India Website: http://www.igau.edu.in/
Laboratory of Aquatic Vegetables	Breeding	Yangzhou University, Yangzhou, 225009, P.R. China Website: http://en.yzu.edu.cn/
Fengshan Tropical Horticultural Experiment Station	Germplasm maintenance, breeding	Taiwan Agricultural Research Institute, Fengshan, Kaohsiung, Taiwan Website: https://www.tari.gov.tw/english/
Department of Botany	Phytochemistry	University of Allahabad, Allahabad, India Website: http://www.allduniv.ac.in/
School of Environment and Life Science	Agronomy	University of Salford, Salford M5 4WT, United Kingdom Website: https://www.salford.ac.uk/
College of Natural Resources	Plant breeding	University of California, Berkeley, CA 94720, USA Website: https://www.berkeley.edu/
Humboldt-University of Berlin	Molecular biology, breeding	Institute of Horticultural Sciences Lentzeallee 75, 14195 Berlin Germany Website: https://www.hu-berlin.de/
Department of Botany	Taxonomy	Dr. B. A. M. University, Aurangabad, (M.S.), India Website: http://www.bamu.ac.in/
Department of Safety and Environmental Engineering	Breeding and molecular biology	Hunan Institute of Technology, Hengyang 421002, China Website: http://www.hnit.edu.cn/
Key Laboratory of Tropical Agro-environment	Agronomy, nutrition	Ministry of Agriculture/South China Agricultural University, Guangzhou 510642, P.R.China Website: http://english.scau.edu.cn/
Resources and Environment College	Pollution-safe cultivar through traditional breeding	Qingdao Agricultural University, Qingdao 266109, China Website: https://www.qau.edu.cn/

(continued)

Institution	Specialization and research activities	Contact information and website
College of Agronomy	Pollution-safe cultivar through traditional breeding	Hunan Agricultural University, Changsha 410128, China Website: http://english.hunau.edu.cn/
Advanced Pharmacognosy Research Laboratory	Pharmacognosy and phytochemistry	Department of Pharmaceutical Technology, Jadavpur University, Kolkata, 700032, India Website: http://www.jaduniv.edu.in/
Department of Horticulture	Breeding, nutrition	Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia, West Bengal 741252 Website: https://www.bckv.edu.in/

Appendix II: Genetic Resources of Water Spinach

Cultivar	Important traits	Cultivation location	Source
Ipomoea aquatica - Variant I	Broad leaved	Southeast Asia	Austin (2007)
I. aquatica - Variant II	Narrow leaved	Southeast Asia	Kaiser Hamid et al. (2011)
Kankoong beeasa	Dark-green leaves and stems and purple flowers	Java	Cornelis et al. (1985)
Kankoong nagree	Yellowish-green leaves, yellowish stems and white flowers	Java	Cornelis et al. (1985)
Pak Quat	White stems	Hong Kong	Pritesh Pandey and Madan Jha (2019)
Ching Quat	Green stems	Hong Kong	Pritesh Pandey and Madan Jha (2019)
cv. QLQ	Low shoot Cd cultivar	China	Xin et al. (2010)
cv. T308	High shoot Cd cultivar	China	Baifei Huang et al. (2014)
Red stem cultivar	Dryland cultivation	China	Austin (2007)
White stem cultivar	Wetland cultivation	China	Austin (2007)

(continued)

Cultivar	Important traits	Cultivation location	Source
Taiwan filiform-leaf *I. aquatica*	High phytoremediation potential	Taiwan	Quan-Ying Cai et al. (2008)
Hong Kong white-skin *I. aquatica*	Low phytoremediation potential	Hong Kong	Saikat Dewanjee et al. (2015)
cv. Taiwan 308	Non-Cadmium-PSC	Taiwan, China	Wang et al. (2009)
Xianggangdaye, Sannongbaigeng, and Jieyangbaigeng	Non-Cadmium-PSC	China	Wang et al. (2009)
cv. Daxingbaigu, Huifengqing, Qiangkunbaigu, Qiangkunqinggu, Shenniuliuye, and Xingtianqinggu	Cadmium-PSCs	China	Wang et al. (2009)
Thaiqinggengliuye water spinach (Liuye)	Non- Arsenic-PSCs	China	Dua et al. (2015)
Hong Kong chunbaidaye water spinach (Daye)	Arsenic-PSCs	Hong Kong, China	Wang et al. (2009)
cv. YQ	Low-Cd-Pb	China	Junliang Xin et al. (2012)
cv. GDB	High-Cd-Pb	China	Baifei Huang et al. (2012)
Salween	Small bamboo-like leaves and Suitable for the hot rainy season	Northern Thailand, Vietnam, southern China	Grubben (2004)
Liao	Bamboo-like leaves for the dry season	Northern Thailand, Vietnam, southern China	Grubben (2004)
Chinwin	Branching cultivar	Northern Thailand, Vietnam, southern China	Cornelis et al. (1985)

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Chapter 6
Watercress (*Nasturtium officinale* R. Br.) Breeding

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Abstract Watercress is an aquatic perennial herb belonging to the family Brassicaceae, which is commonly found in the wild in cool stream margins and ditches as well as cultivated in tanks or moist soil for its edible leaves. The most common and widespread species of the genus *Nasturtium* are *N. officinale* R. Br. and *N. microphyllum* Boenn. Rchb., which originated from Eurasia and North Africa and widely spread in Europe, including Britain, from Sweden and Denmark, and to North America. Watercress is consumed fresh or as an ingredient in soups or other recipes. It contains vitamins, phenolic compounds, folic acid, carotenoids, minerals, fibers, lipids, proteins, and a high level of glucosinolates. It purportedly possesses antioxidant and anti-carcinogenic properties and a possible role in the prevention of other diseases including cardiovascular, neurodegeneration and diabetes. The best site for a watercress bed is on a relatively flat area with a slight slope below a spring that supplies water. The beds should be protected from floods, which could cause severe damage to both the watercress and the beds. It is propagated by seeds and vegetative reproduction. This chapter presents watercress biodiversity and distribution, cultivation practices, conservation, traditional breeding, molecular breeding, functional genomics and genetic engineering.

Keywords Anti-carcinogenic · Aquatic · Glucosinolates · Isothiocyanates · Vegetative reproduction

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6.1 Introduction

Watercress (*Nasturtium* R. Br., *Cardamine* L. and *Rorippa* Scop.) is a common name for aquatic perennial herbs with white crosslike flowers, belonging to the family Brassicaceae (Hedge 1968). Watercress is commonly found in the wild in cool stream margins and ditches as well is cultivated in tanks or moist soil for its edible leaves.

Nasturtium, *Cardamine* and *Rorippa* are three closely related genera, which have been placed in the tribe Arabideae DC. (Al-Shehbaz 1988). The genus *Nasturtium* comprises five species (Al-Shehbaz and Price 1998). The most common and widespread species are *Nasturtium officinale* R. Br. and *Nasturtium microphyllum* Boenn. Rchb., which originated from Eurasia and North Africa and spread widely in Europe, including Britain, from Sweden and Denmark to North America (Al-Shehbaz and Price 1998). Like other members of the Brassicaceae family, *Nasturtium* and *Rorippa* have very similar morphological characteristics, which have resulted in disagreements among taxonomists on the separation of the two genera. However, the combination of morphological identification and the molecular analysis has clearly revealed the relationships among the genera of Brassicaceae (Al-Shehbaz and Warwick 1997; O'Kane and Al-Shehbaz 1997).

Previous research did not recommend the incorporation of *Nasturtium* into *Rorippa* (Franzke et al. 1998; Les 1994). Les (1994) compared sequences of the rbcL chloroplast gene in six species of the cardaminoid group in Brassicaceae. The results supported the separation of *Nasturtium* from *Rorippa*, and indicated that *Nasturtium* is closely related to the cosmopolitan genus *Cardamine* and proposed grouping *N. officinale* with *Cardamine pensylvanica* Muhlenberg ex Willdenow. The two species *R. sylvestris* (L.) Besser (the generic type) and *R. amphibia* (L.) Besser, form a separate clade more closely related to lakecress (*Neobeckia aquatica* (Eaton) E.L. Greene) and horseradish (*Armoracia rusticana* P. Gaertner).

Molecular studies based on the ITS and ndhF and the trnL-F intron and spacer regions also indicate that the endangered species *Nasturtium gambellii* (S. Watson) O.E. Schulz, forms a clade with *N. officinale*, and the genus *Nasturtium* is much more closely related to *Cardamine* than to other genera in the cardaminoid group, including *Armoracia*, *Rorippa* and *Barbarea* R. Brown (Sweeney and Price 2000).

Cardamine can clearly be distinguished from *Nasturtium* by its unique fruits that dehisce explosively, the spirally coiled valves without a distinct midvein, and the flattened replum. However, in *Nasturtium*, the fruits do not dehisce explosively, the valves possess a distinct midvein and do not coil after dehiscence, and the replum is round.

As mentioned above, *Nasturtium* includes five species: *N. officinale* (the type species) and *N. microphyllum*, both native to Eurasia and North Africa and naturalized worldwide, the Moroccan *N. africanum* Braun-Blanquet, the North American *N. gambellii* (California, Mexico), and *N. floridanum* (Al-Shehbaz & Rollins) Al-Shehbaz & R.A. Price. *Nasturtium valdes-bermejoi* Castroviejo was first

described from Spain, but it was later deemed a minor variant of *N. microphyllum* (Valentine 1993).

Nasturtium officinale has been introduced to East and Southeast Asia, Sub-Saharan Africa, the Americas and the Caribbean, Australia, New Zealand and some Pacific islands (USDA-ARS 2014). Howard and Lyon (1952a) proposed that *N. officinale* and *N.* × *sterile* (Airy Shaw) Oefel., the hybrid between the two species (i.e. *N. officinale* × *N. microphyllum*) are more likely to have been introduced than *N. microphyllum*, because the latter is not usually cultivated. However, *N. microphyllum* might have been introduced accidentally as several species of watercress often grow together in the same area.

6.1.1 Diversity and Distribution of Watercress in Iran

Unlike *Nasturtium officinale,* which grows in wet habitats in most parts of the world, *N. microphyllum* is a species that is confined in one identfied locality (Bakhtiari, Belu) (Hedge 1968). In recent years, the latter plant species has been found in several locations in Guilan and Mazandaran provinces. A hybrid taxon between *N. officinale* and *N. microphyllum* occurs in the areas, where the two species overlap. This hybridization event has been reported for the first time in the North of Iran (Naqinezhad 2006).

Jafari and Hassandokht (2012) evaluated 24 wild growing accessions of *Nasturtium officinale* collected from six provinces of Iran using agro-morphological traits. In their study, plants of the Noshahr accession had the minimum number of silique per plant and the shortest length of flowering stems. Plants collected from Sarab and Mehraban showed the greatest plant fresh weight. Plants belonging to two accessions collected from Uromia (Uromieh 1 and Uromieh 2) contained the highest anthocyanin amount. Three kinds of leaf shape were described in the studied accessions. Based on cluster analysis, these accessions were divided into four main groups, including members 13, 8, 2 and 1. They suggested that due to morphological diversity of these studied accessions, Iranian watercress accessions have the potential to be used in breeding programs of this plant.

6.1.2 Taxonomy, Habitat and Chromosome Number

Nasturtium officinale and *N. microphyllum* can be distinguished from each other based on their fruits and seeds. They also differ in the number of chromosomes. The fruits of *N. officinale* (Fig. 6.1) are shorter and wider than those of *N. microphyllum*. *Nasturtium officinale* has coarsely reticulate seeds with less than 60 areolae per side compared to the moderately reticulate seeds of *N. microphyllum* having less than 130 areolae per side. Furthermore, *N. officinale* is a tetraploid species (2n=4x=32) (Fiorini et al. 2017), while *N. microphyllum* is an octaploid (2n = 8x = 64).

Fig. 6.1 The vegetative and reproductive organs of *Nasturtium officinale* (Accession of Meymeh, Ilam, Iran), (**a**) Leaf, (**b**) Stem (with and without anthocyanin), (**c**) Hollow stem, (**d**) Parts of a flower, (**e**) Flowering shoots and fruits, (**f**) Pod (Photos by Sajad Jafari)

The hybrid between these two species is either fertile or sterile. Fully fertile hybrids are morphologically and genotypically intermediate between their parent species; they possess 60–120 areolae on each side of their seed surface (Bleeker et al. 1997). Interestingly, the number of chromosomes in *Nasturtium* × *sterile* is also in an intermediate state (2n = 6x = 48). However, due to non-seed fruits or unripe seeds, it has not been feasible to count the seed areolae in *N.* × *sterile* specimens. This hybrid is commonly found in ditches and small streams, but also in ponds with a high fluctuation of water level in Central Europe (Bleeker and Hurka 1997). Human activities favor the formation and establishment of *N.* × *sterile* and it is mostly found in landscape created by humans. *Nasturtium* × *sterile* is more vigorous than the octaploids and can establish itself fast from cuttings (Manton 1935). On the British Isles, the hybrid has traditionally been cultivated as a crop plant (brown cress). These vegetative capabilities should provide this hybrid with a fitness advantage in ditches which are managed on a regular basis (Bleeker et al. 1999). *Nasturtium* × *sterile* grows within irrigation canals or small brooks within rice fields and forest edges that are manipulated by human and animals.

6.1.3 Economic Importance and Health Benefits

Watercress (*Nasturtium officinale*) is a perennial dicotyledonous herbaceous plant of the family Brassicaceae, which is very similar to other brassicaceous vegetables, e.g., cabbage, broccoli, radish, brussels sprouts, mustard and kale (Voutsina et al. 2016). The members of this plant family are consumed fresh or as an ingredient in soups or other recipes due to a pungent aroma and a spicy or bitter taste (Kristal and Lampe 2002). They are also said to have medicinal value (Basu et al. 2007; Goncalves et al. 2009; Riezzo et al. 2005; Yazdanparast et al. 2008). *Nasturtium officinale* contains, not only different vitamins, e.g., vitamin A, B, C and E, phenolic compounds (Table 6.1), folic acid, carotenoids, minerals e.g., phosphorus, calcium, magnesium, copper, zinc, manganese, iron, and iodine, fibers, lipids, and proteins, but also a high level of glucosinolates, especially gluconasturtiin (Bahramikia and Yazdanparast 2005; Jafari et al. 2014; Jeon et al. 2017) and an antioxidant (Azarmehr et al. 2019).

The antioxidant and putative anti-carcinogenic properties of different brassicaceous plants have been reported (Hecht et al. 2004; Yalcinkaya et al. 2019). Previous studies have also reported the antifungal activity of the glucosinolates content of watercress as a class of sulfur- and nitrogen-containing glycosides (Blazevic et al. 2010; Goralska and Dynowska 2012; Khoobchandani et al. 2010). Moreover, these glucosinolates can be hydrolyzed into isothiocyanates, nitriles and thiocyanates (Mikkelsen et al. 2002), which are reputed to surpress tumor growth (Cavell et al. 2011) and also to have a possible role in the prevention of other diseases including cardiovascular, neurodegeneration and diabetes (Bayrami et al. 2019; Payne et al. 2015). The anti-carcinogenic value of watercress is attributed to the reduction in DNA damage and possible modulation of antioxidant status (Gill et al. 2007; Hecht et al. 1999).

Watercress extract has also shown some antagonistic effects on plant pathogens (Gerard and Celia 2011; Horace 1995; Silva et al. 2020). Methanol extracts of watercress inhibit *Penicillium digitatum*, the causal agent of postharvest citrus fruits decay, resulting in an improved postharvest quality of stored fruits (Jafari et al. 2015).

Table 6.1 The amount of ascorbic acid, phenolic compounds and antioxidant capacity of methanolic extract of some *Nasturtium officinale* accessions from Iran

	Accessions				
Traits	Meymeh	Fashand	Sarab	Dehdasht	Noshahr
Ascorbic acid (mg AA 100 g^{-1} DW)	19.36	28.1	23.4	23.7	24.6
Total phenol (mg g^{-1} GA dry extract)	9.2	11.59	11.11	7.34	8.35
IC_{50} (μg mL^{-1})	197	70	123	375	113

AA ascorbic acid, *GA* gallic acid
Source: Constructed by Sajad Jafari, unpublished data

6.1.4 Domestication, Selection and Early Improvements

The consumption of watercress is increasing worldwide. In the West, it is consumed raw in salads, while in the East, the leaves and stems of the plant are cooked and used in soup (Getahun and Chung 1999). Considering food security and sustainable intensification and increasing pressures on the global food system, the availability of plants with high nutritional value has become very crucial (Martin et al. 2013).

Recent droughts have led to the reduction in water resources, which consequently threatens the survival of watercress germplasm growing in the vicinity of water. This might result in the extinction in the wild of this valuable plant (Jafari and Hassandokht 2012). Therefore, more attempts are being made to maintain the habitats of watercress and its genetic resources by identification, protection, propagation and cultivation. An efficient strategy to protect this plant is to exploit wild germplasms showing more adaptive features, e.g., disease and drought tolerance and greater genetic diversity (Mahjoub et al. 2009). However, limited genetic resources of watercress have been obtained and there is no active breeding program, globally. The whole genome sequencing of watercress is not yet available, but the next generation sequencing (NGS) can create opportunities to study the watercress genome at a cost-efficient level (Davey et al. 2011). However, there is an urgent need to explore the variation in desirable traits in watercress and to characterize the wide germplasms phenotypically to be able to target NGS resources effectively (Payne et al. 2015).

6.2 Current Cultivation Practices and Challenges

6.2.1 Current Cultivation Practices

According to earlier research in several regions of Iran, watercress is likely to exist as a native plant, which has been consumed as a fresh leafy vegetable as well as a medicinal plant and used purportedly to treat kidney diseases (Omidbaiegi 2005). It is also believed to be an excellent body booster with vital detoxifying nutrients, disinfecting the digestive system. In Iran, watercress is not cultivated, but collected by people as a wild plant (Fig. 6.2a) (Jafari and Hassandokht 2012; Jafari et al. 2014). Although it grows in wet soil, it grows best in running water. For commercial purposes, it is grown in unshaded shallow pools of flowing clean water (Fig. 6.2b). Most of the commercial production of watercress is located in districts characterized by limestone rock formations and by springs and brooks of clear water relatively high in lime content.

The best site for a watercress bed is on a relatively flat area with a slight slope below the spring that supplies the water. The beds should be protected from flooding, which could cause severe damage to both the watercress and the beds. A constant flow of water and a complete drainage of the beds is necessary. The areas

Fig. 6.2 (**a**) Watercress wild growth, (**b**) Cultivation of watercress (Photos by Sajad Jafari)

where beds are located should be free of immovable rocks or rock outcrops. After grading, the foundation of the beds should remain firm when immersed in water to afford good footing for workers. The size of an individual bed depends partly on the size of the enterprise to be developed and partly on the contours of the site. The shape of a bed also depends on the contours of the site.

6.2.1.1 Reproductive Biology

Nasturtium officinale is a long-day plant, which flowers in mid to late summer in response to increasing day length (Bleasdale 1964). Flowers are self- or cross-pollinated. The main pollinators are likely insects (Johnson 1974). Seed pods break open and scatter seeds, when they are ripe. Most of the seeds fall near the parent plant. However, some seeds float for 12 h or even longer and disperse to other areas (Howard and Lyon 1952a). Seed production is usually high, at about 29 seeds per fruit and 20 or more fruits per inflorescence (Howard and Lyon 1952b).

Seeds germinate soon after being shed, giving 92–100% germination within a week on moist filter paper in the light. Seeds remain viable for up to approximately five years, when stored dry in packets in the laboratory, but lose their viability with a longer period of storage (Howard and Lyon 1952b). No germination was observed in darkness, however, a short exposure of 5 min to light led to some germination, provided the seeds had absorbed water (Howard and Lyon 1951). Howard and Lyon (1952b) observed that reproduction by seedlings is effective if seeds fall on bare ground, but if the ground is covered by vegetation, vegetative reproduction will be of paramount importance. Asexual propagation through vegetative stems probably increases the plant longevity for several years (Howard and Lyon 1952b).

6.2.1.2 Growth

The growth of *Nasturtium officinale* in natural streams has been studied by several researchers in the UK, North America and New Zealand (Castellano 1977; Howard-Williams et al. 1982; Kaskey and Tindall 1979). *Nasturtium officinale* is a fast-growing plant, particularly in spring to mid-summer, yielding many leaves and roots early in the growing season; however, in late summer to winter most of the dry matter accumulates in the stems. Previous research shows that the biomass of a population of *N. officinale* in New Zealand increased twofold within a short growing period of 12.2 days (Howard-Williams et al. 1982). The leaves of *N. officinale* remain green in autumn, but the plant is frost susceptible in winter and spring.

Watercress develops two types of roots: basal roots that anchor the plant in the bed substrate and adventitious roots developing from the stem that float in the stream of water. Both types are likely to take up nutrients, however, most of the phosphate and potassium is absorbed by adventitious roots of *Nasturtium officinale* (Cumbus and Robinson 1977).

6.2.1.3 Environmental and Nutrition Requirements

Nasturtium officinale is found in slow flowing fresh water in rivers, streams, ditches and springs, but not in still water. It can grow on different substrates such as gravel, sand, silt or clay, but not on acid or alkaline peats (Howard and Lyon 1952b). Going et al. (2008) reported that there was a significant reduction in the total biomass and

root biomass with decreasing light levels. These authors also noted that *N. officinale* showed remarkable morphological plasticity, acclimatizing to low light conditions by increasing leaf area and canopy surface area. This plant is able to overwinter; surviving as long as the water does not freeze. *Nasturtium officinale* usually occurs on calcareous soils (Shaw 1947). A soil pH of 4.3–8.3 favors watercress (Simon et al. 1984).

Nasturtium officinale is a marginal plant and takes up most of its required nutrients from the water through its roots. This plant does not have high nutrient requirements, but cultivated watercress may show signs of potassium, iron or phosphorus deficiencies (Smith 2007). Therefore, to achieve optimum growth, additional nutrients, e.g., NPK fertilizers are applied. Howard and Lyon (1952a) noted that *N. officinale* requires higher amounts of calcium and pH compared to *N. microphyllum*. It was shown that watercress absorbs nitrogen and phosphorus and thus has been considered as a means to strip excessive nutrients from streams (Howard-Williams et al. 1982).

6.2.2 Current Agricultural Challenges

Nasturtium officinale is a perishable crop, mostly sold either directly to dealers in large cities having special handling facilities or directly to hotels and restaurants. With the recent widespread improvement in facilities for handling fresh vegetables, such as plastic bags and refrigerated and humidified display cases, there are opportunities to expand the market for watercress. The possibilities of expanding production, however, are rather limited, due to a scarcity of favorable sites, high cost of establishing commercial beds and the amount of manual labor required to produce the crop.

Several pests and pathogens are associated with *Nasturtium officinale* (Howard and Lyon 1952a). Several fungi, including *Cercospora* leaf spot (*Cercospora nasturii* Sacc. 1876), *Septoria* leaf spot (*Septoria sisymbrii* Henn. and Ranoj.) (Andrianova and Minter 2004), *Sclerotinia sclerotiorum* (Lib.) de Bary and *Spongospora subterranean* (Wallr.) Lagerh. have been reported on watercress.

Among bacterial diseases, black rot (*Xanthomonas campestris* (Pammel 1895) Dowson)) is common in outdoor crops during rainy periods. *Pythium ultimum* Trow, the causal agent of damping off disease, can also damage young and weakened plants.

Yellow spot virus also causes chlorotic spotting and blotching on leaf veins (Tomlinson and Hunt 1987). Watercress yellows is a serious disease of watercress caused by a phytoplasma that results in reduced leaf size, leaf yellowing and crinkling, and witches' broom (Borth et al. 2006).

Several species of aphids, e.g., *Aphis brassicae* (L.) and the green peach aphid (*Myzus persicae* (Sulzer)) have also caused severe damage to the watercress (Thompson 1922). Caterpillars of the diamondback moth (*Plutella xylostella* (L.)) are also considered a destructive species on different brassicaceous crops including

watercress. They feed on leaves and young shoots. Stink bugs (*Nezara viridula* (L.), *Nysius* spp.) and cyclamen mite (*Stenotarsonemus pallidus* (Banks)) are common pests in certain production regions. Slugs and snails, freshwater pulmonate species (*Lymnaea ovata* (L.), *L. stagnalis* (L.), *Physa acuta* (Draparnaud) and *Planorbis leucostoma* (Millet)) have been detected in the watercress bed. If these species are infected with *Fasciola hepatica* (L.), they can cause fascioliasis (liver fluke) in humans (Dreyfuss et al. 2003; WHO 2007).

In hydroponic systems, algae may obstruct water flow, causing overheating of bed systems. In New Zealand, natural watercress beds growing at the edges of rivers are often invaded by weeds such as *Apium nodiflorum* (L.) Lag. and *Mimulus guttatus* (Fisch.) Nesom, which compete with *Nasturtium officinale* for space (USDA-ARS 2014).

6.3 Germplasm Biodiversity and Conservation

6.3.1 Germplasm Diversity

Unravelling genetic diversity and its origins shed new light on the management of plants species (Choudhary et al. 2011; Zhang et al. 2008). Wild populations or germplasm collections often contain beneficial genetic variation to introgress into breeding programs for agronomic and nutritional traits, as well as biotic and abiotic stress tolerant traits (Moose and Mumm 2008). Introgression of such wild alleles has already been studied in breeding programs. Several traits of tomato e.g., drought and salinity tolerance, soluble content and fruit color were improved by introgression of genes from wild relatives (Hajjar and Hodgkin 2007; Levin et al. 2004). Introgression of the alleles from wild relatives into some other crops with a limited genetic diversity, e.g., wheat, barley and rye has also been successful (Feuillet et al. 2008). Earlier research has shown that little genetic variation exists among commercial watercress plants (Sheridan et al. 2001). Therefore, exploring the wild populations may identify useful material for breeding crops with improved traits associated with a higher nutritional profile or frost and disease resistance. Breeding could make great contributions to the preservation of this endangered crop.

Various methods have been employed to preserve species of watercress and many special studies still need to be done on the various watercress species. Research has shown that botanic gardens play a major role in the conservation of watercress wild relatives and endangered species (Benson 1995).

A considerable contribution has been made by the Universidad Politecnica, Madrid, in the collection, taxonomic characterization and storage of wild Brassicaceae species of the Mediterranean and northern Spain (Tsunoda et al. 1980b). The conservation of endangered Brassicaceae species and their habitats has received global attention (Cropper 1987). Micropropagation techniques are also increasingly important for the multiplication of reduced populations of endangered

species, as exemplified by the studies of Iriondo and Perez (1990) concerning the conservation of *Coronopus navasii* Pau. This *Brassica* is protected by the 1982 Berne Agreement for the Conservation of Wildlife and Natural Habitats in Europe. The entire population of the plant is located in two extremely vulnerable habitats in the southeastern region of Spain. Previous research concluded that micropropagation of endangered species is an important tool in both in vitro conservation and in facilitating the bulking up of plantlets, which may, at a later stage, be reintroduced into their natural habitat.

Brassicaceae germplasm storage utilizes seeds, zygotic and somatic embryos, pollen, anthers and anther-derived embryos, shoot tips, callus and cells. For standard conservation strategies, seed storage is still the usual method of choice for *Brassica* genetic resources. In general, seeds may be stored using orthodox methods of controlled dehydration to a minimum moisture content, followed by maintenance at subzero temperatures within the range of −5 to −20 °C (Innes 1985; Takayanagi 1980; Tsunoda et al. 1980a).

Currently, no active breeding program exists for watercress, globally, which is surprising given the important nutrient intensity of this crop relative to others. No germplasm collection exists for watercress presently. Existing information is based on the local data collected by a few researchers, e.g. Jafari and Hassandokht (2012) who studied the genetic diversity of 24 accessions of *Nasturtium officinale* in Iran.

6.3.2 Cultivars Characterization and Genome Size

To date, no cultivars of watercress have been reported, and all cultivated species are varieties and have arisen naturally. The results of the phylogenetic analyses of 56 species, based on complete chloroplast (cp) genomes and common protein-coding genes, confirmed that the genus *Nasturtium* is a sister genus to *Cardamine* in the Cardamineae tribe (Yan et al. 2019). Comparison analyses of chloroplast genomes among seven *Cardamineae* species is presented in Table 6.2.

In the watercress cp genome, codon usage was biased toward A/T-ending, and all the identified RNA editing sites were C-to-U transitions. The genome size, overall structure and gene organization were similar to those of other reported cp genomes of Cardamineae. An evolutionary dynamics analysis of genes in the cp genomes of Cardamineae revealed positive selection of the *ycf2* gene in watercress.

6.3.3 Cytogenetics

To date, three species of watercress have been reported from Europe, including *Rorippa nasturtium-aquaticum* (L.) Hayek (common or green watercress, 2n = 32), *R. sterilis* (Airy Shaw) (brown watercress, 2n = 48) and *R. microphylla* (Boenn.) Hyland (wild watercress, 2n = 64). On the other hand, *R. africana* from North

Table 6.2 Comparison analysis of chloroplast genomes among seven *Cardamineae* species

Genome features	*Nasturtium officinale*	*Cardamine resedifolia*	*C. impatiens*	*C. amara*	*C. oligosperma*	*C. parviflora*	*Barberia verna*
Genome size (bp)	155106	155036	155611	154561	153888	154684	154532
LSC (bp)	84265	84165	84711	84281	83194	83934	83435
SSC (bp)	17831	17867	17948	17706	17768	17732	18095
IR (bp)	26505	26502	26476	26287	26463	26509	26501
GC content (%)	03637	0.363	0.3633	0.364	0.3641	0.3636	0.3643
Total number of genes	113	113	113	112	112	112	112
Protein-coding gene	79	79	79	78	78	78	78
tRNA	30	30	30	30	30	30	30
rRNA	4	4	4	4	4	4	4
Genes duplicated in IR	20	17	17	18	19	19	18

LSC a large single copy region, *SSC* a small single copy region, *IR* inverted repeats, *GC content* guanine-cytosine content
Source: Yan et al. (2019)

Morocco, *R. valdes-bermejoi* (Castrov.) Mart.-Laborde & Castrov. from Spain, and *R. gambellii* (Watson) Schulz and *R. floridanum* (Al-Shehbaz & Rollins) Al-Shehbaz & Price from North America (Al-Shehbaz and Rollins 1988) have been proposed to discriminate *Nasturtium* from *Rorippa* (Al-Shebaz and Price 1998). It is suggested that these species are more closely related to *Cardamine* than *Rorippa*, as evidenced from sequencing of the gene *rbcL* (Les 1994).

6.4 Traditional Breeding

In spite of the importance of watercress because of its distinctive nutritional profile and its global value as a food crop, an active breeding program is yet to be established. Moreover, to date, there are no genetic and genomic resources for this crop and there is limited information about watercress as a source of germplasm for breeding. Some efforts have been made by small farmers as an in-house crop to improve the agronomic traits, e.g., frost or disease resistance. However, until now, no varieties have been bred for commercial production (Palaniswamy et al. 2003; Rothwell and Robinson 1986). Previous research has shown very little genetic variation among commercial watercress (Sheridan et al. 2001).

Recently, more attention is being given to watercress, because of its beneficial effects on human health. Watercress is known to be rich in secondary metabolites (Giallourou et al. 2016). These metabolites, with a broad range of biological activities, transform this edible plant into a medicinal plant with purported anti-carcinogenic features (Pereira et al. 2017). As a result, research has focused on the improvement of phytochemicals of watercress. A recent study identified promising morphological, biochemical and functional genomic variations in 48 accessions of an existing watercress germplasm collection (Payne et al. 2015). This information can be used in future breeding, which aims to enhance the medicinal properties and agronomic traits in this crop.

These studies showed chemical, epigenetic and genetic differences between wild and cultivated watercress based on which they can obviously be distinguished by their phenolic composition and their genetic and epigenetic variations. These differences might be attributed to the growing conditions. The richness of epialleles could help develop tools to manipulate the watercress epigenome in order to develop cultivars producing higher amounts of beneficial bio-products.

Epigenetic modifications are key factors modulating the expression of genes responsible for the synthesis of phytochemicals. The knowledge of plant epigenetic and genetic variations can improve the production of bioactive compounds. These subjects have been little explored in watercress. Gutiérrez-Velázquez et al. (2018) studied the determination and comparison of phenolic composition and epigenetic and genetic variations between wild and cultivated *Rorippa nasturtium* var. *aquaticum*. They found significant differences in the quantitative phenolic composition between wild and cultivated watercress. They used eight primer combinations in the methylation-sensitive amplification polymorphism (MSAP) method, which resulted in different epigenetic status for each watercress type; the cultivated watercress was the most epigenetically variable. The genetic variability shown by the *EcoRI/MspI* amplification profile and also by eight inter-simple sequence repeat (ISSR) primers was different between the two types of watercress. The results of the Mantel test showed that the correlation between genetic and epigenetic variations is reduced in the cultivated type. According to cluster analysis, epigenetic and genetic characterizations obviously distinguished wild watercress from the cultivated one. Chemical, epigenetic, and genetic differences between wild and cultivated watercress can contribute to fingerprint and develop quality control tools for the integral and safety use and the commercialization of watercress. The richness of epialleles could support the development of tools to manipulate the watercress epigenome to develop high bioproduct-generating cultivars.

6.5 Molecular Breeding

The most useful genes, such as pest and disease resistance genes and quality-related genes, are commonly found in genetic diversity centers. Therefore, breeders with the knowledge of the area of origin and distribution of the plants can be more

efficient in exploiting genetic resources and needed hereditary stock. However, it should be noted that not all useful genes are present in diversity centers and some of the genes required are found in particular ecological zones. For example, to find cold-resistant plants, one should search for them in highlands and mountains. Finding desired traits requires identifying native or wild plants in each area that have stored traits for many years.

The careless application of biotechnology can have a significant impact on biodiversity, especially genes that are present only in a limited number of varieties (Farsi and Baghery 2004). On the other hand, biotechnology can have a positive role in biodiversity, by the creation of new organisms and also by providing techniques to increase the efficiency of processing industries. It is usually considered of great importance for the conservation of plant germplasm in important biological research, because some genes in plants may provide resistance to new diseases, pests, environmental conditions or issues related to crop production. Therefore, a few researchers have lately attempted to gain new insights into genetic and genomic resources for watercress, which can be used in future breeding programs.

Jafari (2012) evaluated the genetic diversity of 24 accessions of watercress (*Nasturtium officinale*) collected from eight provinces in Iran by random amplified polymorphic DNA (RAPD) molecular markers (Table 6.3). He reported that these accessions were clustered into four groups (Fig. 6.3). A similarity matrix showed the lowest similarity between accessions Jobshirali and Ghaemshahr. Although no differences were observed between the accessions collected from four areas, including Central and North, North West, West and South West, high variations were found within accessions. Based on the results, Iranian watercress accessions showed high genetic and morphological diversity for breeding purposes of genetic resources. Furthermore, Payne et al. (2015) reported morphological (stem length, stem diameter), biochemical (antioxidant potential) and functional genomic variations across 48 watercress accessions collected from different locations. They identified a set of transcripts involved in the regulation of growth and development as well as those associated with the secondary metabolites. The Affymetrix *Arabidopsis* ATH1 microarray gene chip was used to examine the variation in expression of genes involved in encoding glucosinolates as precursors of phenethyl isothiocyanate, which are related to the reported anti-carcinogenic properties of watercress. This has been considered the first comprehensive analysis of natural variation across the watercress genome, which provided crucial information for future breeding with more emphasis on anti-cancer properties and agronomic traits in watercress.

Voutsina et al. (2016) described first transcriptome of watercress using RNASeq data from 12 watercress accessions and performing differential expression analysis to detect genes related to important phytonutritional traits, i.e., antioxidant capacity and glucosinolate content. Identification of such genes and gathering genomic resources will facilitate the development of molecular markers needed for future breeding activities.

Table 6.3 Combined details of 23 RAPD primers and amplified bands of all the DNA samples obtained from 24 Iranian accessions of *Nasturtium officinale*

Primer	Sequence (5´- 3´)	Total no. of bands	No. of polymorphic bands	Polymorphic percentage (%)	Resolution power
R2	TGC CGA GCT G	10	9	90	2.5
R3	AGT CAG CCA C	3	1	33	0.83
R9	GGG TAA CGC C	20	15	75	7.5
R10	GTG ATC GCA G	20	17	85	5.16
R11	CAA TCG CCG T	18	14	77	3.75
R24	CCG CCC AAA C	23	22	95	11.16
R29	CCC TAC CGA C	20	20	100	9.58
R31	AAT GCC CCA G	10	10	100	2.92
R35	CTC CTG CCA A	6	4	66	1.25
R40	GTG TCG CGA G	15	15	100	6.91
R52	CCT TGA CGC A	18	18	100	8.58
R57	AGG GAA CGA G	6	3	50	1.42
R58	CCA CAG CAG T	10	10	100	2.08
R68	GAG GGG GTG A	13	9	69	3.5
R69	GAG CAC CAG G	7	6	85	0.75
OPH04	GGA AGT CGC C	2	1	50	0.5
OPH12	ACG CGC ATG T	25	23	92	9.42
OPGO3	GAG CCC TCC A	12	8	66	2.83
TIBMBA08	CCA CAG CCG A	7	5	71	2.58
TIBMBB07	GAA GGC TGG G	11	11	100	3.16
TIBMBB13	CTT CGG TGT G	11	8	72	2.66

(continued)

Table 6.3 (continued)

Primer	Sequence (5´- 3´)	Total no. of bands	No. of polymorphic bands	Polymorphic percentage (%)	Resolution power
TIBMBB14	GTG GGA CCT G	11	10	90	2.91
TIBMBD17	GTT CGC TCC C	19	14	73	2.33

Source: Constructed by Sajad Jafari, unpublished data

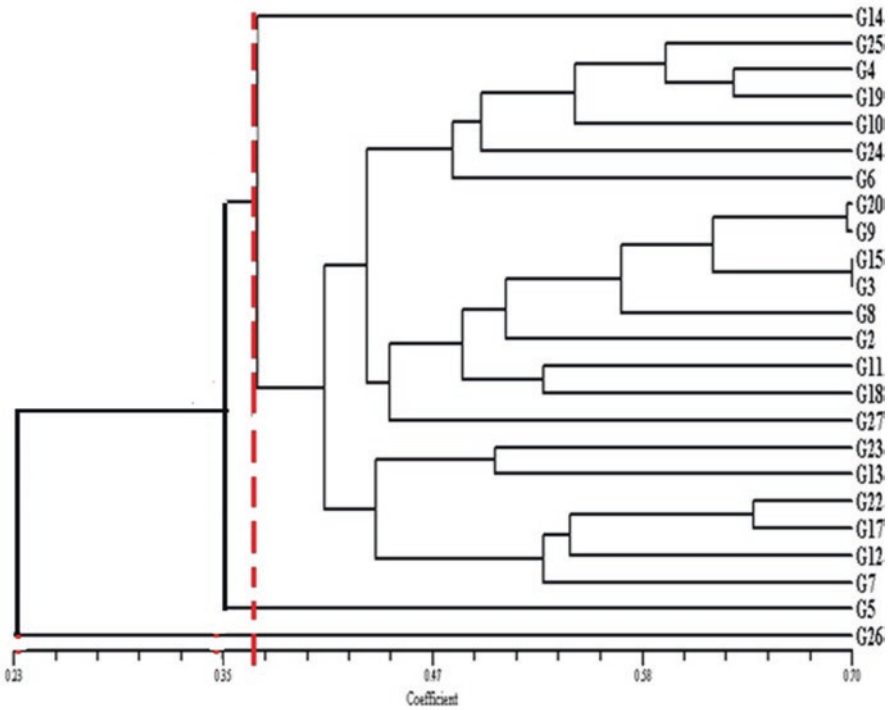

Fig. 6.3 Clustering of 24 collected accessions of watercress from Iran based on RAPD data by 23 primers and UPGMA method (Source: Constructed by Sajad Jafari; unpublished data)

6.6 Functional Genomics

Recently, Yan et al. (2019) sequenced the whole chloroplast genome of *Nasturtium officinale* comprising plentiful phylogenetic information, which can be used for phylogeny reconstruction and population studies in plants. Hence, based on these cp genomes, the phylogenetic position of *N. officinale* and the evolutionary relationships within Brassicaceae were determined. The results revealed that the genome size, general structure and gene organization of this species resembles those of other

members of Cardamineae. Phylogenetic reconstruction based on the complete cp genomes and shared protein-coding genes proved that the genus *Nasturtium* is a sister genus to *Cardamine* in the Cardamineae tribe. In addition, 21 single nucleotide polymorphisms (SNPs) and 27 indels were found, mostly in noncoding sequences, compared with the previous genome available in Gene Bank. This could, to some extent, reveal intraspecific variations of watercress.

Functional genetics programs in the future will include the introduction of cultivars with resistance to common watercress diseases. Important diseases and pests of watercress have been reported in Sect. 6.2.2, above. Other functional genetics perspectives include glucosinolate biosynthetic pathways of this plant have been investigated by Jeon et al. (2017).

6.7 Genetic Engineering

6.7.1 Evaluation of Morphological Diversity

Morphological traits are essential for preliminary assessment of genetic diversity and subsequently, the associated qualitative traits; these can be used as practical markers in breeding programs to develop new commercial cultivars and to conserve genetic resources for future use (Balmer and Blanke 2005; Hrotko et al. 2008; Jafari and Hassandokht 2012).

Jafari and Hassandokht (2012) aimed to evaluate 24 wild growing accessions of *Nasturtium officinale* collected from seven provinces of Iran using agro-morphological traits. Details of measured quantitative and qualitative traits are presented in Table 6.4.

6.7.2 Genetic Transformation Using **Agrobacterium rhizogenes**

There has been increased interest in genetic transformation of plants for molecular breeding to introduce desirable traits into the plant genome. Several methods of plant transformation have been developed to obtain stable expression of transgenes in plants (Gelvin 2009; Rao et al. 2009).

Agrobacterium tumefaciens and *A. rhizogenes* are often used to transform plants with specific genes. The soil bacterium, *A. rhizogenes,* transfers DNA (Ri T-DNA), which induces the formation of root structures known as hairy roots in the plant. The roots induced by *A. rhizogenes* regenerate shoots that carry Ri T-DNA into the whole plant and its progeny (Tepfer 1984). The induced hairy roots are able to synthesize several beneficial metabolites that can be a source of phytochemicals used in pharmaceuticals, cosmetics and food. *Agrobacterium rhizogenes* can also transfer

Table 6.4 Average, minimum, maximum and the variance of measured quantitative and qualitative traits in Iranian watercress accessions

Measured traits	Abbreviation	Unit	Average	Maximum	Minimum	Coefficient of variation
Leaf length	LL	cm	9.27	18.4	4.73	31.98
Leaf width	LW	cm	4.71	13	2.04	45.88
Leaf thickness	LT	mm	0.43	0.88	0.24	24.59
Number of leaflet in leaf	NLL	–	7	9	5	14.57
Fresh weight	FW	gr	19.81	50.73	9.66	52.04
Lateral branch number	NLB	–	4.6	10.33	1.55	42.43
Plant height	PH	cm	41.78	122.6	13.99	45.58
Leaf number	NL	–	22.19	79.93	14.13	58.74
Flower number	NFB	–	84.77	155.49	13.35	50.3
Inflorescence number	NI	–	4.82	8.8	1.97	35.08
Flower number in inflorescence	NFI	–	17.75	35.06	5.97	40
Flower stem length	FSL	cm	7.36	14.6	4.44	28.61
Silique number	NS	–	26.35	59.11	7.93	44.92
Silique length	SL	mm	15.47	35.3	6.51	33.72
Silique width	SW	mm	2.58	16.93	1.28	122.4

Source: Constructed by Sajad Jafari and Hassandokht, unpublished data

genes to change the metabolic pathways, resulting in new bioproducts (Nishikawa and Ishimaru 1997; Zhi-Bi and Min 2006).

As mentioned before, *Nasturtium officinale* contains a large amount of glucosinolates, which can be hydrolyzed by myrosinase to isothiocyanates. The latter is known as a product with anti-carcinogenic properties. Park et al. (2011) transferred DNA segments into plant genomes to produce hairy root cultures using *Agrobacterium rhizogenes*. Afterwards, polymerase chain reaction (PCR) and cytohistochemical staining validated transgenic hairy roots from *N. officinale*. The glucosinolate content of the hairy roots was extracted and analyzed using high-performance liquid chromatography (HPLC) coupled with electrospray ionization (ESI) mass spectrometry. Hairy root culture of watercress is a great achievement for metabolic engineering of glucosinolate in plants.

6.8 Conclusions and Prospects

Watercress has traditionally been used as a medicinal and leafy crop for many centuries. This plant was recently ranked as a top powerhouse of fruits and vegetables, mainly due to its purported role in decreasing the incidence of chronic disease. Several other studies indicate that watercress may have health benefits due to its anti-carcinogenic, anti-inflammatory and anti-aging properties based on in vitro

application of the extracts of watercress in suppressing the growth and metastasis of cancer cells. The other potentially positive effects of watercress are to limit exercise-induced DNA damage and to increase amounts of blood antioxidants. Much medical research remains to be done along these lines.

Considering the nutritional and medicinal value of watercress and its wide distribution throughout the world, more intensive research should be carried out in order to improve the cultivation of watercress, selection of germplasms thorough evaluations and classical and molecular breeding. In the next step, identification of genes associated with important agronomic and medicinal traits, gene transfer and creating new cultivars should be taken into consideration.

Using several different approaches, it is possible to cryopreserve and regenerate plants from a range of diverse tissues and cells derived from watercress. Based on research on the Brassicaceae family, considerable emphasis has been placed on the cryogenic storage of pollen microspores, which are capable of retaining their embryogenic potential and thereby provide a strategic supply of haploid and diploid plants for biotechnology programs. The possibility that some components of the cryopreservation procedure can be manipulated to increase post-freeze recovery of diploid plants is an added advantage of using cryopreservation.

Additionally, global warming and the emission of greenhouse gasses have become very crucial issues. Worldwide, food production accounts for 25% of such greenhouse gasses, of which 18 % is attributed to the rearing of livestock for meat and dairy products. Consequently, recent research focuses on partial substitution of meat with whole grains, legumes, vegetables and fruit, among which the plant species with high nutritional value like watercress that can be of paramount importance in this regard. On the other hand, high temperatures and drought due to climate change will be limiting factors in the growth and survival of watercress growing in the vicinity of water. Hence, more attention will be given to germplasms possessing more adaptive features, e.g., heat and drought tolerance. Moreover, research relevant to the whole genome sequencing of watercress, gene editing and the next generation sequencing (NGS) are essential prospective tools for future breeding programs.

Appendices

Appendix I: The Countries Where Watercress (Nasturtium officinale *R. Br.) Is Present in Various Continents*

Continent/Country	Origin
Africa	
Algeria	Native
Democratic Republic of the Congo	Introduced

(continued)

Continent/Country	Origin
Egypt	Native
Eritrea	Introduced
Ethiopia	Introduced
Kenya	Introduced
Lybia	Native
Morocco	Native
South Africa	Introduced
Tunisia	Native
Uganda	Introduced
Asia	
Afghanistan	Native
Armenia	Native
Azerbaijan	Native
China	Native
Himachal Pradesh	Native
Jammu and Kashmir	Native
Iran	Native
Iraq	Native
Israel	Native
Jordan	Native
Japan	Introduced
Kazakhstan	Native
Kyrgyzstan	Native
Lebanon	Native
Pakistan	Native
Syria	Native
Tajikistan	Native
Turkey	Native
Turkmenistan	Native
Uzbekistan	Native
Yemen	Introduced
Europe	
Albania	Native
Austria	Native
Belgium	Native
Bulgaria	Native
Czechia	Native
Denmark	Native
France	Native
Germany	Native
Greece	Native
Hungary	Native
Ireland	Native
Italy	Native

(continued)

Continent/Country	Origin
Netherlands	Native
Poland	Native
Portugal	Native
Romania	Native
Russia	Native
Serbia and Montenegro	Native
Slovenia	Native
Spain	Native
Sweden	Native
Switzerland	Native
Ukraine	Native
United Kingdom	Native
North America	
Canada	Introduced
British Columbia	Introduced
Manitoba	Introduced
New Brunswick	Introduced
Newfoundland and Labrador	Introduced
Ontario	Introduced
Prince Edward Island	Introduced
Quebec	Introduced
Saint Pierre and Miquelon	Introduced
United States	Introduced
Alabama	Introduced
Alaska	Introduced
Arkansas	Introduced
Georgia	Introduced
Hawaii	Introduced
Idaho	Introduced
Kentucky	Introduced
Maine	Introduced
Massachusetts	Introduced
Michigan	Introduced
Minnesota	Introduced
Mississippi	Introduced
Nebraska	Introduced
New Hampshire	Introduced
New Mexico	Introduced
New York	Introduced
North Carolina	Introduced
Oregon	Introduced
Pennsylvania	Introduced
Tennessee	Introduced
Virginia	Introduced

(continued)

Continent/Country	Origin
Wisconsin	Introduced
South America	
Argentina	Introduced
Chile	Introduced
Oceania	
Australia	Introduced
New South Wales	Introduced
Queensland	Introduced
South Australia	Introduced
Tasmania	Introduced
Victoria	Introduced
New Zealand	Introduced

Source: USDA-ARS (2013)

Appendix II: Research Institutes Relevant to Watercress (Nasturtium officinale R. Br.)

Institution name	Specialization and research activities	Address / Country	Contact information and website
Centre for Biological Sciences, Institute for Life Sciences, University of Southampton	Plant adaptation to the changing environment, genomics studies linked to phenotypic analysis	Southampton, SO17 1BJ, UK	Gail Taylor G.Taylor@soton.ac.uk https://www.southampton.ac.uk/life-sciences/
Vitacress Salads Ltd, Lower Link Farm	Suppliers of fresh produce, specialising in watercress, salads and fresh herbs	St Mary Bourne, Andover, Hampshire, SP11 6DB, UK	Graham Clarkson gjjclarkson@hotmail.com https://www.vitacress.com/
Genetic Resources Unit, Wellesbourne Campus, The University of Warwick, UK Vegetable Genebank	Vegetable Genebank	Wellesbourne, Warwick CV35 9EF, UK	https://warwick.ac.uk/fac/sci/lifesci/wcc/gru/genebank/
Vegetable Crops & Medicinal and Aromatic Plants, Department of Agricultural, Forest and Food Sciences, University of Turin	Medicinal plants	10095 Grugliasco Turin, Italy	Silvana Nicola silvana.nicola@unito.it https://en.unito.it/

(continued)

Institution name	Specialization and research activities	Address / Country	Contact information and website
Idaho Department of Agriculture Idaho Invasive Species Council	Invasive species		Amy Ferriter aferriter@agri.idaho.gov http://invasivespecies.idaho.gov/
Department of Crop Science, College of Agriculture and Life Sciences, Chungnam National University	Agriculture	220 Gung-dong, Yuseong-gu, Daejeon 305–764, Korea	supark@cnu.ac.kr http://plus.cnu.ac.kr/html/kr/intro.html

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Part II
Flowerheads and Green Pods

Chapter 7
Advances in Cauliflower (*Brassica oleracea* var. *botrytis* L.) Breeding, with Emphasis on India

Shrawan Singh and Pritam Kalia

Abstract Among the cole vegetables, cauliflower is a widely grown crop worldwide for its nutrients and flavor. It is a thermosensitive crop for its curd formation and development. Different cultivar groups in cauliflower are known such as Italian or Original, Cornish, Northerns, Roscoff, Angers, Erfurt, Snowball and Indian, based on phylogeny and plant traits. The Indian cauliflower group evolved from European cauliflower and later classified as early, mid-early, mid-late and late, depending upon temperature requirements related to curd initiation and development. A large number of varieties and hybrids have been developed in tropical cauliflower, for different maturity groups and established using a cytoplasmic male sterility (CMS) system for hybrid breeding. Recently, biotechnological tools such as DNA markers, genomics and tissue culture for doubled haploid development, prebreeding for introgressing genes/QTLs from alien brassicas were deployed in cauliflower breeding. Resistant sources identified in cole vegetables for black rot and downy mildew by genetic investigations revealed single dominant gene governance of resistance for both diseases. Cauliflower is one of the best candidate crops for β-carotene biofortification, hence a natural mutant native *Or* gene was introgressed into Indian cauliflower. Besides, transgenesis is underway to develop diamondback moth resistant varieties by stacking *cry 1b* and *1c bt* genes in cauliflower. This chapter highlights recent developments in cauliflower breeding particularly in tropical types.

Keywords Glucosinolates · Hybrid · Indian cauliflower · Male sterility · Molecular markers · Orange cauliflower · Resistance · Self-incompatibility

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7.1 Introduction

Cauliflower (*Brassica oleracea* var. *botrytis* L.; 2n = 2x = 18) is an important cole vegetable belonging to Brassicaceae family. It grows at latitudes varying from 45° S in New Zealand to 65° N in Scandinavian countries. Asia is the leading producer followed by Europe and covers small hectarage in North America, South America, Africa, Australia and New Zealand. Globally, cauliflower is cultivated on 1.38 million ha; India and China together account for about 69% area, of that, India alone contributes nearly 34%. Similarly, both countries share almost equally 75% of global cauliflower production (24.18 million mt) (FAOSTAT 2017). India is the second largest producer of cauliflower in the world after China. Presently, cauliflower occupies 0.45 million ha in India with a production quantity of 8.9 million mt (NHB Database 2017).

In 1822, cauliflower was first cultivated in United Province (now Uttar Pradesh) India during British rule as a choice food by the British. Later, introduced genotypes became adapted to local environments to evolve an entirely new ecotype, grouped as Indian cauliflower or Tropical cauliflower. It has a tolerance to high temperature for vegetative growth (>35 °C) and for curd initiation and development stages (>27 °C) (Gill and Sharma 1996). Cauliflower growing areas expanded into tropical regions and seasons (as extra-early and early crops) in India (Kalia et al. 2016). The earliest varieties of this type were Early and Main Crop Patna and Early and Main Crop Benaras (Gill and Sharma 1996). Since then, major developments have occurred to breed improved varieties/hybrids through conventional and nonconventional breeding methods i.e., selection for simple traits, backcrossing for introgression of resistance or male sterility related genetic mechanisms, pre-breeding for development of genetic stock for novel or complex traits, and use of recent molecular and gene-editing tools. Consequently, cauliflower cultivation area has expanded both temporally and spatially even in non-traditional areas and became established as an important vegetable crop in India as a popular cool season vegetable crop. However, development of tropical types extended the growing period to both extremes of winter season in India and other countries. Cauliflower is popular among growers due to its short crop duration (60–80 days), high crop yield (15–35 mt/ha), low level of disease and insect pest incidence (mainly in the winter season crop) and better returns per unit area and time expended (Kalia et al. 2016). The curd is the edible part of cauliflower, which is made of pre-floral apical meristematic tissues. It is a dome of tissues made up of a mass of proliferated floral meristems at harvest. In some regions, tender leaves are eaten as a leafy vegetable, after boiling, frying or mixing with other vegetables. Consumers prefer cauliflower for its unique taste, diverse delicacies, purported anti-cancer glucosinolates and other essential minerals and vitamins. Nutritionally, cauliflower is a good source of dietary fibers (2%), protein (1.9%) and potassium (299 mg/100 g). It is an ideal candidate crop for biofortification of β-carotene; hence, Kalia et al. (2018) developed the first β-carotene fortified tropical cauliflower, Pusa KesariVitA-1, that contains β-carotene in a range of 8–10 ppm in the edible portion of the curd (Anonymous

2016). It has great potential to challenge widespread deficiency in human populations in developing countries. Consumed singly or in combination with other vegetables, cauliflower is also processed by blanching, pickling or freezing.

For systematic analysis and compilation of information on cauliflower, the present chapter details a holistic presentation of information on important components such as an understanding of evolution using modern tools, development for hybrid breeding, use of molecular markers, developments in breeding for quality traits like glucosinolates and biofortification for β-carotene and anthocyanin content. It also describes resistance breeding for biotic and abiotic stresses along with the use of recent techniques such as transgenics for insect resistance.

7.1.1 History and Evolution of Indian Cauliflower

The origin of cauliflower is the island of Cyprus and the Eastern Mediterranean (Gustafson 1994); it has been cultivated in Europe since the fifteenth century (Grout 1988). It was dispersed to other areas like Syria, Turkey, Egypt, Italy, Spain and northwestern Europe (Boswell 1949), and is now grown worldwide including in parts of tropical regions during cooler months (Fig. 7.1). In India, cauliflower was first introduced in Saharanpur, Uttar Pradesh (then called United Provinces) in 1822 (Swarup and Chatterjee 1972). Afterwards, the local growers initiated production of cauliflower seeds locally, which helped in its early adaptation to Indian climatic condition. Over the period of 1822–2019, introduced cauliflower underwent remarkable adaptation to heat and humidity tolerance as well as other plant traits. The selection for good horticultural traits along with heat tolerance was a major attempt toward the development of Indian cauliflower. It has an early maturing type, satisfactory seed yield (300–400 kg/ha) in north Indian conditions and has wide adaptability to hot and humid weather. The earliest varieties of cauliflower in India were Early and Main crop Patna and Early and Main crop Banaras, developed by Sutton and Sons (Gill and Sharma 1996).

Swarup and Chatterjee (1972) demonstrated close morphological affinity between Indian cauliflower and different western European types like Cornish, Roscoff, Italian, Northern, Angiers and Snowball or Erfurt, though not exactly the same. Giles (1941) opined that Indian cauliflower is a dwarf selection of Erfurt or Snowball types, a view supported by Nieuwhof (1969). He reported that early varieties were selections from Erfurt-Alpha types, which performed better in warmer regions (20 > °C). As per the climatic conditions of north India, the typical Indian cauliflowers are categorized in two groups (I, II), and mature on an average daily temperature 20 > °C. They have a long stalk, open growth habit, exposed yellowish to creamy and uneven cruds, which loosen easily and with strong flavor. Some of their characters are typical of the Cornish cultivar while some leaf and curd characters resemble Roscoff and Italian cultivars. Indian cauliflower genotypes mature during December–January and show some phenotypic affinity with Snowball or Erfurt types (European Summer Group) and Italian autumn cauliflower (Gill and

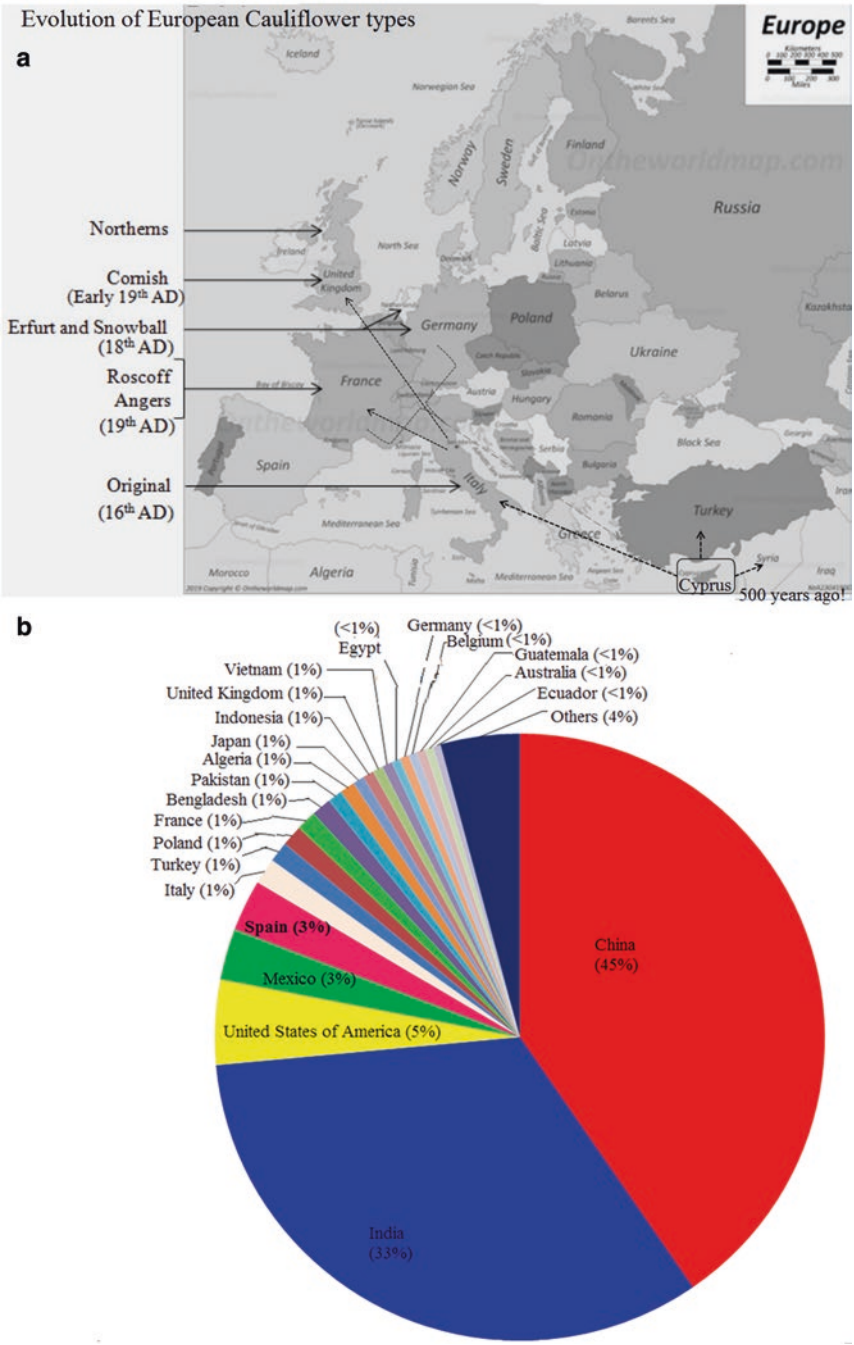


Fig. 7.1 Cauliflower distribution and production data. (**a**) Distribution of cauliflower types, (**b**) Production in the world. (Source: FAOSTAT 2018)

Sharma 1996). Group-I of Indian cauliflower has a high degree of self-incompatibility, high heterosis and resistance to black rot disease (Swarup and Chatterjee 1972). The Cornish type was the earliest introduction to India, which contributed numerous genes to present-day Indian cauliflowers. Indian seedsmen and growers have contributed significantly to the development of Indian cauliflower varieties. The seedsmen of Hajipur (Bihar) specialize in the varieties of group I and II. Foreign seedsmen like Sutton and Sons (which later became Indian) played a significant role in this venture. The *aristocratic* character of European cauliflower has undergone transformation to a *cosmopolitan* status showing flexibility of adaptation to regions from New Zealand to the Scandinavian countries. It is also significant to note that of the total area under cauliflower in India about 40% is represented by hot weather tropical cauliflowers, which includes Groups 1 and II (Seshadhri and Chatterjee 1996). Crisp and Tapsell (1993) proposed the evolutionary development for cauliflower as follows:

(a) A wild, annual, eastern Mediterranean subspecies of *Brassica oleracea* (or *B. nivea*; white flower in cyme, a primitive form of broccoli, with terminal and perhaps lateral shoots of dense buds as the edible portion) was domesticated several years ago.
(b) The introduction of this species took place in the east, and southern China where adaptive changes developed into the only Chinese *endemic* crop of *B. oleracea*, the Chinese broccoli or kale (*B. alboglabra*). It is a branched annual, usually with white flowers (yellow flowers also occur because of introduction of other *B. oleracea* crops and their possible part in natural crossing).
(c) The ancestral broccoli dispersed to the west, where natural hybridization with other wild and cultivated *B. oleracea* group types resulting in many forms around the Mediterranean Region. Notably, hybridization with the yellow-flowered, racemose, biennial western European *wild cabbage* gave rise to biennial types.
(d) Around 500 years ago, selection for increased terminal head size and probably major gene mutation for greatly enlarged, immature floral buttons (i.e. curd) associated with decreased lateral branching from the stem below the curd. This phenotype may have arisen repeatedly within the diverse broccoli gene pool or may have spread by intentional or accidental introgression.

    Over the years, many local types of cauliflower evolved (large terminal curd) and broccoli (large terminal heads of tightly packed flower buds, or with many side branches) became established around the Mediterranean and in Europe. Annual cauliflowers became an important crop in several inland regions, and biennial cauliflower varieties (giving curds from late autumn until early summer) were developed in coastal regions where winter temperatures were buffered by the marine influence.
(e) During British colonization, diverse types of cauliflowers were introduced to India and Australia, where genetic recombination gave rise to distinct types, some adapted to tropical conditions. At least some of the adaptation shown by tropical cauliflowers may have arisen by mutation. It was the single dominant

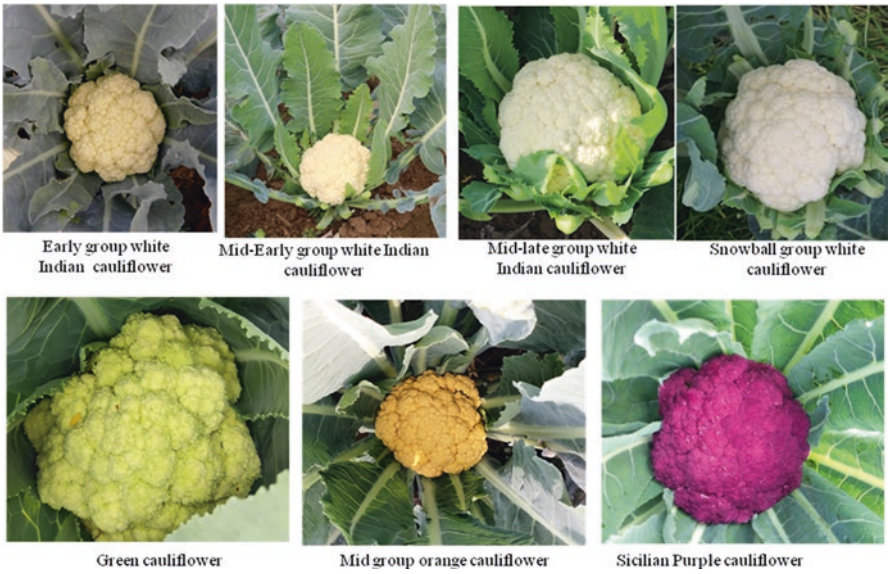


Fig. 7.2 Diversity in curd colors in cauliflower. (Photo is credited to Dr. Shrawan Singh)

gene and cytoplasmic effect, which confers *tropical* characteristics to an Indian cultivar in comparison with a European annual cultivar.

(f) The direction of breeding played a key role in evolution of modern-day cauliflower. In recent years, breeding has concentrated on annual, white-curded types of cauliflower with large, worldwide sales of seed.

In recent years, new colors also gained attention particularly orange (due to β-carotene), purple (anthocyanin) and green (chlorophyll) curding cauliflower (Fig. 7.2). These are showing uniqueness over the traditional cream white to persistent white curding cauliflowers in terms of nutritional and specialty traits.

7.1.2 Curding and Flowering Trait Genetics

In cauliflower, curd consists of a dense mass of arrested inflorescence meristem, only ~10% of which develop into floral primordia and normal flowers. The cauliflower *curd* phenotype in mutants of *Arabidopsis thaliana* is due to a class of flower developmental regulatory genes viz., *APETALA 1* (*AP1*, Mandel et al. 1992) and *CAULIFLOWER* (*CAL*; Kempin et al. 1995) that specify the floral meristem identity (as opposed to the inflorescence meristem) developing reproductive primordia. *Arabidopsis* mutants (*AP1* and *CAL*) are arrested in inflorescence development at the meristem stage and develop into a dense mass similar to cauliflower curd. Orthologous genes *BoCAL* are involved to alter inflorescence in cauliflower (Kempin

et al. 1995). The *BoCAL* allele has a premature termination codon at position 151 (E → stop) which appears to be of recent origin. Alleles carrying this nonsense mutation in exon 5 of *BoCAL* are fixed in cauliflower and broccoli, both of which show evolutionary modifications of inflorescence structures (Purugganan et al. 2000). Hence, specific alleles of *BoCAL* were selected at an early stage of evolutionary domestication of subspecies within the vegetable crops of *Brassica oleracea*.

Based on molecular allelic variation, Smith and King (2000) suggested that heading Calabrese broccoli was the source of modern cauliflower (compact curds) via an intermediate Sicilian crop type which has heads of an intermediate type. Close association of *BoAP1-a* and *BoAP1-c* with the self-incompatibility locus S may have reduced the number of S-alleles within the gene pool. Duclos and Bjorkman (2008) investigated the transcript abundance of *BoFUL* paralogues and *BoLFY*, finding it was highest at inflorescence meristem arrest and maintenance of this arrest is a consequence of suppression of *BoCAL*, *BoAP1-a*, or *BoLFY*, or failure to suppress *BoTFL1* (a strong repressor of flowering in *Arabidopsis*). Li et al. (2017) identified a novel homologous gene containing the Organ Size Related (OSR) domain *CDAG1* (Curd Development Associated Gene 1) in cauliflower. It has higher transcript levels in young tissue and promotes organ growth by increasing cell numbers, which results in a larger organ size and increased biomass. This gene inhibits transcriptional expression of endogenous OSR genes, *ARGOS* and *ARL*. Rosan et al. (2018) studied a genome-based model simulating the development of doubled haploid (DH) lines to time to curd induction and observed $R^2 = 0.40$ for the quantitative traits and $R^2 = 0.48$ for the GS model. Duclos and Bjorkman (2008) reported increased *BoAP1-a* and *BoAP1-c* transcript levels in cauliflower just before floral-primordium initiation. Application of GAs during reproductive development stage does not activate meristem identity genes or A-function genes (Yu et al. 2004). Hence, GAs (GA_3 and GA_{4+7}) can trigger the vegetative-to-reproductive transition in both cauliflower and broccoli resulting in early curd formation (Duclos and Bjorkman 2015). Recently, Singh et al. (2020) studied genetics and expression analysis of anthocyanin accumulation in the curd portion of Sicilian purple to facilitate biofortification of Indian cauliflower.

7.1.3 Cauliflower Groups

Cauliflower evolution continued in different regions depending upon prevalent climatic situations. The evolved groups remained geographically isolated for a long period (except for the Italians or Originals) within insulated populations and restricted breeding. Based on morphological characters, Swarup and Chatterjee (1972) classified present-day cultivars of cauliflower into seven broad groups (Table 7.1) so that a proper understanding and relationship of them is possible. Further, Crisp (1982) also classified cauliflowers according to their phylogeny (Table 7.2). However, further studies were made to separate grouping of the North European annual and Australian types (Chatterjee 1993).

Table 7.1 Broad groups of cauliflower based on origin and morphological characters

Cauliflower types	Area/country of origin	Probable period of first cultivation	Characters
Italians or Original	Mediterranean	Sixteenth century	Plants short, leaves erect broad with rounded tips, bluish green, curds good not protected by leaves
Cornish	England	Early nineteenth century	Plants vigorous, long stalked, leaves loosely arranged, broadly wavy, curds flat, irregular, loose, not protected, yellow, highly flavored
Northerns	England	Nineteenth century	Leaves petiolate, broad, very wavy, serrated, curds good, well protected
Roscoff	France	Nineteenth century	Plants short, leaves long erect, slightly wavy with pointed tip, midrib prominent, bluish green, curds white or creamy, hemispherical, well protected
Angers	France	Nineteenth century	Leaves very wavy, serrated, greyish green; curds solid, white, well protected
Erfurt and Snowball	Germany and Netherlands	Eighteenth century	Plants dwarf; leaves short, erect, glaucous green, curds solid, well protected
Indian cauliflower	India	Late nineteenth century	Plants short, long stalked, leaves loosely arranged, broadly wavy, curds flat, somewhat loose, yellow to creamy, not protected and highly flavored

Source: Adapted from Sharma et al. (2004)

Table 7.2 Grouping of cauliflower according to phylogeny

Group	Chief Characteristics	Common types
Italian	Very diverse, include both annuals and biennials and curds with peculiar conformations and colors	Jezi, Naples (Autumn Giant), Romanesco, Flora Blanca
North-West European biennials	Derived within the last 300 years from Italian material	Old English, Walcheran, Roscoff, Angers, St. Malo
North European annuals	Developed in northern Europe for at least 400 years. Origin unknown, perhaps Italian or Eastern Mediterranean	Lecerf, Alpha, Mechelse, Erfurt, Danish
Asian	Recombinants of European annuals and biennials developed within 250 years, adapted to tropics	Four maturity groups are recognized by Swarup and Chatterjee (1972)
Australian	Recombinants of European annuals and biennials and perhaps Italian stock, developed during the last 200 years	Not yet categorized

Source: Adapted from Sharma et al. (2004)

7.1.4 Indian Cauliflower Classification

Indian (or Asian) cauliflower is classified into four groups viz. early, mid-early, mid-late and late or snowball types, based on thermosensibility (Table 7.3). The first three types include Indian or tropical types and the selections made for this purpose perform well, producing quality curds even during May–June, making it possible to grow cauliflower almost year around (Chatterjee 1993; Singh and Sharma 2001). There are several local cultivars in India of varying maturity, commonly named after the season of curd maturity, such as, Kunwari (September–October), Katki (October–November), Aghani (November), Poosi (December) and Maghi (January). These cultivars are highly heterozygous with respect to all characters, whether vegetative, curd or maturity. These cultivars have short a short stature, bluish green leaves with a waxy bloom and with a very small meristem curd tending to grow loose faster. They are also sensitive to buttoning earlier. Mainly private seed companies of Hajipur (Bihar) and Ayodhya the then Faizabad (Uttar Pradesh) regions market seeds of these local types. Many of these local cultivars are cultivated in Bihar, Uttar Pradesh, Punjab, Haryana, Rajasthan, Madhya Pradesh, Maharashtra and

Table 7.3 Grouping of cauliflower based on temperature requirement for curd initiation and development

Maturity group	Traditional groups	Sowing time	Mean temperature for curd initiation & development	Harvest or period	Cultivar
Early	Kartiki	June	20–27 °C	September–November	Pusa Meghna Pusa Ashwini Pusa Kartiki Pusa kartik Sankar Pusa Deepali
Mid-early	Aghani	End of July–August	16–20 °C	November–December	Pusa Sharad, Pusa Hybrid-2, Improve Japanese Pusa synthetic
Mid-late	Poosi	End of August–September	12–16 °C	December–January	Pusa Himjyoti Pusa Paushja Pusa Shukti
Late	Maghi	September–November	10–16 °C	January–March	Pusa Snowball K-1, Pusa Snowball 1, Pusa Snowball K-25 Pusa Snowball Hybrid-1

Source: Singh et al. (2018)
[a]Under northern Indian plains

Gujarat. The earliest selected local cauliflower varieties, Early and Main crop Patna, Early, and Main Crop Banaras were from M/s Sutton and Sons, India, in 1929. However, the systematic breeding program in Indian cauliflower started around five decades ago and helped in the genetic shift towards desirable traits and germplasm diversification, which, later on, acted as source for breeding programmes across the countries. Over the years, the work of breeding for heat tolerant varieties and temporal shift for earliness in Indian cauliflower has resulted in the development of a range of varieties (Table 7.3). These are open-pollinated varieties, which further served as a source breeding materials for developing varieties for local climatic condition in different regions of the country. Today, a number of varieties and hybrids in the public and private sector are available for different maturity groups. Table 7.3 shows important public sector varieties/hybrids.

7.2 Genetic Diversity and Exploration of Wild Relatives

7.2.1 Genetic Diversity

The greatest genetic diversity of cauliflower is in the Mediterranean gene center including Greece, Syria, Cyprus, Sicily, Italy, Spain and Portugal. However, wild species or subspecies and improved heterogeneous cultivars have been primary genetic resources for various characters available to the breeders. Introgression from wild species or primitive forms of *Brassica oleracea* in the Mediterranean area and northwestern Europe has resulted in the great genetic diversity found in cauliflower. The annual types of cauliflower occur in Italy and surrounding areas. The introduction of improved cultivars and hybrids into Europe has replaced many of the landraces, primitive cultivars and traditional varieties or types. There is extensive genetic erosion but fortunately, many genetic resources are still available in the Mediterranean gene centers. In addition to landraces in Italy, cauliflower genetic diversity also exits in France, UK, Sweden, Denmark and the Netherlands. In Europe, researchers developed cauliflower varieties in the sixteenth century, such as, Originals or Italians (Jezi, Naples, Romanesco, Flora Blanca), Erfurt, Alpha and Snowball in Germany and the Netherlands, Cornish and Northerns in England and Roscoff and Angers in France. The European biennials include Old English, Walcheran, Roscoff, Angers and St. Malo and the annuals like Alpha, Erfurt, Danish, Lecerf and Mechelse. The two other groups of cauliflower suggested are the Indian (or Asian) and Australian, due to recombination of European annuals and biennials.

Genetic diversity is an important factor and pre-requisite for heterosis breeding. Hybrids between genetically-diverse parents manifest greater heterosis than those of closely-related parents. However, Tonguc and Griffiths (2004a) reported a very low extent of genetic diversity, which hinders modern breeders from producing new cauliflower varieties with high yield and specific qualities. Researchers have employed different molecular markers to quantify the genetic diversity level in

cauliflower. Dey et al. (2011) did line × tester analysis in Snowball group of cauliflower using three CMS lines (Ogu1A, Ogu2A and Ogu3A) and nine diverse lines. The number of heterotic hybrids for yield and earliness was low, indicating the narrow genetic base of the Snowball cauliflower. A great extent of variability evolved in tropical early cauliflower (Santhosha et al. 2011). The authors studied 51 genotypes using 16 quantitative characters and reported 14 clusters, of them genotypes of Cluster 8 (IIHR-323-13, IIHR-214-5, IIHR-277-14) and cluster 10 (IIHR-263, IIHR-272) as the best choice for hybridization. Similarly, Astarini et al. (2006) also reported genetic variation and relationships among 8 Indonesia-, Australian- and European-based cultivars and within Indonesia open-pollinated cultivars using RAPD and ISSR markers. The comparison between the two groups showed that Indonesian cultivars evolved to unique genotypes and would be promising sources of genes for future crop improvement. El-Esawi et al. (2016) reported 27.1% genetic variation among accessions while 72.9% within the accessions in cabbage, cauliflower, Brussels sprouts and kale using SSR markers from Ireland. Yousef et al. (2018) characterized 192 cauliflower accessions from the USDA and IPK gene banks with genotyping by sequencing (GBS) which formed two major groups representing the two gene banks. They indicated that the composition and type of accessions have a strong effect on the germplasm structure, although regeneration procedures and local adaptation to regeneration conditions also exert influence. Meanwhile, primary and secondary centers of diversity still have wild relatives and/or original types of cauliflower. However, local landraces are being replaced rapidly by improved cultivars and hybrids due to yield advantages. Hence, in situ conservation of cauliflower genetic resources is currently difficult for cauliflower. However, ex situ conservation is a common approach. The Horticultural Research International, Wellsbourne, Warwick, UK; Instituut voor de Veredeling van Tuinbouwgewassen, Wageningen, Netherlands; Instituto del Germoplasm, Bari, Italy; Indian Agricultural Research Institute, New Delhi; Indian Agricultural Research Institute Regional Station, Katrain, Himachal Pradesh and the National Bureau of Plant Genetic Resources, New Delhi are managing significant collections of cauliflower germplasm. Germplasm conservation through in vitro propagation of cauliflower is feasible using seedling explants (Arora et al. 1997), protoplast culture (Yang et al. 1994) and anther culture (Yang et al. 1992). Culture of curd explants on MS medium with 6-benzyladenine (cytokinin) and gibberellic acid is an effective way to regenerate cauliflower plants (Bhalla and De Weerd 1999).

7.2.2 Pre-breeding for Cauliflower Improvement

Pre-breeding is an important activity in crop improvement and covers all activities designed to (i) identify desirable characteristics and/or genes from nonadapted (exotic or semi-exotic) materials and (ii) transfer these traits into an intermediate set of materials. In case of the diverse cole vegetables, a large number of species have been exploited for CMS or other important traits. The wild or related species for

B. oleracea are canola (*Brassica napus* L. and its hybrids with *B. campestris*), *B. macrocarpa*, *B. villosa*, *B. rupestris* and *B. incana* in addition to turnip (*B. rapa* ssp. *rapa* L.), *B. campestris* L., *B. napobrassica* L., *B. nigra* Koch, *B. juncea* (L.) Czern. and *B. carinata*. Sharma et al. (2016) initiated introgression of black rot resistance *Xca1bc* locus (on B7 chromosome) from *B. carinata* to *B. oleracea* var. *botrytis* using ILPAt1g70610 marker and embryo rescue. Dey et al. (2015) attempted introgression of black rot resistance (for both race 1 and 4) from *B. carinata* into Snowball cauliflower using embryo rescue.

7.2.3 Embryo Rescue

Embryo rescue can help overcome natural reproductive barriers in the development of interspecific hybrids in *Brassica* (Ayotte et al. 1987; Hansen and Earle 1995; Momotaz et al. 1998; Niemann et al. 2013; Weerakoon et al. 2009). Wide crosses between crop plants and their wild relatives have become routinely, possible by the embryo rescue technique. Different techniques of plant cell and tissue culture, such as ovary, ovule and embryo culture as well as protoplast fusion, have proved useful for production of interspecific hybrids. Rescue of hybrid embryos and their culture in vitro helps to overcome post-fertilization barriers in interspecific crosses. Since its first use in *Brassica* by Nishi et al. (1959), extensive investigations to improve the techniques for obtaining higher seed set have been carried out by Inomata (1993, 2002) and Zhang et al. (2003, 2004). The successful application of this technique depends on the stage of the rescued embryo cultured in vitro. Several attempts have been made to transfer desirable gene(s) from alien *Brassica* spp. to *B. oleracea*, such as powdery mildew resistance (Tonguc and Griffiths 2004b), downy mildew (Chiang et al. 1977), male sterility (Chiang and Crete 1987) and atrazine resistance (Jourdan et al. 1989). Progress toward marker-assisted *Xcc* resistance gene transfer from *B. carinata* to cauliflower has been very slow (Tonguc and Griffiths 2004c; Tonguc et al. 2003).

7.2.4 Conservation Strategies

Sporophytic self-incompatibility (SI) in cauliflower prevents pollination of flowers on the same plant. In cauliflower, it is active only after anthesis; hence, the germplasm having SI needs to be maintained at bud stage by bud-pollination at 2–4 days prior to anthesis (Kalia 2009). Singh and Vidyasagar (2012) reported that NaCl sprays (3–5%) are effective in temporarily breaking down self-incompatibility in cabbage. However, strong SI lines and male sterile lines can also be maintained by tissue culture (Bhalla and De Weerd 1999; Bhatia et al. 2014).

7.3 Breeding Objectives

In cauliflower, the most important objectives of breeding new varieties/hybrids are high commercial quality, including adequate curd size and shape, good and attractive color, compact and firm curds and uniformity of field appearance of plant type (Kalia 1994). A high level of uniformity is difficult to achieve in the case of open-pollinated varieties and in F_1 hybrids using conventionally developed inbred lines due to the cross-pollinating nature of cauliflower. Hence, use of doubled haploid (DH) lines is needed to produce F_1 hybrids with a great extent of uniformity and in reduced time span. The different kinds of CMS system established in cauliflower genotypes for use in hybrid breeding can be used to tap the heterotic potential of cauliflower groups. Development of varieties/hybrids resistant to diseases and insect pests is an important objective both to reduce pesticide load on food and in the environment. Quality traits such as curd flavor, persistent white color, curd texture and compactness and shape need adequate attention. In cauliflower, selective approach for glucosinolates is required because some have harmful health effects while there are glucosinolates, which possibly counter cancerous agents. Novel or *specialty traits* desired by certain consumers such as orange, green and purple curds in cauliflower could be tackled for better consumer health and premium price for farmers. Further, in a changing climatic scenario, development of resilient open pollinated/hybrids varieties enabled with traits such as reduced crop period, extended reproductive phase, elongated root length and better blanching habit are desirable. On the basis of genetic stocks studied in Indian cauliflowers, the ideotype of cauliflower should possess: (i) stem length: 12–25 cm, (ii) plant type: No. 3, (iii) frame (spread): 35–45 cm, (iv) leaf number per plant: 18–22, (v) leaf length: 50–55 cm, (vi) curd shape: hemispherical, (vii) curd diameter: 15–18 cm, curd weight: 750–1000 g, (viii) curd color: retentive white, (ix) resistance to: black rot, curd and inflorescence blight and (x) curding period: better plasticity for extended growing period.

7.4 Divergence in Important Characters

7.4.1 Maturity of Curd

Chaterjee and Swarup (1972) classified Indian cauliflowers into three maturity groups: (i) Maturity group I – Curds harvested from September to early November, curds loose, cream to yellow and strong flavored; (ii) Maturity group II – Curds harvested from mid-November to early December, curds are somewhat loose, cream white and have strong flavor and (iii) Maturity group III – Curds harvested from mid-December to mid-January, curds are more compact and somewhat whiter curds not so strongly flavored and has Plant type 3 features.

Group I and II have Plant Type 2 and characteristic typical features of the Cornish type. However, intensive development in the past three decades transformed Indian type with development of varieties having short-to-medium stalk length, white, compact and partial dome shape with curds covered partially. The varieties of Maturity group I with partial covering and good curds are Pusa Deepali, Pusa Ashwini and Pusa Kartiki.

7.4.2 Plant Type

On the basis of growth habit, three plant types were identified: (i) Plant type No. 1 – long stalk, curds completely exposed with flat leaves; (ii) Plant type No. 4 – completely erect habit of leaves with covered curds and (iii) Plant types No. 2 and 3 are intermediates, the former being close to No. 1 and the latter approaching No. 4 (Swarup and Chatterjee 1972). Among them, Plant type No. 3 was considered best as it had long, erect leaves with or without blanching habit and medium-sized curds. It corresponds to the plant type of Snowball. Plant type No. 2 is the most common among Indian cauliflowers.

7.4.3 Stem Length

There is large variation in stem length of different cauliflower germplasm. Chatterjee and Swarup (1972) classified Indian cauliflower into three groups based on stem length: (i) shoot (<15 cm); (ii) medium (16–20 cm) and (iii) long (21 > cm). Stalk duration was longer in Maturity Groups I (September to Early November) and Group II (Mid November to Early December) than Group III (Mid December to Mid January). Long stem was only present in Group I. Medium stem length was predominant in Maturity Group II. Maturity group III mostly had a short-stalk (54.5%) and medium-length stalk (45.5%).

7.4.4 Stem Pigmentation

The stem may be green or pigmented as in the Snowball group. However, the intensity of pigmentation may vary in different types of cauliflower. Stem pigmentation is important as a dominant marker gene character. Purple pigmentation appears in the apical region of some genotypes but that does not persist in later growth and curding stages.

7.4.5 Leaf Characters

The leaves may be long and narrow, long and broad, and short and broad. The leaf margins are straight or broadly wavy. Leaf color varies from bluish green and wavy green to glossy green. A recessive gene governs the glossy leaf. The number of leaves ranges from 18 to 50 during the curding stage. Generally, there are more leaves in early-maturing types than in the late-maturing types.

7.4.6 Self-Blanching Character

When the inner leaf whorls are joined at the top of the curd, the plant is known as a self-blanching type. Cauliflower types vary greatly in this character i.e., not covered (early group), partly covered (mid group) and covered (Snowball group). However, partial covering also has been introgressed in some early varieties.

7.4.7 Curd Shape

The shape of cauliflower curd may be circular (Pusa Himjyoti), broad elliptic (mid and Snowball groups) and narrow elliptic (early group). Further, curd doming is another trait which appears to be weak (early group), medium (mid and Snowball groups) and strong (Pusa Paushja).

7.4.8 Curd Size and Weight

Cauliflower germplasm has broad genetic variation concerning size and weight of the curds. Polar diameter is defined as small (<15 cm), medium (15–20 cm) and large (20 > cm) curds. Similarly, equatorial diameters are also categorized as small (<15 cm), medium (15–20 cm) and large (20 > cm) curds. Most early varieties are grouped in the small category, while Snowball types have large curds.

7.4.9 Curd Compactness and Texture

Loose, medium and compact are common categories of curd compactness while fine and coarse are two categories of curd texture. Loose curds are defined as having a surface which feels spongy to the touch, sometimes caused by wilting and also because the curd has thin interstitial branches or the segments of curd have

elongated due to its maturity before the subtending leaves have folded back to expose the curd. Early type varieties have loose to medium compact curds with fine texture while the Snowball type produces compact curds with fine texture. Bracting in curd ranges from being barely visible to several centimeters in length, from being few in numbers to several thousand, from white to green and with somewhat purple tips. The appearance of bracts in curds is under genetic control but their color (white or green) and size (small to large) are influenced by temperature factors. Riciness, ricyness, or wooliness is due to the appearance of miniature floral buds as outgrowths about 1 mm in diameter above the curd surface, which is clearly visible under a microscope.

7.4.10 Curd Color

Curd colors in Indian cauliflowers have different shades, which range from yellow to bright white. The bright white curds, as in Snowball, have wide market preference. The early-maturing hot weather cauliflowers are mostly yellowish to somewhat creamy white. Curd color is influenced by the blanching habit of the variety and growing temperature. Additionally, orange (β-carotene), green (chlorophyll) and purple (anthocyanin) colors are not uncommon in cauliflower. One semidominant gene *Or* determines carotenoid accumulation in the curd portion, giving an orange color. Green curd color curd is governed by high chlorophyll content and controlled by two genes and bleaching (white) genes perhaps three in number causing curds to remain white in the presence of sunlight, probably due to lower peroxidase activity. Yellow or pink discoloration may transiently appear in curds when unexposed to sun and may persist after maturity. They are probably due to flavonoids; however, genotypes with a waxy coating to the leaf consistently show pink curds.

7.4.11 Curd Maturity

Based on curd initiation (i.e. days to 50% of the plants with curd initiation from sowing of seed), cauliflower varieties are grouped into three categories Early (<75 days), Medium (75–100 days) and Late (100 > days). Most of the early types are early curd-forming varieties while Snowball group varieties are late curd-forming varieties.

7.5 Gene Action

Cauliflower was first morphologically distinguished based on a few gene differences (Giles 1941). These crops have same chromosome number (n = 9) and there are almost no differences in chromosome morphology. *Brassica oleracea* is triple tetrasomic with the genomic formula A BB CC D EE F with six basic chromosomes, which show some secondary pairing (Robbelen 1960). The cauliflower genome size is 584.60 Mb, contained 47,772 genes and 56.65% of the genome is composed of repetitive sequences (Sun et al. 2019).

In cauliflower, the transformation from vegetative phase to curding is governed by temperature; varieties differ in optimum temperature requirement for curd formation (Haine 1959; Kato 1964; Sadik 1967). Hence, any study on this character must take into consideration the suitability of prevailing temperatures for curding of the varieties. Watts (1964) made two series of diallel crosses between (a) eight varieties of autumn cauliflower (intervarietal) and (b) six inbreds of a single variety of early-summer type. He found no F_1 (in either series), which was earlier than the early parent was. Further, in autumn types, he noted additive effects and those varieties with early mid-maturity possessed dominant polygenes while those with late maturity possessed recessive polygenes. In the early-summer type (intravarietal diallel), some interaction was noted. There was an association between early curding and low leaf number and between later curding and high leaf number. Swarup and Pal (1966), in a similar study of late-maturing cauliflower, found that dominance and epistasis contributed most towards the inheritance of curd maturity. Heterosis was manifested in earliness.

Nieuwhof and Garetsen (1961) reported that curd compactness or solidity is controlled by polygenic factors. They observed a negative correlation between the firmness of curd and seed yield. Combining ability analysis of seven inbred lines of curd maturity group III (mid-December to mid-January) indicated that nonadditive effects were more important in the expression of plant height, plant spread, curd maturity, curd weight and curd size index (Lal et al. 1978). The nature of gene effects was studied in 36 cross combinations obtained by crossing 6 inbred lines of maturity group II and 6 of maturity group III. Lal et al. (1979) concluded that dominance and epistasis were quite high in the expression of curd weight and curd size indices. The crosses showing high performance for these characters may be utilized for heterosis breeding. Some crosses also revealed a significant additive component of variation indicating the possibility of improvement in these characters by selection. High heritability and genetic advance were observed for traits such as net curd weight, total plant weight, harvest index, curd size index, curd diameter, stalk length and leaf length, respectively, in Indian cauliflower (Dubey et al. 2003).

Several cauliflower researchers have reported the genetics of qualitative and quantitative traits (Table 7.4), genetic advance, heritability and combining ability. Ahluwalia et al. (1977) described the inheritance of qualitative characters in Indian cauliflower in detail.

Table 7.4 Genetics of quantitative characters in cauliflower

Character	Nature of gene action	References
Curd weight	Dominance and epistasis Pronounced overdominance and epistasis Additive and dominance gene action	Gangopadhayay et al. (1997), Jyoti and Vashistha (1986), Sharma et al. (1988), Singh et al. (1975, 1976a) and Swarup and Pal (1966)
Curd: Plant ratio	Partial dominance	Kale et al. (1979)
Curd diameter	Predominance of dominance gene action	Lal et al. (1979)
Curd size index	Pronounced overdominance and epistasis Dominance and epistasis Additive dominant gene action Partial dominance	Kale et al. (1979), Lal et al. (1979), Sharma et al. (1988), Singh et al. (1975, 1976a) and Swarup and Pal (1966)
Curd angle	Pronounced additive gene action Additive and dominant gene action	Chand (1980), Dadlani (1977) and Lal et al. (1979)
Curd compactness	Polygenic Dominance and additive gene action Additive	Lal et al. (1979), Nieuwhof and Garretsen (1961) and Vashistha et al. (1985)
Maturity earliness	Partially dominant gene action Dominance and epistasis Predominance of additive gene action Additive gene action Additive and dominant gene action	Gangopadhayay et al. (1997), Kale et al. (1979), Lal et al. (1979), Mahajan et al. (1996), Sandhu and Singh (1977), Sharma et al. (1988), Singh et al. (1975, 1976b), Swarup and Pal (1966) and Watts (1964)
Maturity lateness	Recessive polygenes	Watts (1963)

Source: Sharma et al. (2004)

The information on genetics of traits is a prerequisite for their improvement. Genes for other traits such as downy mildew and black rot resistance were investigated and symbols given were *Ppa3* and *Xca1bo,* respectively. In the selection of parental/inbred lines for improvement of traits of significance, it is essential to know the gene action for the particular trait. Classical studies of cauliflower genetics reviewed by Bose et al. (2003) found that the gene action of curd weight is dominant and due to epistatic or partial dominance, additive gene action or additive and dominant. For curd to plant ratio, gene action was reported as partially dominant while for curd depth it is additive and dominant gene action. Curd size index is governed by epistasis or overdominance and epistasis, additive gene action or partial dominance. The gene action for curd angle is due to additive gene action, and additive and dominance gene action. Polygenic or two genes *Co1* and *Co2* or additive gene action was reported for curd compactness while the earliness (maturity) trait is controlled by recessive polygenes while traits such as plant height, plant expansion, number of leaf blades and heading stage are governed by nonadditive gene action.

Several plant characters in cauliflower have simple inheritance, such as plant type, leaf characters (petiolate, leaf apex, margin, arrangement, glossiness), stalk length, curd color and flower color (Table 7.5). The important quantitative traits governed by polygenes in cauliflower include curd diameter, compactness, maturity, weight, depth, size-index, shape and yield (Table 7.6). The loose, bracted (small light to dark green or slightly purple leafiness in curd portion) and ricey defects (uneven lengthening of peduncles of prefloral buds on curd surface leading to a condition known as *ricey* or *riceyness*) in cauliflower curds are perhaps polygenic characters greatly influenced by environment. Possibly looseness and riceyness are highly heritable, hence they need proper tracking during breeding.

Table 7.5 Important simple inherited characters in cauliflower

Plant characters	Inheritance pattern
Plant type	Single gene, erect dominant (EE) Three major genes-additive, dominant and epistasis
Leaf characters	
Petiolate Leaf apex Leaf margin	Single gene, dominant (PET) Single gene, dominant for round apex (RO) Single gene, dominant for wavy (WY)
Leaf arrangement around curd	Two genes, dominant for semi-blanched (Bl_1, Bl_2)
Glossy leaf	Single gene, recessive (gl) Two genes, inhibitory (IG)
Stalk length	Single gene, long stalk dominant
Curd color	
Orange Green Purple Retentive white	Single semidominant gene *Or* Two genes Single semi-dominant gene *Pr* Three genes for bleaching, controlling peroxidase activity
Flower color (white, yellow, cream)	Two independent genes, dominant, epistatic interaction with few modifiers

Source: Swarup (2006)

Table 7.6 Inheritance of important quantitative traits in cauliflower

Character	Inheritance
Curd maturity	Polygenic, predominance of additive gene action; earliness partially dominant gene action additive and dominant gene action; lateness controlled by recessive polygenes, dominance towards earliness
Curd diameter	Polygenic, predominance of dominant gene action; additive and dominant gene action; low heritability
Curd depth	Polygenic, additive genetic variance; dominant and additive gene action
Curd-size index (diameter/depth)	Polygenic, epistasis; overdominant and epistasis; dominance and epistasis; partial dominance and additive gene action; partial dominance; highly complex inheritance
Curd compactness	Polygenic, dominant and additive gene action; two major genes (Co_1, Co_2) in which Co_1 is epistatic to Co_2; semi-compactness controlled by both recessive genes
Curd weight	Polygenic, dominant and epistasis in Snowball group epistasis; partial dominance and additive gene action; overdominance and epistasis; additive and dominant gene action, in tropical cauliflower
Curd shape	Polygenic, partial dominance for smooth curds in Italian types

Source: Swarup (2006)

7.6 Genetic Mechanisms for Hybrid Breeding

In cauliflower, the extent of heterosis in terms of yield has a range of 15–50% depending upon the crop. In cauliflower, heterosis of hybrids over open-pollinated cultivars may be only 10%, but a high degree of natural outcrossing and greater uniformity in yield and quality relative to open-pollinated varieties make hybrids the preferred choice for cultivation. The superiority of hybrids over the mean parental value depends directly on the existence of dominance and indirectly through interactions involving the dominance effect at different loci. There are several helpful biometrical procedures available to understand the heterosis in terms of actions and interactions at a variable number of loci. This procedure allows the partitioning of heterosis based on the relative roles of additive, dominance, epistasis, linkages, maternal effect and genotype x environmental interactions. It is also observed that the heterosis in cauliflower finds support for its physiological basis of faster growth rate, higher leaf area index, stout stem and root portions and greater biomass production (Sharma et al. 2004).

Cauliflower has a good amount of diversity with an adequate level of heterosis. However, hybrid breeding has constraints because of (i) lack of stable self-incompatible lines/cytoplasmic male sterile lines which results in sib-mating within the parental lines; (ii) nonsynchronous of flowering time between male and female genotypes; (iii) shorter period of flowering flush in cauliflower due to cymose inflorescence which leads to nonsynchrony of flowering of parent inbreds and (iv) minor heterosis for curd size in some combinations in comparison to other brassicas. Heterosis was exploited in cauliflower in the development of Pusa Hybrid-2

(November–December maturity group) for earliness, high yield, bigger curd size, better curd quality, uniform maturity and disease resistance (Singh et al. 1994).

Investigations on degree of heterosis in cauliflower revealed variation for adaptive trait such as for days to curd maturity (−3.92–16.3%), plant height (−10.40–31.33%), plant spread (−10.68% to −29.52%) and number of leaves/plant (−10.44% to −39.27%) (Garg and Lal 2005). The heterotic combinations have better performance for quality traits such as curd compactness (−36.37–0.58%) and color. High heterosis was recorded for yield traits, which ranged from −51.77% to 24.25%. Better hybrid performance against abiotic stresses like heat and humidity could be due to changes in the salicylic acid- and auxin-regulated pathways (Groszmann et al. 2015). These authors indicated that hybrids with larger leaves have greater capacity for energy production to support increased growth vigor and seed yields of the hybrids. Sheemar et al. (2012) observed that net curd weight had significant positive correlation with total plant weight and leaf width. Yield attributes such as size and weight of curds, harvest index and yield per hectare are considered when evaluating heterotic combinations. The heterobeltiosis for harvest index ranged from −47.59% to 15.0%; for curd diameter from −22.22% to 35.63% and for net curd weight, it ranged from 11.19% to 45.38% (Singh et al. 2009). For days to harvest, negative heterosis was reported in all heterotic combination in the range of −4.59% to −1.46%. However, information on the extent of heterosis on flowering behavior, seed production traits, growth attributes such as leaf area, leaf number, canopy parameters, erectness, blanching habit, leaf shape and orientation and plant spread is still not clear. Dey et al. (2014) reported heterosis for important vitamins and antioxidant plant pigments in Snowball cauliflower. They observed high a SCA effect and a predominant role of nonadditive gene action for most of the quality traits in heterotic hybrid combinations. Kumar (1983) reported maximum heterosis for survival percentage of seedlings and total minerals.

7.6.1 Self-Incompatibility

Self-incompatibility (SI) is the inability of a plant to set seed when self-pollinated, even though it can form normal zygotes when cross-pollinated and its pollen can fertilize other plants. The SI system is a genetically controlled mechanism, which favors cross-pollination and is commonly used in hybrid seed production of cole crops. All *Brassica* vegetables have sporophytic SI systems, being strongest in kale and weakest in (European) summer cauliflower. Cauliflower has homomorphic sporophytic SI with trinucleate pollen and pollen germination inhibition occurs at the stigmatic surface. In this system, inhibition of self-incompatible pollen takes place on the surface of the papilla and deposition of callose takes place inside the papillae. A detailed investigation of Indian cauliflower self-incompatibility revealed that inbred lines of maturity group I have the strongest self-incompatibility followed by maturity group II; group III showed weak self-incompatibility (Sharma et al. 2003). However, some reports indicated a strong self-incompatibility in all the maturity

groups of Indian cauliflower while maturity Group II exhibited an intermediate position for self-incompatibility (Chatterjee and Swarup 1984). Singh et al. (2002) reported a high level of self-incompatibility in 13 genotypes from different groups such as in Group I, Group-II, Group III and Group-IV. Sharma et al. (2003) investigated SI level in early group genotypes in Punjab and found that Early Kumari and NDC-1 were strongly self- incompatible. Being a natural mechanism, self-incompatibility has no adverse side effects, such as those often found with cytoplasmic or chemically-induced sterility.

Bud pollination and spraying with 3% NaCl solution (Kucera et al. 2006) are used to break SI to maintain SI lines. In cauliflower, cvs. Pusa Kartik Sankar and Pusa Hybrid-2 and in cabbage cv. Pusa Cabbage Hybrid-1 have been developed using SI lines and released for commercial cultivation in India (Sharma et al. 2004). Hadj-Arab et al. (2010) studied variability of the SI response in homozygous plants in cauliflower and reported continuous phenotypic variation for SI response in offspring plants. They observed that SI levels decreased during the life of the flower. This is mainly due to two key genes S-locus receptor kinase (SRK) and S-locus cysteine-rich (SCR/SP11) genes. Zeng and Cheng (2014) cloned yellow mustard *S*-locus genes of SI lines using the *S*-locus gene-specific primers from *Brassica rapa* and *B. oleracea*. The study indicated that self-incompatibility was dominant over self-compatibility and controlled by a one-gene locus. The authors developed dominant and codominant markers in yellow mustard which may be useful in cauliflower. Verma et al. (2017) characterized SI lines of early and mid-maturity Indian cauliflowers using quantitative and molecular analyses and reported higher diversity in the mid-maturity group.

7.6.2 Cytoplasmic Male Sterility (CMS)

The *Ogura* cytoplasm of the radish genus *Raphanus* is the most important source of sterile cytoplasm used in cauliflower. Cybrids were utilized to transfer CMS to cauliflower from *Ogura*. However, there were problems of temperature sensitivity and chlorosis in hybrid plants, which were overcome by protoplast fusion. CMS male sterility, especially *Ogura*, has been established in different groups of cauliflower and is being explored for F_1 hybrid development at the Indian Agricultural Research Institute (IARI), New Delhi. The transfer of sterile *Anand* cytoplasm from *Brassica rapa,* originally derived from the wild species, *B. tournefortii* via *B. napus* into cauliflower is also being explored as a new source to facilitate F_1 hybrid breeding. A recessive *ms* gene in cauliflower has been tagged by using RAPD and RFLP markers to accelerate hybrid breeding.

The transgenes, *Barnase, Bar* and *Barstar* are being utilized to develop transgenic cauliflower hybrids. The male sterility transgene *Barnase* is also in *Bacillus amyloliquifaciens,* in which ribonucleases destroy the tapetum layer in the pollen to produce stable male sterile plants. The male sterility *ms* gene is linked to the

herbicide resistance gene, *bar*. The restorer line for the *barnase* sterile lines can be obtained by expressing the gene coding for *barstar*.

Cauliflower has a SI system, which favors outcrossing but has limitations of breakdown and maintaining SI inbreds. Hence, the search for other mechanism such as cytoplasmic male sterility (CMS) in its own germplasm or related species was done. The CMS system is the most reliable for hybrid seed production and various types of CMS have been developed to breed vegetable crops. CMS is a maternally-inherited trait encoded in the mitochondrial genome. No CMS system is yet reported in *Brassica oleracea*, however, *Ogura* sterile cytoplasm was first introduced into cauliflower (Ogura 1968). Later, it was transferred into heat-tolerant Indian cauliflowers from kale and broccoli. Four lines (MS-91, MS-51, MS-11, MS-110) were used to transfer *Ogura* CMS via kale into five lines (MS-01, MS-04, MS-05, MS-09, MS-10); this CMS system was transferred from broccoli for use in heterosis (Sharma and Vinod 2002). Use of male sterile lines not only extended the range of heterosis but also improved the quality and efficiency of hybrid seed production. Ruffio-Chable et al. (1993) reported the influence of temperature on the male sterile phenotype, while Kaminski et al. (2012) observed the presence of atypically developed plants with chimeral generative stacks or partially-fertile flowers among segregating test cross progeny. In brassicas, several other CMS systems (*oxyrrhina, polima, tournefortii*, *erucastrum, moricandia*) are being investigated, but so far, these could not be successfully used for hybrid seed production due to various limitations viz., breakdown of male sterility, chlorosis and abnormalities in petals, poor nectarie function and lack of appropriate restorer lines, all of which need more attention. However, the cytoplasmic male sterility (CMS) system has been introgressed into tropical types of cauliflower genotypes for use in hybrid breeding. The CMS system is much more effective than the SI system due to its stable genetic mechanism, because the SI system is comparatively weak within the mid-group of cauliflower. Besides, the open-pollinated varieties are a better choice for nonconventional areas and improvement of land races by appropriate selection methods. Some of the traits like black rot, yellowish and loose curds, advanced earliness and stability in performance need more attention for further improvement of tropical cauliflowers. Jourdan et al. (1985) reported high regeneration capacity from cultured mesophyll cells in a cauliflower line carrying *Ogura* CMS. Further, the cell fusion technique is also used to produce male sterile lines from wild species not used in interspecific and intergeneric sexual hybridization. Liu et al. (2006, 2007) reported use of antisense RNA or RNAi to silence relevant gene expression of pollen development related gene *BcMF3* and *BcMF4* from Chinese cabbage pakchoi to inhibit development of pollen.

Dey et al. (2011) reported development of *Ogura*-ddbased improved CMS lines of snowball cauliflower viz., Ogu1A, Ogu2A and Ogu3A through conventional backcrossing. Chamola et al. (2013) transferred cytoplasmic male sterility from alloplasmic *Brassica juncea* and *B. napus* to cauliflower through interspecific hybridization and embryo culture. The CMS system has been used in commercial F_1 hybrid production in *B. oleracea* using an improved *Ogura* cytoplasm (Pelletier et al. 1989). Introgression of *Ogura* cytoplasm also altered important quality traits in *Ogura* cybrid cytoplasm-based cauliflower CMS lines (Dey et al. 2017a). Dey

et al. (2014) did not observe significant differences among A and B lines for most of the vegetative traits but they varied in curd maturity, leaf number, leaf size and plant height. They also investigated 25 CMS lines for different agronomic and floral traits along with combining ability and SSR marker analyses (Dey et al. 2017b). Bhatia et al. (2014) developed a protocol for in vitro maintenance of *Ogura* CMS lines of cauliflower using hypocotyls and curds as explants by using MS medium supplemented with 2.5 mg/l kinetin, 0.2 mg/l NAA and 0.2 mg/l GA_3.

7.6.3 Combining Ability for Exploiting Heterosis

The selection of inbreds of varieties for use in heterosis breeding should be based on their combining-ability performance. Combining ability is effective for the selection of excellent parents in early generations, because evaluation of all possible crosses is time consuming and laborious in a breeding program. Generally, SCA values of the cross give better predictive information than GCA of the parents. Single or three-way crosses can provide the SCA analysis while polycrossing is used for GCA analysis, and top and diallel crosses for analysis of both SCA and GCA. The lines with high GCA values are useful in a hybridization program to develop improved lines and those with better SCA for hybrid breeding.

A number of investigations have been carried out on identification of inbreds or varieties for use in heterosis breeding through SCA in cauliflower. Dixit et al. (2004) reported sufficient heterosis for early maturity, net curd weight, curd size index and curd yield. Earliness is an important trait of tropical cauliflower, which has sufficient heterosis (Gangopadhyay et al. 1997; Sharma et al. 1983). Dey et al. (2014) reported that the CMS line Ogu12A of cauliflower was a good general combiner (GCA effect) for most of the important vitamins and antioxidant pigments. The proportions of gca/σ2sca were <1 in 40 hybrids indicated for the presence of nonadditive gene action for the traits. The study suggested that high heterosis for ascorbic acid, anthocyanin and carotenoids in cauliflower indicated the scope for development of F_1 hybrids rich in phytonutrients. Thakur et al. (2004) investigated the extent of heterosis for curd compactness and revealed appreciable heterosis over the better parent. Saha et al. (2015) reported that overdominance had a predominant role for marketable curd weight, curd diameter and curd depth. For marketable curd weight, dominance (h) and dominance × dominance (l) components with duplicate type of epistasis were present. Lines IHR3, IHR4, IHR9 and IHR36 were good combiners for most of the characters. Sheemar et al. (2012) reported that the net curd weight correlated significantly and positively with total plant weight, and total plant weight had the highest positive direct effect on net curd weight, harvest index and curd depth. They also reported that the net curd weight, curd depth and curd diameter were significantly correlated with days to 50% curd maturity, and the net curd weight with total plant weight and leaf width.

Varalakshmi (2009) performed line × tester analysis involving four lines and five testers in early cauliflower and reported predominance of nonadditive gene action

for days to 50% curd initiation, 50% curd maturity, leaf number, leaf weight, stalk weight, curd size and curd weight. In Snowball cauliflower, Ram et al. (2017) found a wide range of heterosis for important dietary minerals and identified CMS lines with good combining ability. Verma and Kalia (2017) analyzed genetic component of variance and reported the preponderance of dominant variance and nonadditive gene action for leaf, plant and curd traits. In hybrids, the contribution of lines was higher over the testers for all traits. They also analyzed genetic diversity and its relation to heterosis in early- and mid-maturity groups of Indian cauliflower (Verma and Kalia 2017). Additive genetic effect is more important than nonadditive effects in the expression of resistance to diseases, average curd mass, curd color and hollow stalk incidence in Brazilian cauliflower materials (Arashida et al. 2017).

7.6.4 DNA Markers for Heterosis Breeding

Research is heading towards detection and mapping of heterosis quantitative trait loci (Heterosis QTLs; hQTLs). For this, identified heterotic groups based on marker data and complementary groups are crossed to produce hybrids. After this, genomic regions involved in heterosis are identified and the target regions introgressed into appropriate inbreds to enhance hybrid performance. Marker-based estimates of genetic diversity between the parents would predict heterosis more precisely than that of phenotypic diversity, but this expectation is not yet realized in cauliflower. Molecular markers assign the inbred lines to appropriate heterotic groups and identify the heterotic loci. A detailed analysis of these loci may provide a better insight into the genetic basis of heterosis and afford a more reliable heterosis prediction.

For heterosis breeding, identification of the useful hybrid combinations based purely on field evaluation is expensive and quite time demanding. The use of robust DNA markers linked to the hQTLs is quite interesting. These markers can be identified with standard protocols of marker development or identification. To search for hQTLs, the F_2 population is ideal because it provides estimates of different components of genetic variance. The doubled haploid (DH) population is very suitable for mapping of economic traits but not for identification of hQTLs because it consists of only homozygous plants, which carry only additive and additive x additive interaction genetic variances.

Heterotic genes are now also being sought using genomics, however, there is no report on the use of molecular markers or transcriptomics to understand the hQTLs. Chétritl et al. (1984) constructed a physical map of the cauliflower mitochondrial DNA with the restriction endonucleases Sall, Kpnl and Bgll. The 26S and 18S – 5S ribosomal RNA genes appeared to be separated by about 75 kb in this map. However, further use of such information in male sterility is not clear. Gu et al. (2008) constructed a genetic linkage map (668.4 cM) of cauliflower using 234 AFLP and 21 nucleotide binding site (NBS) markers with an average distance of 2.9 cM between adjacent mapped markers, in order to identify potential molecular markers linked to important agronomic traits that could be useful in crop improvement. Li and Garvin

(2003) mapped the *Or* gene in cauliflower by using AFLP markers which later converted to RFLP and SCAR markers. The *Or* gene is semi-dominant in nature which induces accumulation of β-carotene in plant tissue and turns it orange. This has triggered interest in cauliflower breeders to use the technique for rapid introgression of the *Or* gene in commercial cauliflower varieties or developed hybrids to counter human vitamin A deficiency. Saxena et al. (2009) identified two RAPD markers D-3_{450} (5' GGACCCAACC 3') and C-20_{350} (5' ACTTCGCCAC 3') flanking the stalk rot (*Sclerotinia sclerotiorum*) resistance gene in cauliflower with a distance of 2.7 cM, and 4.2 cM, respectively, in a F_2 mapping population of Olympus (R) × Pusa Snowball (S). *Purple* (*Pr*) gene mutation in cauliflower confers anthocyanin accumulation and intense purple color in the curds. Chiu et al. (2010) isolated the *Pr* gene via a combination of candidate gene analysis and fine mapping which offers a genetic resource for development of new varieties in cauliflower with enhanced health-promoting properties and visual appeal. These reports suggest systematic efforts on the development of DNA markers to understand the heterotic genes for better yield and superior quality. However, due to limitations in marker-based genotyping approaches and high similarity among cauliflower genotypes, the development of high polymorphic marker systems such as new sequence based markers linked to CMS locus could be useful tool for hybrid breeding.

Morphological traits and isozyme markers have been used for analysis of genetic diversity and relatedness in cauliflower germplasm, but they have several disadvantages, such as their limited number, environmental dependence and temporal and spatial expression; hence, DNA markers could be a useful tool to predict the genetic divergence in the parents in testing for heterosis. DNA markers are more efficient tools for rapid detection of genetic purity of commercial hybrids than the conventional grow-out test (GOT) method, due to environmental independence and a lesser time requirement (Nicholas et al. 2012). Pattanaik et al. (2018) reported promises of simple sequence repeat (SSR) markers in cauliflower hybrid purity.

DNA marker use in cultivar identification, diversity analysis, construction of genetic maps and tagging agronomically-important genes is reliable. The markers are used to correlate genetic diversity and heterosis in several crops such as maize (Kiula et al. 2008), pearl millet (Singh and Gupta 2019), rice (Zhang et al. 1996) and wheat (El-Maghraby et al. 2005). Hence, this approach can predict heterosis in hybrids, reducing labor and the time needed to evaluate hybrids for heterosis or combining ability in the field.

7.6.5 Heterosis Fixation

Heterosis declines in successive generations because of meiotic recombination during gamete formation and genetic segregation. This requires constant renewal of hybrid seeds and proper maintenance of parental stocks. It avoids unwanted seed progeny and minimizes the cost of seed production. Heterosis fixation can employ doubled haploids, apomixes and mass propagation of hybrids. The DH technology

is more useful in cauliflower but apomixes is not yet reported in this crop. Mass propagation is also feasible but has limited application. In mass production of hybrids, plants can be propagated asexually on a large scale under in vitro conditions either directly from apical, axially adventitious buds, or indirectly through somatic embryogenesis.

7.7 Open Pollinated and Hybrid Variety Development in India

Breeders the world over serve and need to satisfy two constituencies: growers and consumers. From the growers' viewpoint, the breeding aims for cauliflower improvement are: (a) increased crop productivity; (b) reduced losses due to diseases, insect pests and physiological disorders; (c) heat tolerance; (d) improved curd/plant weight ratio and (e) uniformity in appearance and maturity. From the consumers' perspective, quality is the main concern, in terms of curd whiteness, structure (density, surface texture, suitability for floreting), freedom from diseases, nutrient rich and good sensory traits.

Previously, there was a lack of useful SI alleles limiting hybrid breeding in cauliflower. However, the advent of cytoplasmic male sterility has stimulated hybrid development in cauliflower in both the public and private sectors.

In India, the main emphasis in cauliflower improvement, of late, is on development of cultivars and hybrids (SI and CMS system-based) with heat tolerance and resistance to diseases (black rot, downy mildew, *Alternaria* leaf spot, *Sclerotinia* rot) and insect pests (diamondback moth, cabbage butterfly). Earlier emphases were on breeding heat-tolerant early-maturing varieties and hybrids. As a result, improved varieties namely Pusa Meghna, OPusa Early synthetic and F_1 hybrid Pusa Kartik Sankar (CC × DC 41-5) were developed and released for cultivation. Earlier, Pusa hybrid 2 (CC × Sel 1-3-18-19) was the first hybrid developed in cauliflower in India using the SI mechanism in the November–December maturity group (Singh et al. 1994).

An emphasis on resistance breeding led to the isolation of multiple resistance sources viz., Kn-81 (DM, SR, Alt), BR-2 (DM, BR, DMB), Lawyana (BR, SR, DBM) and Armel (BR, SR, DM). These were involved in hybridization with commercial consumer-acceptable land varieties/lines of each maturity group to transfer resistance. The improved elite material was achieved in advanced F_5/F_6 generation stages, which are being evaluated for resistance to diseases/insect pests, yield potential and other horticultural traits.

In India, cauliflower breeding is being carried out at the ICAR institutes namely IARI, New Delhi (tropical cauliflower) IARI Regional Station, Katrain (Snowball group), Indian Institute of Vegetable Research, (IIVR), Varanasi and Indian Institute of Horticultural Research (IIHR), Bangalore. The State Agricultural Universities (SAUs) working on cauliflower are located at Ludhiana, Pantnagar, Hisar, Solan, Sabour and Palampur. Table 7.7 provides details of the cauliflower varieties developed for different maturity groups.

Table 7.7 Varieties of Indian cauliflower developed and released in different maturity groups

Variety	Pedigree	Source	Year	Maturity	Yield (mt/ha)	Remarks
September maturity group (Sowing: end of May; transplanting: mid July; temperature for curding: 20–30 °C)						
Pusa Meghna	Selection	IARI, New Delhi	2004	End of September	12	Compact, cream white curds
October maturity group (Sowing: mid June; transplanting: mid July onwards; temperature for curding: 20–25 °C)						
Pusa Kartiki	Selection	IARI, New Delhi	2015	October 2nd fortnight	22	Compact, white curds
Pusa Ashwini	Selection	IARI, New Delhi	2015	October 1st fortnight	18	Compact, white curds
Sabour Agrim	Selection	BAU, Sabour	2013	Mid October	15	Compact white curds
Kashi Kunwari	Selection	IIVR, Varanasi	2005	Mid October	16	White, compact
Pusa Kartik Sankar (F_1)	F_1 hybrid (CC x DC 41-5)	IARI, New Delhi	2004	Mid October	16	Compact, white curd
Pant Gobhi 3		GBPAUT, Pantnagar	1993	Mid October	10	Cream, compact curds
Pusa Deepali	Selection from local cultivar	IARI, New Delhi	1975	End of October	12	White, self blanching
November-December maturity (Sowing: end of July; transplanting: end of August; temperature for curding: 15–20 °C)						
Kashi Agahani	Selection	IIVR, Varanasi	2008	End of December	22	White, compact curd
Pusa Sharad	Selection	IARI, New Delhi	2004	Mid November	24	White, compact curds
Pusa Hybrid 2 (F_1)	CC 32 × DC 18-19	IARI, New Delhi	1994	Mid December	23	White, compact
Pant Gobhi 4	Recurrent selection in local collection	GBPAUT, Pantnagar	1993	November	12	Creamy white compact
Hisar 1	Selection	HAU, Hisar	NA	End of November	12	Cream, compact curds
December-January maturity group (Sowing: end of August; transplanting: end of September to mid-October; temperature for curding: 12–15 °C)						
Pusa Shukti	Recurrent breeding	IARI, New Delhi	2011	January	35	White, compact
Pusa Paushja	Recurrent breeding	IARI, New Delhi	2008	December	30	White, compact

(continued)

Table 7.7 (continued)

Variety	Pedigree	Source	Year	Maturity	Yield (mt/ha)	Remarks
Pusa Synthetic	Synthetic, involving 7 inbred lines	IARI, New Delhi		December	24	Plants erect, curds creamy white, tolerant to curd and flower blight
Snowball or late maturity group (Sowing: end of September to mid-October; transplanting: beginning of October; temperature for curding: 16–20 °C)						
Pusa Snowball-1	Selection from EC 1203 × EC 1202	IARI Regional Station (RS), Katrain		February	28	Leaves upright, self-blanched, curds white, compact
Pusa Snowball K-1	Selection	IARI RS, Katrain		February	30	White compact
Pusa Snowball KT-25	Selection from EC 103576 × Pusa Snowball-1	IARI RS, Katrain		February-March	34	White, compact
Pusa Snowball Hybrid-1	CMS based hybrid	IARI RS, Katrain	2015	February	35	White compact

7.7.1 Breeding Open-Pollinated Varieties

IARI first released a tropical cauliflower variety, Pusa Katki, in 1954, which was suitable for October maturity. Later on, Pusa Meghna was released for September–October maturity and in recent years, Pusa Ashwini and Pusa Kartiki were also added to the early group varieties (Kalia et al. 2016) These two varieties mature in the second fortnight of October and the end of October, respectively, in sequence, even on the same date of transplanting. Pusa Sharad is the only mid-season variety developed by IARI, New Delhi (Sharma et al. 1999) while Pusa Paushja and Pusa Shukti are in the mid-late group which maturing in December–January (Kalia et al. 2016).

7.7.2 Heterosis Breeding

Although, Jones (1932) first reported heterosis in cauliflower it took a long time to tap its potential at a commercial scale. In India, Swarup and Pal (1966) and Pal and Swarup (1966) found appreciable heterosis in Snowball cauliflower for earliness (5–7 days), curd weight (24.5–28.2%), curd size index (22.54–34.85%) over the better parent. Later, a number of reports indicated an appreciable amount of heterosis in different maturity groups of Indian cauliflowers (Swarup and Chatterjee 1972,

1974; Deshpande 1975; Gangopadhayay et al. 1997; Kumaran 1971; Sandhu et al. 1977; Singh et al. 1975; Swarup and Chatterjee 1972). Hoser-Krauze et al. (1982) used three SI lines of Indian cauliflower and three SI lines of temperate cauliflower and reported heterosis for earliness, curd diameter, curd weight and quality.

7.7.3 Synthetic Varieties

About four to six inbred lines were used to develop synthetics. The inbred lines after testing their general combining ability in a diallel cross, polycross or in a top cross, can be synthesized to form a synthetic variety. Exploiting the pronounced additive genetic variance can develop a synthetic variety. A variety produced in this manner has a threefold advantage: (1) its seeds can be easily produced from open pollination and maintained by the farmer; (2) it is useful particularly in locations no commercial seed industry exists and (3) it is broad-based and, therefore, better adapted to changing growing environments. Bhatia et al. (1978) reported that S_1, S_2 and S_3 generations of early maturing synthetic cauliflower showed 72.0%, 37.0% and 20.8% increase in curd weight, respectively, over that of the standard variety, Improved Japanese. This indicated a high-yield potential of synthetics evolved from Indian cauliflowers. In India, Pusa Early Synthetic and Pusa Synthetic cauliflower varieties were developed by synthesizing 6 and 7 parents, respectively (Gill 1993; Singh et al. 1997).

7.7.4 Intervarietal Crosses for Yield Improvement

Selection in F_2 generation of intervarietal crosses can recombine and fix favorable yield genes. A well-pronounced additive variance for yield or other characters in the elite F_2 generation offers great potential for improvement in such characters through appropriate selection procedures. The occurrence of transgressive segregants (i.e., individual plants, the performance of which is better than that of both the parents of the cross) towards favorable directions for many desirable economic characters in the F_2 generation of intervarietal crosses of cauliflower was reported by Swarup and Pal (1966). Such segregants can serve to develop superior lines/varieties.

7.7.5 Mutation Breeding

Mutation is a useful technique to generate variability for rare or unavailable traits in usable germplasm. Cauliflower, as a member of the Brassicaceae family, has vast diversity. However, the search for traits such as resistance to black rot, *Alternaria* leaf spot, insect resistance and quality traits needs to explore mutations to create

favorable alleles. Narayanaswamy (1988) treated Pusa Deepali and Early Kunwari seeds with ethyl methanesulfonate (EMS) and identified LD_{50} 0.77% and 0.70% for black rot resistance; however, none of the M_1 plant were found to be resistant. While observing the mutation in seeds taken into space by satellite, there was reportedly significant phenotypical changes in both the size of the plant and the weight of the flower head in cauliflower, whereas no major change was noted in broccoli except for a single plant. However, they did report black rot resistance in cauliflower.

7.7.6 Doubled Haploids

The DH technique is very useful for the development of homozygous and homogenous inbred lines within a short time period. These lines can be used as parents in hybrid breeding. Wijnker et al. (2007) used *reverse breeding* by which they identified superior hybrid genotypes in segregating populations and introduced a gene through genetic transformation for induced suppression of meiotic recombination and developed several DH lines. Further, Dey et al. (2014) suggested that in vitro maintenance of CMS lines of Indian cauliflowers can be used as an alternative method for conventional CMS-based hybrid seed production.

7.8 Breeding for Improved Quality

The extrinsic quality characters in cauliflower are curd color, solidity, free of bracting, pinking and riceyness. The cauliflower curd consists of a mass of short peduncles bearing many thousands of apical meristems. These meristems normally develop to bear flowers, but a large proportion of them (usually over 90%) abort before or occasionally during the floral phase. The curd is, thus, a precociously developed floral button, and its appearance at the marketable stage is affected by the normal ontogeny of flowering and by the death of excess floral material.

The quality of the curd, in terms of cosmetic appeal to consumers is largely determined by the timing of the morphological changes associated with flowering in relation to the curd reaching a marketable size. The distinct features characteristic of poor cosmetic quality are disfiguring defects like elongation of the peduncles, precocious development of apical meristems into flower buds, growth of bracts and leaves from the peduncles through the surface of the curd and development of pink or purple colorations in the curd. The first two of these defects, known respectively as looseness and riceyness, are essential parts of the flowering process, and selection against them is largely a matter of ensuring that they appear as late as possible after the curd has reached a marketable size. Bracting and pinking, do not appear to be essential for flowering and, therefore, selection against them is for their complete elimination.

7.8.1 Curd Appearance

The environment affects the expression of bracting, pinking and riceyness, but these may be completely absent in some seasons while a serious problem in others. Comparison of the morphology of curds when aseptically cultured in nutrient solution, with the effects of their genotype on bracting and pinking as shown by a progeny test, Crisp et al. (1975a, b) found that the formation of bract-like structures in culture was a reflection of the genotypic tendency to do this in the field. Thus, assessment in culture as well as in the field increased the selection pressure against bracting. The results with pinking are even more useful because while the assessments of purple colorations in the field and in culture were phenotypically independent, both were genotypically related to the appearance of the defect in the field, possibly owing to different genes governing the same phenotype. With respect to riceyness, which has been found to have inherent association with endogenous synthesis of auxins, the screening of genotypes can be done at an early stage by growing young cotyledons aseptically on auxin-free nutrient medium where ricey susceptible genotypes will strike roots within a week (Kalia 1994).

This suggests that a two-tier system can be applied to selecting against bracting, pinking and riceyness in cauliflower to produce potential new cultivars with bract-free, ricey-free non-pink curds. In addition, the assessment of bracts in artificial culture media may be carried out for screening of anthocyanin production (Crisp et al. 1975a, b) and for vegetative propagation of selected plants (Crisp and Walkey 1974) which may be necessary in a breeding program. Teakle (2004) reported two MADS-box regulatory genes (*BoAP1-a*, *BoCAL-a*) that are present at loci having key roles in determining the formation of cauliflowers. A number of cDNA for different MADS-box genes have been isolated from cauliflowers, which include both floral promoters and repressors. The expression pattern and genetic map position of these genes will help predict their potential relationship with cauliflower quality i.e. bracting and riceyness.

In cauliflower, major emphasis is given to curd quality because traits like higher yield, disease resistance and wider adaptability become meaningless unless the curd of the variety has good marketability. Hence, the efforts to breed for correction of physiological abnormalities of the curd are fully justifiable. Proper use of nutrients and moisture along with the correct selection of a variety for specific season prevents these abnormalities, but they can be corrected genetically as well. This is because the expression of most of these defects is under genetic control. Loose curd, curd bracting and precocious flower bud formation appear to be due to high apparent genotype × environment interactions. Crisp et al. (1975a, b) estimated and recorded heritability of the appearance of bracts through the surface of the cauliflower curd to be 0.73 ± 0.10 under field conditions.

7.8.2 Dietary Nutrients

Cauliflower curd is a good source of dietary fiber and microelements. The intrinsic quality traits include nutrition-related parameters like vitamins, minerals, protein, carbohydrates, fats and flavor. Inherently, cauliflower is not very rich in nutritional traits, but since the Indian cauliflowers represent a new group with wide variability for horticultural traits, which has been exploited for varietal improvement, the variability with respect to nutritional traits needs to be investigated and exploited in the present scenario of nutritional security. Cauliflower flavor is very delicate which increases its popularity, therefore breeding for flavor will also draw the attention of breeders in future programs.

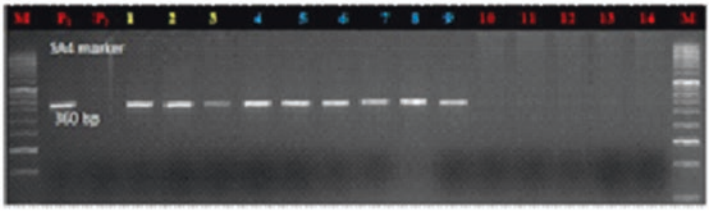
7.8.3 Curd Flavor

Cauliflower contains glucosinolates which on hydrolysis (by myrosinase) gives characteristics volatile flavor products i.e., nitriles and isothiocyanates. Broccoli and Brussels sprout have strong flavor due to glucosinolates. Breeding for glucosinolates in cauliflower has difficulties of poor repeatability of their estimations and varies with individual plant, which may be fixed at an early life stage, perhaps by environmental factors (Sones et al. 1984). Varietal difference in both individual and total glucosinolate content is found in cauliflower (Sones et al. 1984). Hill et al. (1984) estimated appreciable additive heritability of 0.32 for the total glucosinolate content. Breeding for different glucosinolate contents may, therefore, be possible.

7.8.4 Curd Color

Ahluwalia et al. (1977) studied inheritance of various traits including curd color in Indian cauliflowers and gene symbols were assigned for curd color-yellow Y and white- y. The first ever beta-carotene rich variety Pusa KesariVitA-1 in the mid maturity group of Indian cauliflowers using marker-assisted selection was developed by IARI, New Delhi (Fig. 7.3) (Anonymous 2016). This variety has great prospect in programs to mitigate human vitamin A deficiency in tropical regions, particularly India. The commonly-used breeding methods for quality traits are selection, backcrossing, hybridization and hybrid breeding. The successful examples of transfer of quality-enhancing genes in prominent varieties using backcrossing is introgression of the *Or* gene in Indian cauliflower (Kalia et al. 2018; Muthukumar et al. 2017). The β-carotene content in promising lines showing more than 10 μg g^{-1} β-carotene content in the curd portion were identified by Kalia et al. (2018).

Identified molecular markers for foreground selection of Or gene



M- Marker ladder 50bp, P_1 - EC625883 homozygous Or inbred line, P_2 –DC 309 homozygous white, F_1-1-4 Dark orange,, 5-9 BC_2F_1 Dark orange individuals, 10-14 BC_2F_1 white individual. The fragments were seperated on 3.0% metaphor agaraose gel.

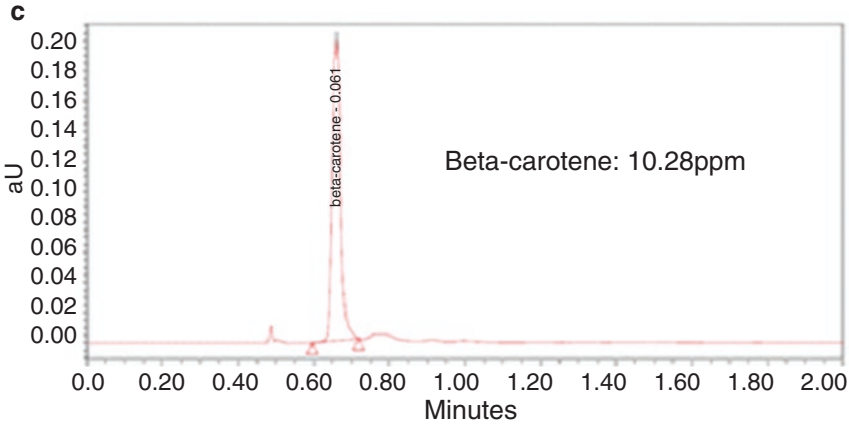


Fig. 7.3 (**a**) Pusa KesariVitA1 harvested marketable cauliflower curds, (**b**) Marker assisted foreground selection of *Or* gene, (**c**) HPLC peak of betacarotene. (Photos are credited to Dr. P. Muthukumar)

7.8.5 Glucosinolates

Natural variability in cauliflower (European) for total glucosinolate content in leaves was reported to be 46–87 µmol/g dry weight (Menard et al. 1999) and 19.5–42.6 mg/100 g fw (Ciska et al. 2000). They also reported wide variation in

individual glucosinolates such as sinigrin (5.7–12.9 μmol/g dw), glucoiberin (0.5–6.6 mg/100 g fw), glucoibervirin (0.6–2.9 mg/100 g fw) and indole (15.2–24.9 mg/100 g fw) which are comparable with total glucosinolate content (0.6–35.6 mg/100 g fw) and glucoraphanin (0.8–21.7 μmol/g dw) and indole (0.4–6.2 μmol/g dw) in green broccoli (Kushad et al. 1999). The genetics of the glucosinolate content in cauliflower is governed by quantitative factors with environmental influence (Hirani et al. 2012). The glucosinolate content also varies considerably among plant ontogenetic stages and plant organs (Van Leur et al. 2006). Variation in cauliflower genotypes for total glucosinolates (19.5–42.6 mg 100 g^{-1} fw) (Verkerk et al. 2009) indicate great scope for its improvement through breeding. Vanlalneihi (2016) analyzed sinigrin in curd and leaf parts of 48 inbred lines of cauliflower comprising early (16), mid-to-early (15) and mid-to-late (17) at IARI, New Delhi. The author reported the highest sinigrin content in curds of DC 41–5 (16.37 μmol 100 g^{-1} fw) and leaves of CC 13 (15.43 μmol 100 g^{-1} fw) in the early group. The highest GCV (57.22%) and PCV (57.25%) were recorded for curd sinigrin. In the mid-maturity group, DC 326 had the highest curd sinigrin (36.93 μmol 100 g^{-1} fw) whereas leaf sinigrin was highest in DC 306 (39.50 μmol 100 g^{-1} fw). Vanlalneihi et al. (2019a, b) analyzed curd and leaf sinigrin which were estimated to be highest for Pant Gobhi 2 and Selection 1-2 with 16.45, 17.56 μmol 100 g^{-1} fw, respectively. This study concluded that the mid-early maturity group genotypes had maximum sinigrin content. Neelavathi et al. (2014) analyzed glucosinolates in 2 cauliflower varieties Pusa Sharad (149.27 μmol/100 g), Pusa Himjyoti (85.44 μmol/100 g) and a hybrid Pusa Hybrid-2 (63.74 μmol/100 g). Pusa Sharad was harvested in November while Pusa Hybrid-2 and Pusa Himjyoti in December were subjected to variation in temperature to affect the glucosinolate content in these varieties but there is no confirmation of evidence to support the role of environment or genotype for this great extent of variation. This kind of information is scare in Indian cauliflowers particularly under Indian growing condition. Therefore, to initiate a breeding program for development of varieties, it is essential to know the variation in glucosinolate content in available germplasm, its genetic control and to develop closely-linked markers.

7.9 Breeding for Climate Resilience

Cauliflower is thermosensitive and temperature plays a key role in curd initiation and development. The ideal temperature for seedling growth is around 23 °C, which can be 10–20 °C at later stages. The seedlings of early Indian cauliflowers grow well even at higher temperature during May–June in north India but need partial shade (50–70%). Cauliflower seedlings cease to grow at temperature slightly above 0 °C. The Indian cauliflowers can grow under high temperature (>35 °C) during vegetative stage but 15–20 °C is favorable for plant growth. The plants remain in a vegetative stage, if temperature remains higher than required for curding in specific cultivars. In contrast, early-group varieties form small size curd *buttons*, if

temperatures remain lower than required for curding. Any fluctuation in temperature at the time of the development stage adversely affects curd quality. Curd disorders such as riceyness, bracting or leafyness occur due to lower and higher temperatures than required for curding, respectively. Hence, Indian cauliflowers form curds in the range of 12–27 °C while Snowball type form curds at 10–16 °C. Based on the temperature requirement for curd initiation and development, cauliflower cultivars are divided into four maturity groups (Table 7.3). These maturity groups are also known by the name of Hindu calendar months for their maturity and arrival in market *viz*., Kunwari (September–October), Kataki (October–November), Agahani (November) and Poosi (December) and/or Maghi (January). Snowball types belong to the late group. Earlier, cauliflower formerly grown only during winter season but now cultivated from May to March and curds are available from the end of August to March under North Indian conditions. Specific cultivars are available with the ability to form curd at a temperature range of 10–27 °C. If the early cultivars are planted late then instead of normal curd, they form small buttons and ricey curds. Similarly, if late types are planted early in the season, they would continue to grow vegetatively-forming curds only when the required temperature range is reached.

7.10 Breeding for Biotic Stress Resistance

7.10.1 Common Diseases and Insect Pests

Downy mildew [*Hyaloperonospora parasitica* Constant (Pers.:Fr) Fr.], black spot (*Alternaria brassicae*, *A. brassicicola* and *A. alternate*), *Sclerotinia* rot [*Sclerotinia sclerotiorum* (Lib) deBarry] and black rot [*Xanthomonas campestris* pv. *campestris* (Pam.) Dowson] and bacterial soft rot (*Erwinia carotovora*) are common diseases infecting cauliflower. Inheritance of downy mildew resistance in cauliflower is governed by a single dominant gene *Ppa3* (Singh et al. 2013) and black rot resistance by a single dominant gene (Saha et al. 2015). Stalk rot or white mould in Snowball cauliflower is polygenically inherited (Thakur 2013).

Diamondback moth (*Plutella xylostella* L.), tobacco caterpillar [*Spodoptera litura* (F.)], cabbage butterflywhite (*Pieris rapae* L.), cabbage head borer [*Hellula undalis* (F.)], Bihar hairy caterpillar (*Spilosoma oblique* Walker, cabbage aphids [*Brevicoryne brassicae* (L.)] and painted bug [*Bagrada hilaris* (Burmeister) (cruciferarum)] are important insect pests of cauliflower. Pesticide residues pose a major health problem; therefore, placing emphasis on host plant resistance is important. Identified resistant sources and an understanding of genetics of resistance for a particular disease and insect pest is essential.

7.10.2 Disease Resistance Breeding

Resistance breeding has resulted in various successful resistant varieties. The development of resistant varieties requires thorough understanding of evolutionary interrelationship of host and pathogen. The success of resistance breeding depends on selection of right genetic sources of resistance, racial composition of pathogen and genetic basis of host-pathogen interaction. It is also essential to have the knowledge and scope of manipulation of host-pathogen interaction. Resistance is a relative term reflecting hereditary capability of the host to reduce the development of pathogen after its infection so that the severity of disease is minimized (Chahal and Ghosal 2002). The strategy for resistance breeding depends on knowledge of gene-for-gene relationship and host-pathogen interaction for efficient deployment of resistance genes in alternate forms. Fehr (1984) categorized three alternate strategies such as: (i) development of cultivars with single major gene against the prevalent pest; (ii) combining genes controlling prevalent and minor races of pests in the form of mixture of different genotypes especially as multiline varieties and (iii) stacking genes controlling prevalent and minor races into a single cultivar i.e., pyramiding of resistance genes. Investigations indicate that a single dominant gene governs the resistance in cauliflower for downy mildew and *Sclerotinia* rot, hence their manipulation is easy. Hybrid breeding, backcross breeding and recurrent selection are common methods employed in cauliflower resistance breeding. In the case of black rot, four races have been reported and deployment of resistance genes for each race in a cultivated variety can be done through gene pyramiding. The steps in resistance breeding are: (i) Collection and maintenance of resistance genes for use in breeding programme. The sources of R gene may be in advance breeding lines, or new genetic stocks developed through pre-breeding, commercial varieties, landraces or primitive cultivars and wild relatives in the form of original progenitors or related species; (ii) Incorporation of one of the resistance gene by incorporation of a resistance parent in hybridization program. This method does not disturb the overall genetic constitution of the recipient commercial variety. The monogenic dominant resistance to downy mildew and black rot can be transferred into cultivated varieties by backcrossing. Further, use of one resistant parent having desirable horticultural traits in hybrid breeding can result in resistant hybrids against these pathogens. The gene pyramiding approach can also be employed to develop varieties having resistance to both the diseases. The breeding efforts in cauliflower are briefly summarized by disease below.

7.10.2.1 Downy Mildew

Downy mildew [*Hyaloperonospora parasitica* (Pers.) Constant. 2002] is a devastating disease of mid-maturity Indian cauliflowers. It is an obligate fungal parasite and systemic in nature. Its infection occurs at seedling stage to seed stage but is most devastating during curd stage (Crute and Gordon 1987). Among Indian

cauliflowers, indigenous genotypes BR-2, CC and 3-5-1-1; and exotic genotypes EC177283, EC191150, EC191157, Kibigiant, Merogiant, EC191140, EC191190, EC191179 and Noveimbrina have been found to be resistant (Mahajan et al. 1991; Singh et al. 1987). MGS2-3, 1-6-1-4,1-6-1-2 and 12C (Chatterjee 1993); KT-9 (Sharma et al. 1991) Early Winter Adam's White Head (Sharma et al. 1995); CC-13, KT-8, xx, 3-5-1-1,CC (Trivedi et al. 2000); Perfection, K1079, K102, 9311 F1 and 9306 F1 (Jensen et al. 1999); Kunwari-7, Kunwari-8, Kunwari-4 and First Early Luxmi (Pandey et al. 2001) are reportedly resistant to moderately-resistant. Pusa Hybrid-2 (Singh et al. 1994), Indian cauliflower, and Pusa snowball K-25, Snowball type, with resistance to downy mildew, were released for commercial cultivation in India. Resistance to downy mildew has been ascribed to a single dominant gene (Jensen et al. 1999; Mahajan et al. 1995; Sharma et al. 1991), single gene with recessive effects (Mahajan et al. 1995) or several genes (Hoser-Krauze et al. 1995). Singh et al. (2013) identified the seven most resistant genotypes: BR-2, CCm, 3-5-1-1, CCm-6, CCm-5, MGS-2-3 and cc-12 among Indian cauliflowers.

7.10.2.2 *Sclerotinia* Rot

The causal organism of this disease is *Sclerotinia sclerotiorum*. This disease has a wide host range infecting most dicot crops, but is more severe in the seed crop of cauliflower, although it may attack the crop at an early growth stage as well. Moderate resistance to this pathogen is reported in EC131592, Janavon, EC103576, Kn-81, Early Winter Adam's White Head, EC162587, EC177283 (Baswana et al. 1991; Kapoor 1986; Sharma et al. 1995, 1997; Singh and Kalda 1995). Resistance is polygenically controled and recessive in nature (Baswana et al. 1993; Sharma et al. 1997). Pusa Snowball K-25 developed by using EC103576 as a resistant source with Pusa Snowball-1 possessing field resistance to *Sclerotinia* rot. Pandey et al. (2003) reported moderately-resistant lines of early cauliflower to *Sclerotinia* rot, namely Kataki-6, Kataki-13, Patna Kataki, Deep Malika, Suryamukhi, Pusa Himkaran, Early Laxmi and PDVR early. However, Kataki-13 and Kataki-6 showed a high degree of tolerance. Saxena et al. (2009) reported the polygenic nature of *Sclerotinia* resistance and identified two RAPD markers D-3450 (5' GGACCCAACC 3') and C-20350 (5' ACTTCGCCAC 3') flanking the stalk rot resistance gene at a distance of 2.7 cM and 4.2 cM, respectively, in the resistant genotype Olympus.

7.10.2.3 Black Rot

Xanthomonas campestris (Pam) Dawson bacterium is the causal organism of this disease. Symptoms begin as yellowing of leaves from leaf margin and extending in the direction of the midrib, followed by blackening of veins (vascular bundles). Cauliflower lines reported as resistant sources are Sn 445, Pua kea and MGS2-3 (Sharma et al. 1972); RBS-1, EC162587 and Lawyana (Sharma et al. 1995); Sel-12 (Gill et al. 1983); Sel-6-1-2-1 and Sel-1-6-1-4 (Chatterjee 1993) and Avans and

Igloory (Dua et al. 1978). Some of the above sources have been used in the development of resistant varieties. Pusa Shubhra was developed, using Pua Kea and MGS2-3 lines and recommended for commercial cultivation (Singh et al. 1993). Pusa Snowball K-1 was also reported to be field resistant to black rot (Gill et al. 1983). The resistance was dominant and governed by polygenes and the dominance components of variation were more pronounced than additive (Sharma et al. 1972). However, Jamwal and Sharma (1986) reported that a single gene governs dominant resistance. Of the 54 accessions wound-inoculated with 4 isolates of Xcc race 4 at the juvenile stage, A 19182 and A 19183 exhibited no symptoms, and the accessions including PI 199947, PI 199949 and PI 194256 segregated for resistance to Xcc race 4 (Tunguc and Griffiths 2004a, b, c). Tonguc et al. (2003) analyzed 3 segregating F_2 populations for black rot resistance along with 8 polymorphic RAPD markers. Segregation of markers with black rot resistance indicates that a single, dominant major gene controls black rot resistance in these plants. Stability of this black rot resistance gene in populations derived from 11B-1-12 may complicate introgression into *Brassica oleracea* genotypes for hybrid production. Recently, Saha et al. (2015) identified new resistance sources for black rot pathogen Xcc race 1 in Indian cauliflower namely BR-207, BR-1, BR-202-2 and AL-15.

7.10.2.4 Black Leaf Spot

In cole vegetables, the black leaf spot disease is caused by *Alternaria brassicae* or *A. brassicicola*. Brown to black, small to elongated spots appear on leaves and stem. In younger plants, it may cause symptoms like that of *Rhizoctonia solani*. When the fungus infects the curd, especially in the case of seed crop, the disease is referred to as inflorescence blight. Resistance was found in Indian cauliflower lines, MGS2-3, Pua Kea and 246-4 (Sharma et al. 1972), 23-7, 466, MS98, 210-21, Sel-9, 443-7 (Trivedi et al. 2000) and Snowball KT-9 (Sharma et al. 1991). Resistance to curd blight is dominant in nature, polygenically inherited, and in general additive effects were found more pronounced than dominant ones (Sharma et al. 1975). Pusa Shubhra having resistance to curd blight has been released for commercial cultivation (Singh et al. 1993). Both additive and dominant gene action played a role in resistance but partial dominance is more important (King and Dickson 1994).

7.10.3 Breeding for Insect Pest Resistance

Dickson et al. (1986) identified a glossy-leaved cauliflower which exhibited high resistance to diamondback moth. Resistance to cabbage head borer (*Hellula undalis* L. *fabricius*) is reported in cauliflower genotypes ES-97, ES-96, Katiki (J.B), KW-5, KW-8, KW-10, Kunwari (RB), Kathmandu Local, Early Patna, EMS-30 and PSK-16 (Lal et al. 1991). Lal et al. (1994) also found resistance under field conditions in Indian cauliflower F_1 hybrids like aa X ES102, aa × Kataki (JB), aa × First Early, aa

× First Crop, aa × Sel.100, aa × Sel.41 and aa × 824 to Bihar hairy caterpillar (S*pilosoma oblique*). Aphids cause major losses to cole crops. The aphid species responsible for economic losses in cauliflower and other cole crops are cabbage aphid (*Brevicoryne brassicae*), green peach aphid (*Myzus persicae*) and turnip aphid (*Lipaphis erysimi*). Resistance to cabbage aphid is reported in NY 13816, NY101181, NYIr 9602 and NYIR 9605, but work on cauliflower is very scanty. Naturally occurring compounds like glucosinolates, pipecolic acid and β-nitroprionic acid in the tissue of *Brassica* plants are responsible for resistance to cabbage looper and the imported cabbageworm. Breeding resistant varieties in cauliflower and other cole crops for most insect pests and some diseases remains elusive because hardly any germplasm source with a desirable degree of resistance is available.

7.11 Molecular Markers

Marker-assisted selection (MAS) is an indirect process where selection is based on a marker instead of the trait itself. The successful application of MAS relies on the tight association between the marker and the major gene or QTL responsible for the trait (Singh and Singh 2015). They ensure a reasonable likelihood that the genotype combining favorable alleles is present in the population (Ishii and Yonezawa 2007). Kalia et al. (2017) identified two closely linked (1.6 cM) markers (RAPD-OPO-04833 and ISSR-11635) to black rot resistance locus (*Xca1bo)* and converted them into sequence characterized amplified region (SCAR) markers. These two SCAR markers, ScOPO-04833 and ScPKPS-11635, were linked with black rot resistance locus *Xca1bo*. Singh et al. (2012) mapped the RAPD and ISSR markers with downy mildew resistance gene (*Ppa3*) in cauliflower. They also investigated 20 polymorphic primers in bulked segregant analysis and identified seven as putatively linked markers between resistant and susceptible bulks generated from F_2 population developed for downy mildew in Indian cauliflowers (Singh et al. 2015). However, only three (OPC141186, OPE141881, and ISSR231103) of distinguished resistant and susceptible individuals of respective bulbs, hence used for genotyping of mapping population and development of linkage map with *PPa3* gene.

To find markers, the SNPs discovery was performed by genotyping by sequencing (GBS) in cauliflower and broccoli by Stansell et al. (2018) while investigating phylogenetic patterns, population structure and domestication footprints, with and without reliance upon a reference genome, and produced 141,317 and 20,815 filtered SNPs, respectively. Further, Sun et al. (2019) sequenced and assembled cauliflower genome (584.60 Mb) with a contig N50 of 2.11 Mb, and contained 47,772 genes. According to them, chromosome number 03 of cauliflower shared the most syntenic blocks with the A, B (*Brassica* species) and C (*B. oleracea*) genomes indicating that this is the most ancient one in the cauliflower genome.

7.11.1 Marker-Assisted Improvement of Quantitative Traits

Early inheritance studies focused on morphological traits, although complex inheritance was often observed, suggesting that many genes controlled the traits. Lan and Paterson (2000) attempted to resolve these complex inheritance patterns i.e., a series of QTLs associated with cauliflower curd traits using 3 F_2 populations. Segregating populations of F_1 derived doubled haploid (DH) lines from divergent parents had been used to for construction of linkage map of the *Brassica oleracea* genome and identification of QTLs controlling developmental characteristic (Sebastian et al. 1991, 2002). Genetic linkage maps of *B. oleracea* created at the then HRI, Wellesbourne (UK), comprise over 600 molecular markers (King 2004).

7.11.2 Genomic Selection

Genomic selection is a recent approach that also relies on marker-assisted selection. It enables the simultaneous selection for tens or hundreds of thousands of markers, which cover the entire genome. It is thought to provide the key in maximizing the full potential of MAS, especially for breeding complex traits. Genomic selection requires the availability of phenotypic and genotypic data for the reference population. The objective of genomic selection is to predict the breeding values of each individual instead of identifying QTLs for use in a traditional marker-assisted selection. GWS makes use of genomic estimated breeding values (GEBVs) as a selection parameter, rather than the estimated breeding values.

In cauliflower, Thorwarth et al. (2017) found genomic selection effective in predicting the QTLs for improving curd-related traits.

7.11.3 Association Studies

Association mapping uses natural genome-wide distribution of various genes together with other detectable loci/markers in predicting the marker-trait associations (Singh and Singh 2015). However, such studies were not explored in Indian cauliflowers, although Thorwarth et al. (2017) performed genome-wide association studies (GWAS) for genomic prediction to improve curd-related traits in cauliflower and identified a total of 24 significant associations for curd-related traits with prediction abilities ranged from 0.10 to 0.66 for different traits and did not differ between prediction methods. Matschegewski et al. (2015) also performed GWAS for genetic dissection of temperature-dependent curd induction in cauliflower using a panel of 111 cauliflower commercial parent lines and identified 18 QTLs for curding time localized on 7 different chromosomes. They also done transcriptional profiling of flowering genes *FLOWERING LOCUS C* (*BoFLC*) and *VERNALIZATION*

2 (*BoVRN2*) and observed the increased expression levels of *BoVRN2* in genotypes with faster curding. Rosan et al. (2018) tried QTL and GS models to predict time to curd induction, and one of them generated slightly better results (R^2 = 0.52–0.61).

7.12 Transgenesis and Gene Editing

The transgenic approach was performed by Lu et al. (2006) in cauliflower and transformed plants with *Or* transgenesis associated with a cellular process that triggers differentiation of proplastids or other non-colored plastids into chromoplasts for carotenoids accumulation. A successful protocol for genetic transformation of cauliflower employing the process of agroinfection was proposed by Kowalczyk et al. (2018) with variety Pioneer transformation via *Rhizobium rhizogenes* (ATCC 18534, A4) with higher (72%) transformation efficiency GUS assay (55%). In the absence of availability of resistance in the cauliflower germplasm, especially for diamondback moth, genetic engineering offers the same and a lasting solution. Kalia et al. (2020) investigated insecticidal efficacy of *Cry1B/Cry1C* genes in transgenic cauliflower, assessed by feeding neonates of diamondback moth on detached leaves. From a large number of transformed lines analyzed, it is obvious that the *Cry1B/Cry1C* genes potentially exhibited insecticidal activity. During this, they developed a regeneration protocol for Indian cauliflower variety Pusa Meghna (Fig. 7.4). Chakrabarty et al. (2002) evaluated a number of factors that influence genetic transformation to optimize *Agrobacterium*-mediated transformation of hypocotyl explants of cauliflower variety Pusa Snowball K-1. They mobilized synthetic *cryIA(b)* gene into cauliflower and observed effectiveness of the transgene against infestation by diamondback moth (*Plutella xylostella*) larvae. Chikkala et al. (2013) reported production of transgenic cauliflower with plastid division gene *BoMinD* by using PEG mediated transformation of mesophyll protoplasts which has had abnormally-shaped chloroplasts but devoid of true macrochloroplast or minichloroplast phenotype. Ding et al. (1998) performed *Agrobacterium*-mediated transformation of cauliflower with a trypsin inhibitor gene TI gene from a sweet potato cultivar and reported in planta resistance to local insects to which the control plants were vulnerable. Chen et al. (2008) cloned three putative chlorophyllase genes; of them, only *BoCLH1* transcribed during postharvest senescence and antisense *BoCLH1* transcripts showed positive correlations with slower postharvest yellowing. Russell et al. (2017) reviewed progress on deployment of pyramided *Bt* genes *cry1B* and *cry1C* for the control of *Plutella xylostella*, *Crocidolomia pavonana*, *Hellula undalis* and *Pieris* spp. in cauliflower. Recently, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) were reported as an efficient and recent tool for genome editing (Cong et al. 2013). It consists of a nuclease (Cas9) and two short single-strand RNAs (crRNA and tracrRNA) which are fused to form single-guide RNA (sgRNA), for genome editing. Cas9 and a gRNA form a ribonucleoprotein complex and bind to genomic DNA. In cole vegetables, Jansson (2018) was the first to describe the gene editing

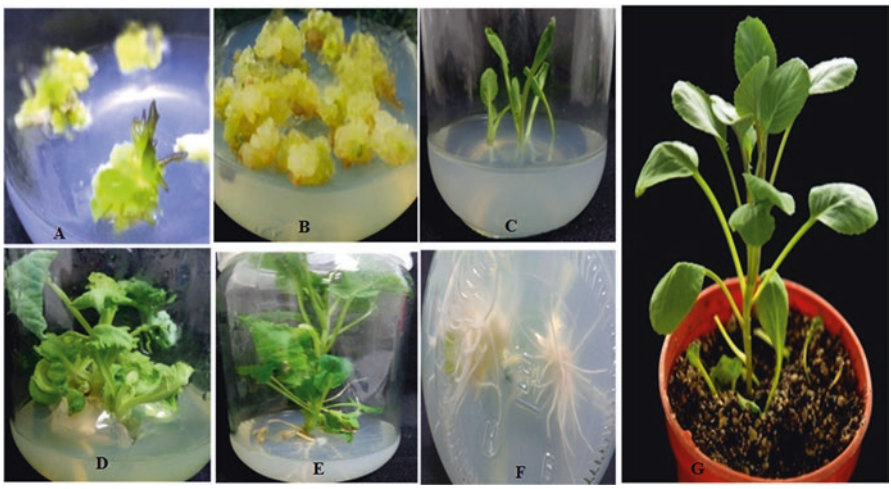


Fig. 7.4 Standardization of protocol for tissue culture of Pusa Meghna cauliflower. (**a–b**) Callusing and shoot emergence, (**c**) Shooting, (**d–e**) Shoot growth, (**f**) Rooting, (**g**) Ex vitro plant. (Photos are credited to late Ms. P. Choudhary)

using CRISPR-Cas9 (a *Brassica* deletion mutant) in cabbage as model plant and PsbS as target gene.

7.13 Conclusion and Prospects

Cauliflower originated in Cyprus and around the Mediterranean coast and evolved into different phylogenic groups in the European region; however the highest share (75%) in current global production is from China and India. This could have happened due to development of tropical types that expanded the growing regions and seasons. In India, cauliflower has four maturity groups which expanded the harvest season from September to March. Cauliflower has diverse germplasm due to diverse groups worldwide but the use of exotic brassicas is also possible. The wild/related species have effectively been exploited to transfer different types of cytoplasmic male sterility systems in cauliflower. Similar attempts are under way for introgression of resistance to diseases such as black rot using *Brassica caritana* and *B. juncea*. The use of novel tools and techniques such as molecular breeding, genomics, association mapping, genomic selection, TILLING, transgenic and CRISPR/Cas9 have great prospect in cauliflower breeding for handling complex traits for yield, stress tolerance and climate change.

Appendices

Appendix I: Research Institutes Relevant to Cauliflower

Institution name	Specialization and research activities	Address	Website
ICAR- Indian Agricultural Research Institute	Tropical cauliflower improvement	ICAR – Indian Agricultural Research Institute, New Delhi-110,012, India	https://www.iari.res.in
ICAR- Indian Agricultural Research Institute Regional Station	Snowball cauliflower improvement	Head, ICAR – Indian Agricultural Research Institute Regional Station, Katrain- 175,129, India	https://www.iari.res.in
ICAR-Indian Institute of Vegetable Research (IIVR)	Improvement and production technology development for cauliflower for eastern region of India	ICAR – Indian Institute of Vegetable Research Post Bag No. 01; P. O. Jakhini (Shahanshapur) Varanasi – 221 305, Uttar Pradesh, India	https://www.iivr.org.in
Himachal Pradesh Krishi Vishwa Vidyalaya	Improvement and production technology development for cauliflower for hill region of India	Himachal Pradesh Krishi Vishwa Vidyalaya Palampur – 176062 (HP), India	http://www.hillagric.ac.in
Dr. Yashwant Singh Parmar University of Horticulture and Forestry	Improvement and production technology development for cauliflower for hill region of India	Dr. Yashwant Singh Parmar University of Horticulture and Forestry Nauni, Himachal Pradesh 173230, India	http://www.yspuniversity.ac.in/
Bihar Agricultural University	Improvement and production technology development for cauliflower for Bihar region, India	Bihar Agricultural University Bhagalpur Rd, Sabour, Bihar 813210, India	http://www.bausabour.ac.in/
ICAR- National Institute of Plant Biotechnology	Research on biotechnology aspects such as Introgression of novel traits in *Brassica oleracea*	ICAR – National Research Centre for Plant Biotechnology, New Delhi-110012, India	http://www.nrcpb.res.in/
Govind Ballabh Pant *University* of *Agriculture* and Technology	Genetic improvement and production technology of cauliflower for lower hill and Trai region	Govind Ballabh Pant *University* of *Agriculture* and Technology*, Pantnagar* – 263145, India	http://www.gbpuat.ac.in/

Appendix II: Genetic Resources of Cauliflower

Cultivar	Important traits	Cultivation location
Pusa Meghna	Early maturity group, heat and humidity tolerant, plants are dwarf, bluish green leaves, maturity end of September to first week of October, curd size 380–450 g, yield 12 mt/ha	India
Sabour Agrim	Early maturity group, heat and humidity tolerant, plants are dwarf, compact white curds, curd weight of 440–500 g, curd yield 13 mt/ha	India
Kashi Kunwari	Early group variety with cream white curds of 400 g and curd yield around 16 mt/ha.	India
Pusa Ashwini	Early maturity group, heat and humidity tolerant, plants are medium vigorous, bluish green leaves, maturity in second fortnight of October, curd size 500–650 g, yield 16–18 mt/ha	India
Pusa Kartiki	Early maturity group, heat and humidity tolerant, plants are medium vigorous, bluish green leaves, curd size 500–650 g, yield 20–22 mt/ha	India
Pusa Kartik Sankar	Early maturity group, heat and humidity tolerant, plants are medium vigorous, bluish green leaves, maturity in mid October, curd size 500–650 g, yield 18 mt/ha, self-incompatibility based hybrid	India
Pusa Deepali	Early maturity group, heat and humidity tolerant, plants are medium vigorous, bluish green leaves, partially self-blanched, maturity at end of October, curd size 500–550 g, yield 14 mt/ha	India
Pusa Sharad	Mid-early maturity group, plants are medium vigorous, bluish green leaves, maturity in mid October, curd size 700–800 g, yield 24 mt/ha, self-incompatibility based hybrid	North India
Pant Gobhi 4	Mid group, creamy white compact curds, yield is around 12 mt/ha	Lower hills in North India
Kashi Aghani	White, compact curds with average yield of 22 mt/ha	India
Pusa Hybrid-2	Mid maturity group, plants are medium vigorous, bluish green leaves, maturity in mid October, curd size 750–850 g, yield 23–25 mt/ha, self-incompatibility based hybrid	North India
Pusa Paushja	Mid-late maturity group, curds are white, compact, 800–950 g, plants are bluish green and medium vigorous, average yield 32 mt/ha	North India
Pusa Shukti	Mid-late maturity group, curds are white, compact, 850–950 g, plants are green and vigorous, semi-erect, average yield 31 mt/ha	North India
Palam Uphar	Mid-late maturity group, curds are white, compact, 800–1000 g curds, curd yield 28 mt/ha	Lower hills
Pusa Snowball-1	Late maturity group, leaves upright, self-blanched, curds white, compact, curd weight ranges from 900 to 950 g, curd yield is 28 mt/ha	Hills and plains of India

(continued)

Cultivar	Important traits	Cultivation location
Pusa Snowball K-1	Late maturity group, leaves upright, self-blanched, white compact curd with average weight of 950–1000 g, curd yield is 30 mt/ha	Hills and plains of India
Pusa Snowball KT-25	Late maturity group, leaves upright, self-blanched, white, compact, curd yield is around 34 mt/ha	Hills and plains of India
Pusa Snowball Hybrid-1	Late maturity group, leaves upright, self-blanched, white compact, average yield is 35 mt/ha	Hills and plains of India

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Chapter 8
Globe Artichoke (*Cynara cardunculus* var. *scolymus* L.) Breeding

Fernando López-Anido and Eugenia Martin

Abstract Globe artichoke (*Cynara cardunculus* var. *scolymus* L.; Asteraceae) is a diploid (2n = 2× = 34), perennial, mostly cross-pollinated species native to the Mediterranean Basin. It represents an important component of the agricultural economy of southern Europe, and is grown for its large immature inflorescences, called capitula or heads. Artichokes have recognized nutraceutical properties for human health. Its commercial production is based mainly on perennial vegetatively-propagated clones. Recently its cultivation has been shifted toward seed-propagation of hybrids. Italy holds the richest biodiversity of cultivated *Cynara*, which has resulted in the culture of varieties and landraces adapted to specific local climatic conditions and markets. Cultivar-groups comprise early and late types, but also spiny, violet, Romanesco and Catanese types. Traditionally selections have been made within a given clone, removing off-types. Due to its heterozygous nature, a great variability is seen after crossing or selfings, promoting the selection of new cloned varieties. Seed-propagated hybrids are feasible upon the use of genic male sterility. In the past 20 years new technologies have been applied to broaden the knowledge of the molecular basis inherent, from the first genetic linkage map, the identifications of QTL for yield and related traits, up to the recent whole-genome sequence.

Keywords Capitula · Breeding · *Cynara* · Clones · Hybrids · Mediterranean

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8.1 Introduction

Cynara cardunculus L. is a diploid (2n = 2× = 34), perennial, mostly cross-pollinated species that belongs to the Asteraceae family, native to the Mediterranean Basin. The wild taxon [var. *sylvestris* (Lamk) Fiori] is recognized as the ancestor of globe artichoke [var. *scolymus* (L.) Fiori, ssp. *scolymus* (L.) Hegi] and the leafy cultivated cardoon (var. *altilis* DC) (Rottenberg and Zohary 1996). Globe artichoke represents an important component of the agricultural economy of southern Europe, and it is grown for its large immature inflorescences, called capitula or head (Bianco 1990). Fresh artichokes commonly are steamed or boiled, after which the fleshy bases of the outer bracts, the inner bracts, the receptacle and portion of the floral stem may be eaten (Fig. 8.1). Its commercial production is based mainly on perennial cultivation of vegetatively-propagated clones. However, in the last 20 years there has been a steady increase in the availability of new seed-propagated cultivars and hybrids, mostly from private seed companies. This has changed the situation, turning artichoke into an annual crop. Artichokes also have nonfood uses as their leaves are a source of antioxidant compounds, such as luteolin and dicaffeoylquinic acids (cynarin) (Di Venere et al. 2005; Gebhardt 1997) and the roots contain inulin, an oligosaccharide known to have a positive effect on human intestinal flora, thus on health (Raccuia and Melilli 2004). The cultivated cardoon is a minor seed-propagated crop, grown for its fleshy stems and leaf stalks, with some regional importance in Italy, Spain and southern France (Dellacecca 1990). For the past 20 years, the potential use of the species as an energy crop through its biomass production has been emphasized (Cravero et al. 2012; Foti et al. 1999; Gominho et al. 2018; Raccuia and Melilli 2010).

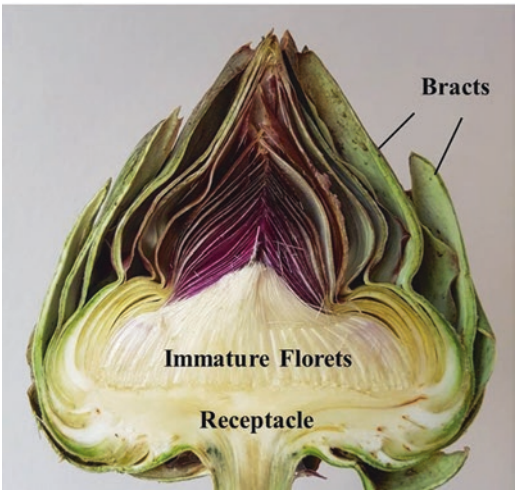

Fig. 8.1 Longitudinal section at the harvest stage of globe artichoke. (Source: Trizek 2018)

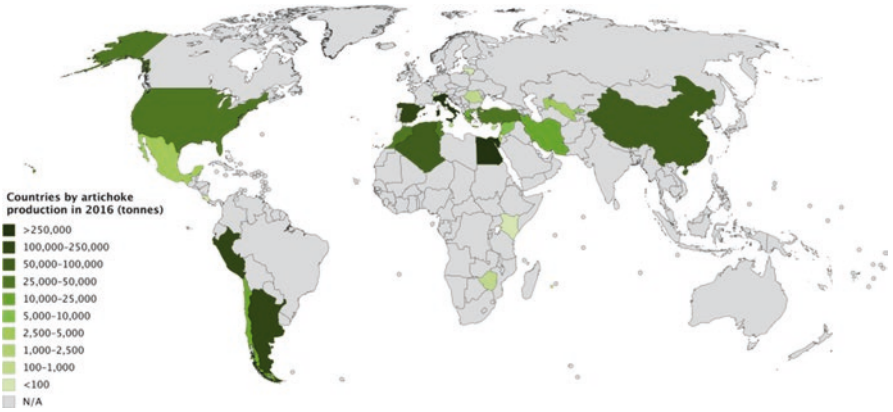

Fig. 8.2 Countries by globe artichoke production in 2016. (Source: JackintheBox 2018)

8.1.1 Economic Importance

Traditionally globe artichoke is cultivated in the Mediterranean countries, which account for almost 80% of the world's globe artichoke growing areas, where Italy and, in recent years, Egypt are major producers (Pesce and Mauromicale 2019). Other countries with a significant production outside the Mediterranean Basin are Peru, China and Argentina (Fig. 8.2). Spain is the most important exporter either as fresh or processed, accounting for about 60% of the traded market (Calabrese 2019).

8.1.2 Germplasm and Domestication

Eight species make up the genus *Cynara* by Wiklund (1992): *C. cardunculus* L., *C. syriaca* Boiss., *C. auranitica* Post, *C. cornigera* Lindley, *C. algarbiensis* Cosson, *C. baetica* (Spreng.) Pau, *C. Cyrenaica* Maire et Weiller and *C. humilis* L. This author placed *C. tournefortii* Boiss. et Reuter in another genus, but it was later included by Robba et al. (2005). More recently, natural spontaneous hybrid plants between *C. tournefortii* and *C. cardunculus* have been described in a sympatric wild population of both species (Blanca and Sánchez-Carrión 2014), supporting this inclusion. All wild species are perennial and the genus is characterized by large spiny leaves and heads. *Cynara algarbiensis*, *C. baetica*, *C. humilis* and *C. tournefortii* are principally of Western Mediterranean distribution, while *C. cornigera*, *C. Cyrenaica* and *C. syriaca* are distributed in the eastern part of the Mediterranean. *Cynara cardunculus* (hereafter wild cardoon), is present in almost all the Mediterranean, and reported to contain two wild subspecies, namely ssp. *cardunculus* and ssp. *flavescens* Wiklund; differing in their bract characters and geographical distribution: the former is distributed from Cyprus to Greece, Central and Southern

Italy, Sicily and Sardinia, and the latter is dispersed in Iberia and the Macaronesian Region (Wiklund 1992).

The domestication of these crops (globe artichoke and cultivated cardoon) is not yet fully understood, and when and where it occurred is still unknown. In a first hypothesis it was believed that both crops resulted from human selection pressure for either large, non-spiny heads (globe artichoke) or non-spiny, large stalked tender leaves (cultivated cardoon) in a single domestication event (Basnizki and Zohary 1994). Applying a molecular clock model, the divergence of cultivated cardoon dates to the beginning of the second millennium AD. Sonnante et al. (2002, 2004) using other molecular markers, such as random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP), indicated that wild cardoon germplasm appears genetically closer to leafy cardoon than to artichoke. The similarity of wild cardoon to cultivated leafy cardoon is also indicated by a number of nucleotide substitutions that are synapomorphic to these two taxa, especially in the ITS regions (Sonnante et al. 2007). In a parsimony consensus tree the wild cardoon accessions were placed closer to the domesticated cardoon than to the globe artichoke (Fig. 8.3). This is in agreement with the hypothesis that a second round of domestication took place in the northern/western range of the Mediterranean, leading to a seed-propagated crop, mostly utilized for its leaves (cultivated cardoon). The lower number of varieties present in cultivated cardoon (Dellacecca 1990) as compared to artichoke (Bianco 1990) is a further indication of a more recent domestication.

Hybridization experiments have demonstrated that wild cardoon and both cultivated species are genetically cohesive since they are completely interfertile and, therefore, they belong to the same gene pool (Basnizki and Zohary 1994; Rottenberg and Zohary 1996, 2005). Other wild *Cynara* species, in particular *C. algarbiensis* and *C. syriaca*, exhibit only limited capacity to set seeds and produce viable hybrids when crossed to the cultigen, while the remaining wild allies showed almost complete genetic isolation (Rottenberg and Zohary 1996).

8.2 Current Cultivation and Reproduction

8.2.1 Growth Habit and Inflorescence Development

The artichoke plant is a typical rosette, with leaf morphology varying in color and shape, depending on cultivar and position in the plant. Roots can penetrate up to 120 cm into the soil, and leaf number, thus size of the rosette, will be determined by the length of the vegetative stage. Floral induction requires a minimum accumulation of low temperatures coupled with long-day photoperiod (Basnizki 1985). The induction requirements will vary with the cultivar. During stem elongation, leaves become progressively narrower and shorter. The stem arises from the center of the rosette, whereas cultivation as perennial may produce several rosettes. The main

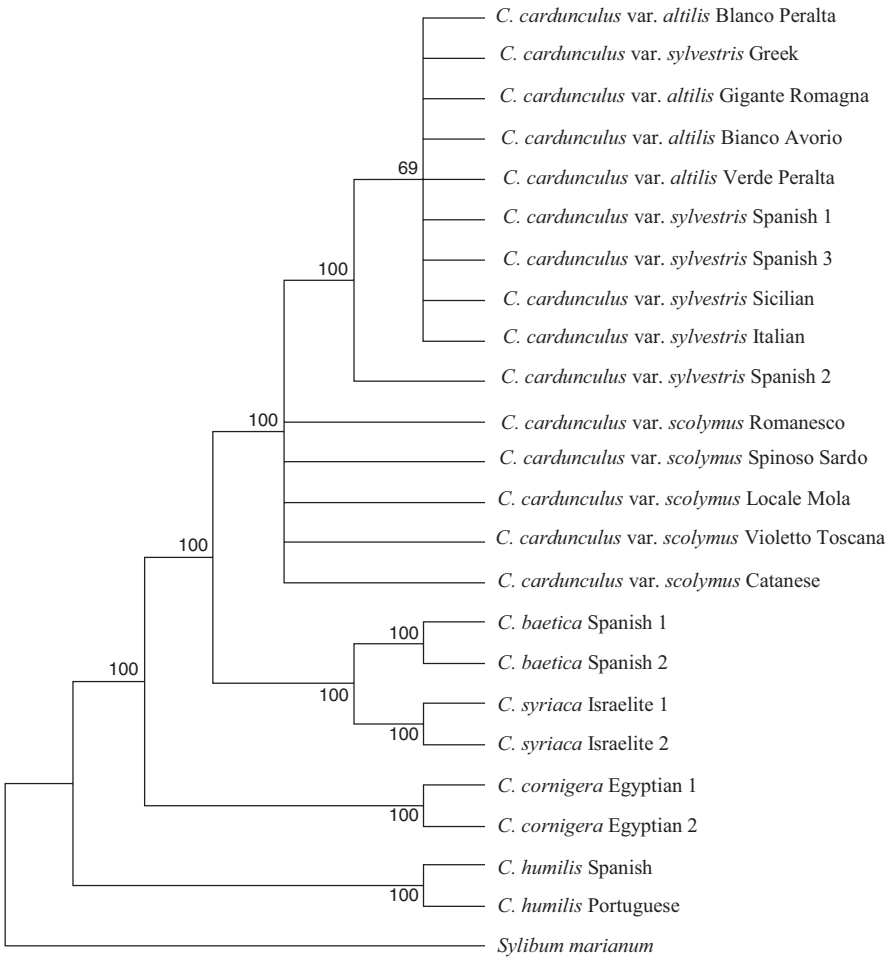

Fig. 8.3 ITS (internal transcribed spacers) parsimony consensus tree showing phylogenetic relationships among cultivated and wild *Cynara* accessions. (Source: Sonnante et al. 2007)

stem bears the main capitulum and ramifications terminated by capitula. The main or central capitulum is always the most voluminous, other capitula become smaller along the ramifications. Under dry summer of the Mediterranean environment, the aerial plant will desiccate after harvest is finished, and the underground stem will remain dormant until rain or irrigation is provided. Under temperate climate, growth from axillary non-induced buds will continue through the summer (Basnizki 1985).

8.2.2 Vegetative Propagation

Three methods of division are customarily practiced in vegetative propagation, which are related to different countries and climates. After the summer drought in the Mediterranean area, axillary dormant or semi-dormant buds borne on short swollen protuberances, with a limited root system, partially attached to the stump of the previous season plant, are separated and are referred as *ovoli*. The use of ovoli to establish new plantations of clonal varieties is widespread in Italy (Ryder et al. 1983). In Spain, it is preferred to divide the stump in pieces called *zuecas*, which contain dormant buds and attached roots. In temperate climates such as France and Argentina, axillary vegetative shoots that emerge from the base of the stump, with some root development, are called *suckers* or *hijuelos*, which are detached and used as propagules.

8.2.3 Floral Biology

The floral biology of globe artichoke has been studied in detail by Foury (1967). It is a predominately a cross-pollinated plant. Cross-fertilization is promoted by protandry. Selfing can be simply accomplished. On most cultivars a first order capitulum contains 800–1400 florets. Anthesis begins with the peripheral florets and along the subsequent 2–3 days progresses centripetally. In each floret the anthers mature first and the unreceptive style pushes the pollen upward. At this time, if crosses or selfings are pursued, pollen collections should be conducted daily between midmornings until noon, in previously bagged capitula. The two stigmatic surfaces of the florets mature 2–3 days after the last central florets finish shedding pollen. At this point, previously collected pollen should be applied using a paint brush in order to obtain selfed or crossed seeds, always maintaining the receptive capitula bagged so as to avoid pollen contamination. Pollen brushing can be repeated for 2 days in order to assure seed set. Pollen remains viable at 2–4 °C for up to 10 days (Foury 1967).

8.2.4 Seed Cultivation

Research aimed at the possibility of obtaining seed-planted materials of globe artichoke has been conducted over the past 50 years (Foury 1979; Pécaut et al. 1981; Porchard et al. 1969). The shift towards seed reproduction was highlighted in order to prevent the spread of soil-borne pathogens and viruses, commonly propagated with the stem pieces and buds; improve soil exploration by long vertical taproots secured by seed-derived plants; convert the crop into an annual and facilitate rotations and develop seed and nursery industry. Due to the high heterozygosity of the

clonal varieties, a great array of segregation appears after selfings, which hinders the attainment of seed-propagated varieties with similar head characteristics and precocity, to the well-adapted vegetatively-cloned varieties (Basnizki and Zohary 1994). Also inbreeding depression for plant vigor and pollen production counteracts the advancement of inbred lines beyond three or four generations of selfings (Foury 1979). However, in crosses, the recovery of hybrid vigor for yield attributes (Pécaut and Foury 1992), and the detection of genic male sterility (Basnizki and Zohary 1998; Principe 1984) has encouraged, in recent years, seed companies to create hybrid cultivars (Big Heart Seed Co 2019; Nunhems 2019).

8.3 Cultivated Gene Pool

Italy holds the richest biodiversity of cultivated *Cynara*, which has resulted in the local culture of many types of varieties and landraces, very often well adapted to specific local climatic conditions and markets (Dellacecca et al. 1976; Elia and Miccolis 1996; Pagnotta and Noorani 2014). This stability of phenotypes make clonal systems attractive for domestication, as any novelty could be fixed by the asexual reproduction. In grapes (*Vitis vinifera*), a species also with a great array of adapted clones, it has been demonstrated that along the domestication history, new variants accrued mostly hemizygously and are locked-in by clonal propagation (Allaby 2019).

8.3.1 Cultivar Groups

Many studies have been conducted to classify the great number of artichoke landraces or clones available (Dellacecca et al. 1976; Elia and Miccolis 1996; Lanteri et al. 2004; Pagnotta et al. 2017). A first criterion is the harvesting time or cycle, which divides the cultivars into early or late types. The early types are harvested in autumn and spring, and are considered remontant or re-blooming, that means that they have two flowering periods, one in autumn and the second in spring. Typical of this group are Violet de Provence, Catenese, Violet d'Algerie, Tudela, Spinoso Sardo and Sakiz. The late types produce only in spring, Romanesco, Green Globe, Camus de Bretagne, Violetto di Toscana, among others, belong to this group (Dellacecca et al. 1976; Lanteri et al. 2004).

The second criterion classifies cultivars on the basis of the morphology of the capitula. In particular, the characteristics taken into consideration are: shape, color and the presence of spines (Fig. 8.4). It is therefore possible to identify four groups: Spiny with long sharp spines on both bracts and leaves, Violet with violet-colored capitula, Romanesco with spherical or sub-spherical non-spiny capitula and Catanese with relatively small, elongated non-spiny capitula. The Spiny and Catanese types are normally of the re-blooming typology, while Violet and

Fig. 8.4 Different morphology of capitula. (**a**) Spherical green, (**b**) Spherical plain violet, (**c**) Spiny elongated, (**d**) Sub-spherical variegated. (Photos **a**, **b** and **d** by F. López-Anido; Photo **c** Source: Radiuk 2013)

Romanesco varieties are usually harvested in spring (Pagnotta et al. 2017). Figure 8.5 presents the results from a cluster analysis conducted in order to classify accessions by phylogenetic groups based on diversity (Lanteri et al. 2004).

8.3.2 Variation in Early Mediterranean Cultivars

Natural occurring variation in some early cultivars was first reported by Porchard et al. (1969). It was considered chimeric, which produces the so-called Bull and Pastel variants. In cv. Violet de Provence, of normal elongated capitula, plants derived from the first mutation, have almost spherical heads and less pinnatifid leaves. On the contrary, Pastel plants possess noticeable pinnatifid leaves and are late bolting (spring). These variations were later described as emerging after in vitro multiplication of early cultivars, where the Bull type resulted from the tetraploid nature. The sexual progeny failed to maintain entire leaves, and resulted in all of the Pastel type (Pecaut and Martin 1993). Recently, Cerruti et al. (2019) identified different methylation patterns between true-to-type and off-type leaves of plants from Spinoso Sardo, another early Mediterranean cultivar, and suggested an epigenetic control related to differences in precocity and vegetative development. It is

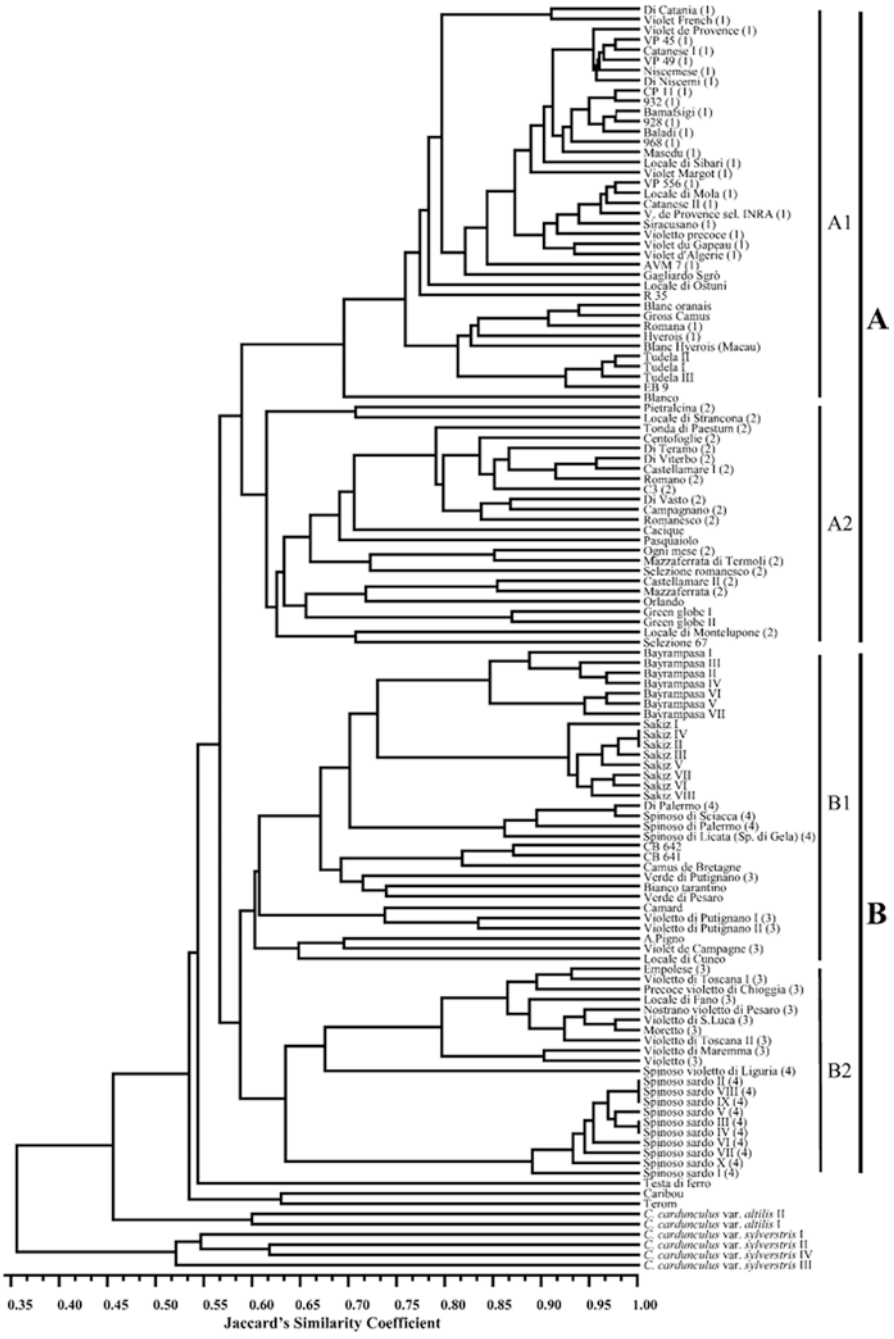

Fig. 8.5 Dendrogram obtained from UPGMA cluster analysis of AFLP data. Subcluster (**a1**) Catanesi type, (**a2**) Romanesco type, (**b1**) Violet type and (**b2**) Spiny. (Source: Lanteri et al. (2004))

interesting from the domestication point of view how this early variant emerged in different areas and as types of cultivated capitula (green, violet, spiny). In grapes, concurrent selections have also been evidence of a domestication syndrome trait (Zhou et al. 2019).

8.4 Germplasm Conservation

8.4.1 Living Collections

The characterization and conservation of *Cynara cardunculus* germplasm is now of international concern. Addressing this, the European Commission (Directorate-General for Agriculture and Rural Development under Council Regulation (EC) No 870/2004) sponsored the European Project CYNARES (Pagnotta and Noorani 2014). The project (2008–2012) has seven partners from France, Spain and Italy (Table 8.1), who share European germplasm collections which have been assessed at the morphological, biochemical and molecular levels, as well as for disease resistance. Conservation of *C. cardunculus* germplasm has been undertaken based on the

Table 8.1 Main collections and accessions held of *Cynara*

Collection centers	Living accessions held
Collection at University of Tuscia and ENE, Italy http://www.unitus.it/ http://www.old.enea.it/com/ingl/default.htm	Artichoke: Romanesco, Montelupone A, Montelupone B, Jesino, Ascolano, Bianco di Pertosa, Tondo Rosso di Paestum, wild and cultivated cardoon
Collection at Cartagena University, Spain https://www.upct.es/	Artichoke: INIA-D, INIA-B, ITGA, Clon 303 and Cabeza de Gato, Spinoso Sardo, Moretto, Salambo, Violet de Provence and Macau; cultivated cardoon: Blanco de Valencia, Blanco de Huerva, Sarramian, Verde Calahorra, Blanco Peralta, Del Cortijo, Verde de Huerva, Lleno de España, Rojo de Agreda and Verde Peralta
Collection at ITGA, Spain https://dialnet.unirioja.es/	Artichoke: ITGA selection, Cabeza Gato, INIA D, PAT-89, Carderas, Camus de Bretagne, Salanquet, Crysantheme, Camerys, Macau, France, Calico Rojo, Calico Verde, C-3, VP-41, Hydes, Apolo, Brindisi, Campagnano, Hysponos, Italiana, Masedu, Moretto and Mutación Romanesco, Criolla; cultivated cardoon: Acequi, Blanco de la Huerva, Blanco de la tierra, Blanco de Peralta, Blanco de Valencia, Blanco Francés de Valencia, C-001-Rosa de Agreda, C-002-Blanco, C-003-Cadrete, Cimbro, Del Cortijo-Arsenio
Collection at Groupe d'Etudes et de Contrôle des Variétés et des Semences (GEVES), France https://www.geves.fr/catalogue-france/	Artichoke: Gros Vert de Laon, Petre, Vert de Provence, Chrysanteme, Camus de Bretagne, Blanc Hyerois, Violet de Provence, Cardinal, Calico, Popvert, Salambo

Convention on Biological Diversity, the FAO Global Plan of Action for Plant Genetic Resources for Food and Agriculture and the International Treaty on Plant Genetic Resources for Food and Agriculture. France, Italy and Spain were key project partners as traditional artichoke cultivars are grown predominantly in these Mediterranean countries.

8.4.2 In Vitro Conservation

Conservation of globe artichoke by tissue culture offers an alternative method to produce large, homogeneous and disease-free populations, and enabling in vitro storage of selected genotypes (Bekheet and Sota 2019). Ancora et al. (1981) and Morone-Fortunato et al. (2005) developed in vitro techniques for micropropagation and short-term conservation of cv. Romanesco. Bedini et al. (2012) optimized in vitro cultures of four Tuscan globe artichoke cultivars: Terom, Violetto di Toscana, Chiusure and Empolese. Moreover, in vitro medium-term storage of globe artichoke cv. Balady was recognized (Bekheet 2007). Shoot bud cultures were stored under aseptic conditions at 5 °C or osmotic stress. The results indicated that storage at the low temperature was obviously effective compared with storage on medium containing osmotic stress agents. For long-term storage, cryopreservation, i.e. storage at ultra-low temperature, usually in liquid nitrogen (−196 °C), is the current method. At this temperature, all cellular divisions and metabolic processes are stopped (Engelmann 2010).

8.5 Traditional Breeding

Compared to other vegetables, globe artichoke breeding has a limited recorded history. The different approaches followed so far are enumerated below.

8.5.1 Intraclonal Selection

Most clonal varieties are landraces maintained over time by vegetative means, where any spontaneous somatic mutations in buds can be passed to the next clonal progeny. As discussed above, in the early Mediterranean cultivars, the occurrence of such variations was frequent, thus many breeding efforts have been aimed at purging clonal varieties of these off-type plants (Abbate and Noto 1981; Deidda 1967; Pécaut 1983). However, the progress that can be accomplished is limited by the average performance of the clone variety itself.

8.5.2 Interclonal Hybridization

Due to the high heterozygosity nature of the clonal varieties, after selfing or hybridization, a tremendous array of variation appears. In the progeny, any plant combining desirable attributes can be cloned, and given enough time constitute a new clonal cultivar. In this context efforts have been aimed at improving earliness, capitula yield and quality (Foury 1969; López-Anido et al. 2005; Miller 1975; Scarascia-Mugnozza and Pacucci 1976; Tesi 1976). Most of the productive characters are of quantitative gene inheritance, thus in a segregant population relatively good progress could be attained. López-Anido et al. (1998) estimated that in a reference population composed of different cloned varieties, and selection intensity of the best 5% clones, the expected selection gain could be greater than 30% of the population mean for weight of secondary capitula, weight of the bottom of the first capitulum, harvest period and total capitula yield.

8.5.3 Breeding Seed Grown Varieties

As already mentioned, in recent years great efforts have been made to implement a seed-cultivation system. The first seed varieties were the result of inbreeding and selection, attempting to create a true-breeding form comparable, in vigor and yield, to the parent stock (Basnizki and Zohary 1994). Talpiot (Basnizki and Zohary 1987) and Imperial Star (Schrader and Mayberry 1992) were the first seed-planted cultivars available, both released through public breeding programs. Later hybrid seed-planted varieties appeared as the result of programs by private seed companies (Big Heart Seed Co 2019; Nunhems 2019). Regarding the component of variation that is involved when dealing with selection and hybridization, for the majority of the productive characters, the general combining ability variance (additive effects) turned out to be of greatest importance. The specific combining ability variance (non-additive effects) was only significant in the case of the weight of the main capitulum and receptacle (Cravero et al. 2004). Another approach relative to seed-planted cultivars is the search for an open-pollinated variety by means of recurrent selection of a given population. This seems plausible in the case where the original population has already been fixed for quality characters like spinelessness or color characteristics (green, violet), and further selection could be applied to capitula shape, cycle or yield itself (Martin et al. 2010).

8.6 Molecular Breeding

8.6.1 Molecular Markers

Molecular studies applied to understand the genome and genomic sequence of globe artichoke started about 20 years ago. Initial studies focused on evaluation of the genetic variability in germplasm collections composed of several globe artichoke varieties and landraces. Usually, these studies included the evaluation of accessions of cultivated cardoon and wild cardoon, since they are considered botanical varieties of the same species. Several molecular markers such as RAPD (Lanteri et al. 2001; Sonnante et al. 2002), AFLP (Acquadro et al. 2009; Lanteri et al. 2004; Pagnotta et al. 2004; Portis et al. 2005), microsatellite (Acquadro et al. 2003, 2005; Sonnante et al. 2008), sequence-related amplified polymorphism (SRAP) (Casadevall et al. 2011; Cravero et al. 2007, 2019) and indels (Scaglione et al. 2009) were applied in genetic diversity studies. In general, these studies showed significant genetic distances among and within cultivars as the result of the multiclonal origin of most landraces, as discussed previously. Moreover, certain research results revealed that some varieties, called by different names at different locations, are duplications with the same molecular characterization.

Breeding approaches using molecular techniques were applied by Martin et al. (2008) to identify molecular markers linked to important traits such as capitula color and earliness. Applying SRAP markers in a F_2 segregating population and a BSA (bulk segregant analysis) strategy, they reported two molecular markers associated with both traits. The marker Me4-Em4 (~850 bp) was associated with green head color, whereas Me3-Em5 (~520 bp) was linked to late production. The same type of molecular marker in combination with simple sequence repeat (SSR) were applied by Reolon da Costa et al. (2016a) to evaluate the homozygosity advance rate after two cycles of recurrent selection in Brazilian artichoke germplasm.

8.6.2 Genetic Linkage Maps

To understand the genome and genomic position of important agronomic traits in *Cynara cardunculus*, based on molecular marker technologies, a number of studies have been carried out to develop genetic linkage maps of the species. Up to now, five linkage maps of the species have been developed as a scaffold of localized important agronomic traits and to understand the genome of the species. Segregating populations were obtained by initial crosses between diverse genotypes of the three botanical varieties of the species (globe artichoke, cultivated cardoon, wild cardoon) following a double pseudo testcross mapping strategy, which is considered the most efficient way to construct genetic linkage maps in outcrossing species. The first linkage map was reported by Lanteri et al. (2006), using AFLP, microsatellite-amplified fragment length polymorphism (M-AFLP) and sequence-specific

amplification polymorphism (S-SAP) markers to genotype 94 individuals of a F_1 population generated from a cross between two globe artichoke clones (Romanesco C3 x Spinoso di Palermo). A total of 204 loci were mapped in the female map with a covered 1330.5 cM, whereas the male map comprised 1239.4 cM with 180 loci mapped. The same maps were enhanced by Acquadro et al. (2009) using a set of microsatellites developed for the species. Moreover, genes related to the caffeolquinic acid pathway and associated single nucleotide polymorphisms (SNP) markers were included in the linkage maps (Comino et al. 2007, 2009; Menin et al. 2010, 2012; Moglia et al. 2009). A second segregating population obtained by crossing Romanesco C3 artichoke clone with the cultivated cardoon Altilis 41 was developed by Portis et al. (2009) and linkage maps presented for both progenitors. The Romanesco C3 map included 326 loci with an overall length of 1486.8 cM, whereas the Altilis 41 map was 1015.5 cM and included 176 loci. Based on the two maps available for the globe artichoke clone Romanesco C3, a reference linkage map of the species was developed (Portis et al. 2012) including 172 microsatellite-derived expressed sequences (Scaglione et al. 2009). Following the same mapping strategy, other segregating populations were generated. Sonnante et al. (2011) developed a linkage map from an initial cross between the globe artichoke Mola and the wild cardoon Tolfa, both genotypes from the Mediterranean Region. The set of 192 F_1 individuals was genotyped by SSR, AFLP and SNP markers related to genes involved in the chlorogenic acid synthesis. The integrated map included 337 loci and covered 1488.8 cM. Using Argentinean genotypes of wild cardoon and Estrella del Sur artichoke, Martin et al. (2013) developed new linkage maps of the species (Fig. 8.6). The main backbone of the maps was SRAP markers and other type of markers were included such as SSR, AFLP and SNP linked to genes involved in the caffeolquinic acid pathway, in order to compare these maps with the reference maps reported by Portis et al. (2012). The female linkage map (wild cardoon) was 1465.6 cM and included a total of 214 loci, whereas the male map was 910.1 cM and comprised 141 loci. A comparison between both mapped parents showed that the wild cardoon map was some 50% longer that the globe artichoke one, reflecting that the heterozygous level in the wild cardoon was higher than in the domesticated form. On the other hand, Estrella del Sur is an open-pollinated commercial variety stabilized for some key agronomic traits, and this selection process would have reduced its heterozygosity.

8.6.3 Identification of Genome Regions Linked to Important Traits

8.6.3.1 Single Locus and Quantitative Trait Loci

Most linkage maps of the species were used to identify and localize regions of certain agronomic important traits, from single locus to quantitative trait loci (QTLs). Loci encodings of single traits such as presence of spines in capitula, leaves and

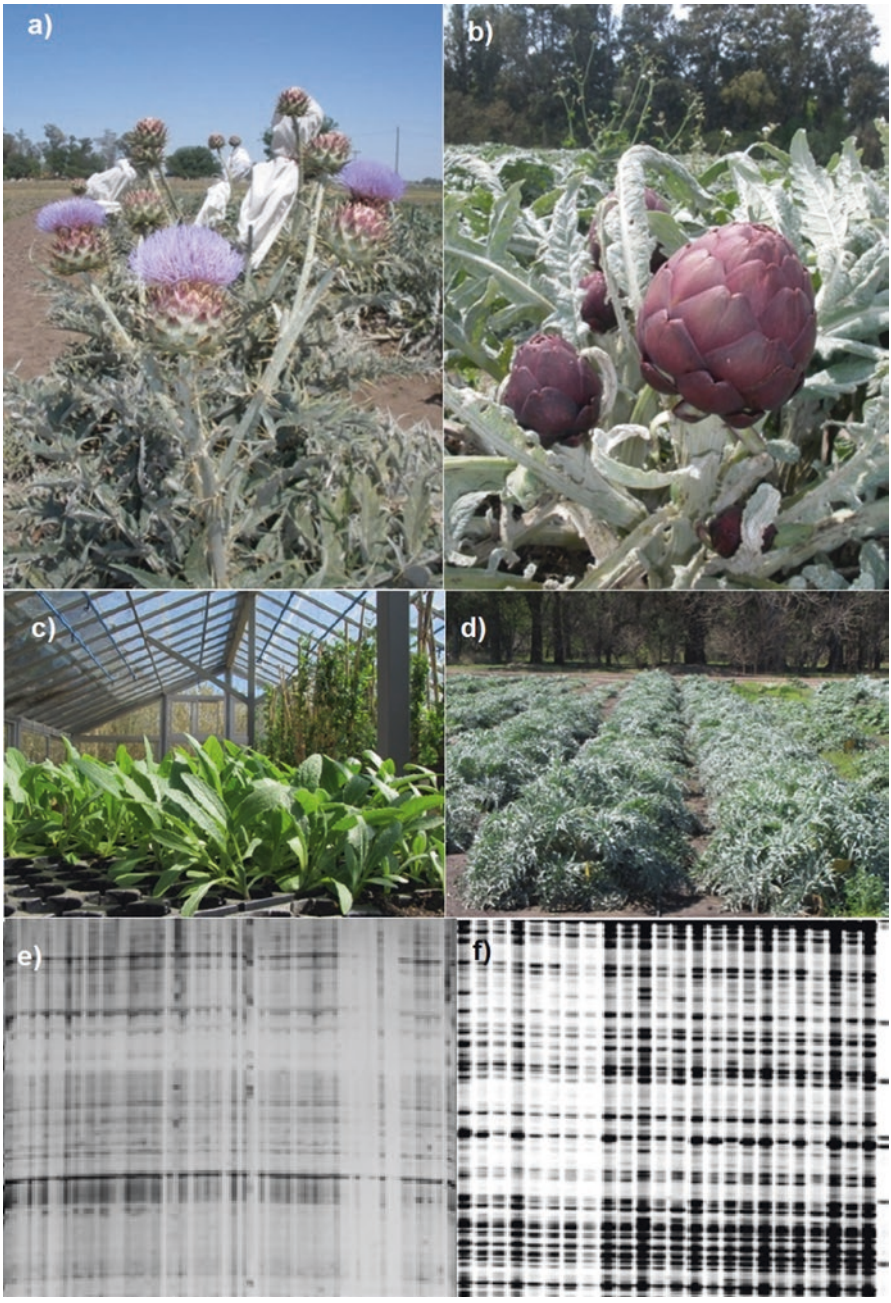

Fig. 8.6 (**a**) Argentinean genotypes of wild cardoon, (**b**) Estrella del Sur globe artichoke used as a progenitor to develop new genetic maps of the species (Martin et al. 2013), (**c**) F_1 mapping population at nursery, (**d**) Same F_1 at field of the Experimental Station of Agronomy College of the Rosario National University, (**e**) Molecular analysis of the segregating SRAP loci, (**f**) AFLP loci. Photos by Eugenia Martin

head color were identified and mapped. Lanteri et al. (2004) reported a segregation ratio of 1:1 for the spines on leaves and bracts of capitula, which was reported to be controlled by a single locus with two alternative alleles: non-spiny (*Sp*) dominant to spiny wild allele (*sp*) (Basnizki and Zohary 1994). The locus was localized on the LG 16 of the maternal map, flanked by the two SSR (CMAFLP-08, CMAFLP-07). The presence/absence of spines on capitula was evaluated by Sonnante et al. (2011) and they observed the same 1:1 segregation ratio. The spiny locus was localized only in the maternal map and at 26.5 cM from the molecular marker CyEM-188. Martin et al. (2013) evaluated the spines in leaves and capitula separately. They observed that each trait is controlled by a single locus with two alternative alleles, one for spines in leaves (SpLeaf) and the other for spines on the capitula (SpHead). The two loci were localized at the LG 8 of the male map, at a distance of 6.5 CM between them, and at 9.0 cM from the SRAP marker Me4-Em3.685. The same authors evaluated head colors and observed a segregation consistent with a monogenic 1:1 ratio for purple-green versus purple capitula. The locus responsible for head color (ColorHead) was located at LG 5 of the female parent, at 17.5 cM from the SRAP marker Me4Em3.350. These results are in agreement with Cravero et al. (2005), who found a recessive allele (*p*) acting epistatically over a second locus *U*. In homozygosis *pp* determines green head whatever allele is present in the *u* locus. Otherwise, the capitula are variegated (Fig. 8.4) when a dominant allele *U* is present or plain violet, when recessive homozygosis (*uu*). The locus mapped by Martin et al. (2013) is the *U* locus of Cravero et al. (2005). Earliness was evaluated by Portis et al. (2012), in the F_1 progeny from Romanesco C3 x Altilis 41. Both parents and F_1 progeny were assessed over two seasons, 19 QTLs were identified and mapped in 7 regions of the consensus map; however, no individual earlier than the globe artichoke parent was found.

Another key important trait is yield and its components. QTLs associated with yield were reported by Portis et al. (2014) and Martin et al. (2016a) in different background crosses. Strong positive correlations between diameter, length and fresh weight of the main capitulum and second order heads were observed. These results suggest that indirect selection might be applied for heavier first order capitulum. Moreover, some transgressive individuals were observed in both mapped populations for most of the evaluated traits. Several QTLs per trait were identified and QTL for correlated traits were frequently co-localized, presumably due to pleiotropy. Since some QTLs identified by Martin et al. (2016a) (Fig. 8.7) were co-localized in the same mapping regions and coincided with those reported by Portis et al. (2012), these regions should be further targeted in order to assist breeding programs aimed at yield increase.

8.6.3.2 Male Sterility Loci

As already discussed, in recent years the artichoke seed market has been trending toward seed-propagated hybrids. In this context, since hand-emasculation is impractical, it is necessary to have an efficient method to produce hybrid seeds by male

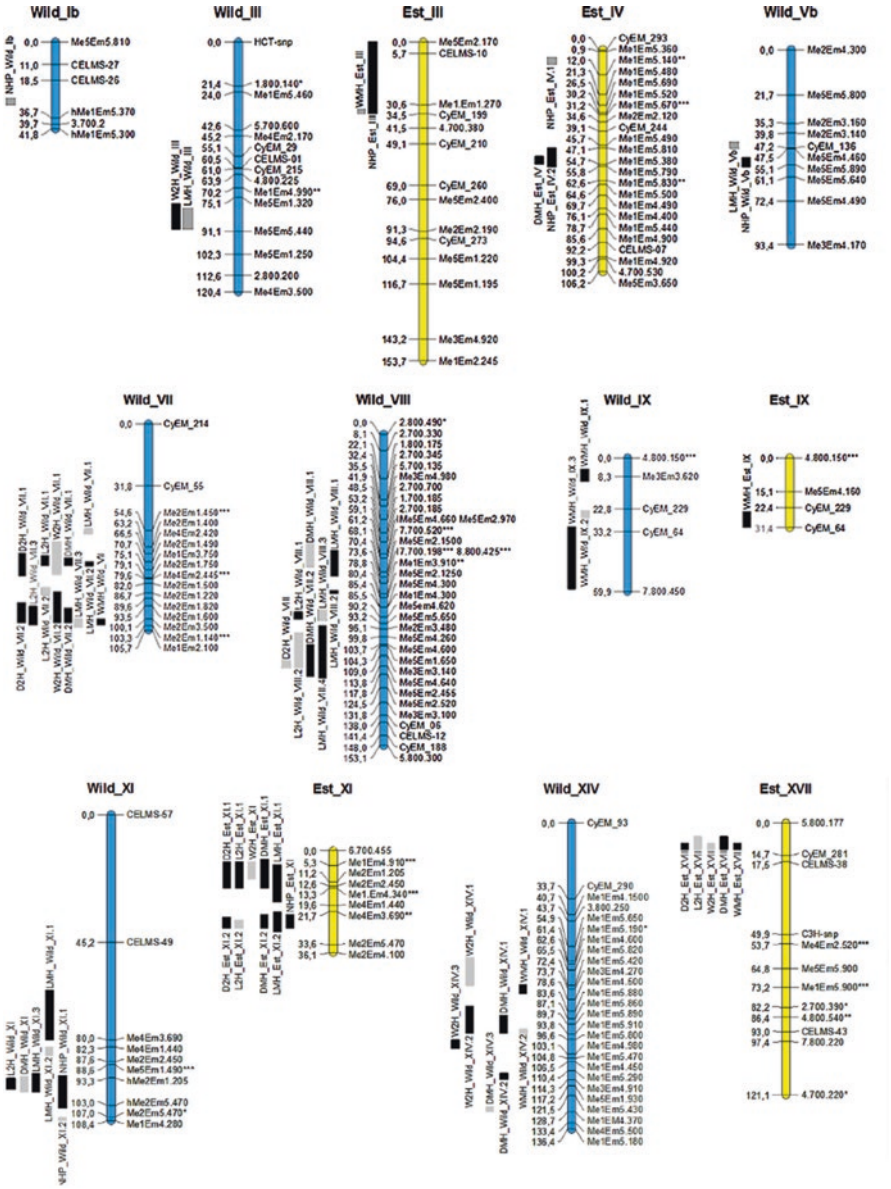

Fig. 8.7 QTLs for yield-related traits identified and mapped by Martin et al. (2016a, used with permission). Number of head per plant (NHP), fresh weight, diameter and length of the main head (WMH, DMH, LMH) and fresh weight, diameter and length of the second heads (W2H, D2H, L2H). Wild cardoon LGs in blue, globe artichoke Estrella del Sur LGs in yellow. Markers showing significant levels of segregation distortion are indicated by asterisks. Each QTL is represented by a bar, black bars show QTL detected in both seasons, while grey bars show QTL only expressed in the first season

sterility (MS). Although nuclear male sterility was reported in the species, the genetic base of this phenomenon is not clear. Principe (1984), in a half-sib family of a selected parent of unknown origin proposed a single recessive model of inheritance, where the male-sterile plants are in a homozygous recessive stage (ms1ms1). Two additional genes related to male sterility were reported by Basnizki and Zohary (1994) and designated ms2 and ms3. The first one was found in a progeny of Cavo origin and produced aborted pollen grains with an irregular form, similar to that observed for ms1, whereas ms3 was from a Tudela progeny, producing totally sterile anthers (Basnizki and Zohary 1998). Stamigna et al. (2004) and López-Anido et al. (2016) found in F_2 populations from French MS and Italian fertile clones a digenic inheritance, fitting a 15 fertile: 1 male sterile segregating plants. In this context, Zayas et al. (2020) extended the analysis incorporating SRAP markers and the BSA approach to identify associations with the ms loci. The evaluation included field screening of all the plants for pollen production over two seasons, quantification of pollen grain by microscopy and viability testing by using lactophenol-aniline blue stain (Fig. 8.8). Applying SRAP markers, they detected three associated with male-sterility (SRAP 7-10.1174, SRAP 4-9.700, SRAP 4-9.332) at a distance of 0.5 cM, 13.9 cM and 4.3 cM, respectively, from the ms locus. The markers were generated by the non-specific primer combinations Me7-Em10 (SRAP 7-10.1174) and Me4-Em9 (SRAP 4-9.332 and SRAP 4-9.700), with a size of 1774 pb for SRAP 7-10.1174, 700 bp for SRAP 7-10.1174 and 332 bp for SRAP 4-9.332.

8.6.3.3 Nutritional Quality

The genus *Cynara* has been proposed as a source of biocompounds with pharmaceutical and nutraceutical properties due to its polyphenolic contents (Ceccarelli et al. 2010; Reolon da Costa et al. 2016b; Rotondo et al. 2020). The polyphenolic content in leaves and capitula were reported for several commercial varieties and segregating populations (García et al. 2016; Moglia et al. 2008; Pandino et al. 2011, 2012). Although the polyphenols content is strongly influenced by the growing environment and the plant developmental stage, some genetic determination can be identified and used to improve new cultivars with higher levels of these compounds. Pandino et al. (2015) observed transgressive segregation for dicaffeolquinic acid in leaves of a F_1 population from a cross between globe artichoke and cultivated cardoon of the Mediterranean Region. The transgressive individuals were selected and evaluated over two growing seasons for its polyphenol profile, including dicaffeolquinic acid, apigenin, luteolin and narirturin, and were genotyped with microsatellite markers. From the selected individuals, two plants were identified that accumulate dicaffeolquinic acid and, if properly cloned, these genotypes could be used to source pharmaceutical compounds. A first approach to elucidated QTL association to the content of some polyphenols, chlorogenic acid and cynarin, in capitula, was reported by Martin et al. (2018), in a segregating population obtained by crossing Argentinean genotypes of wild cardoon x Estrella del Sur. Through a regression model, 11 SRAP markers associated with chlorogenic acid, and 11 SRAP

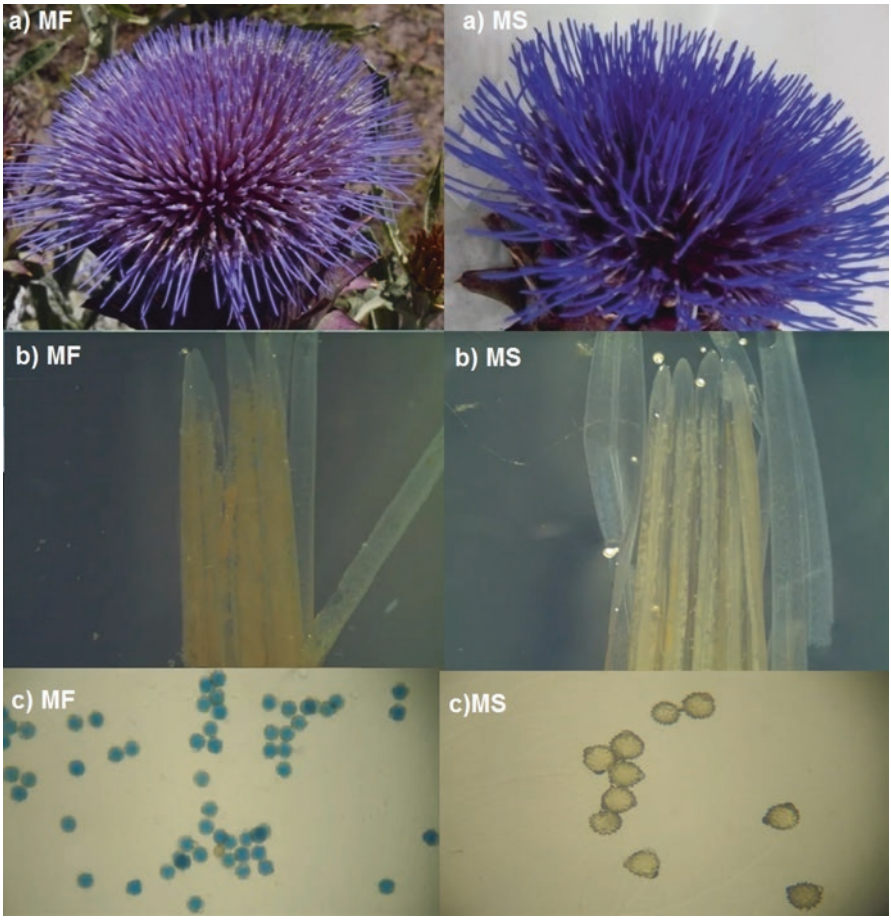

Fig. 8.8 Evaluation of male sterility in *Cynara cardunculus*. (**a**) Male fertile (left) and male sterile (right) capitula at anthesis, (**b**) Anthers of male fertile (right) and male sterile plants (left), (**c**) Pollen viability test by lactophenol-aniline blue stain, MF (male fertile plant), MS (male sterile plant). Photos by Aldana Zayas and Marta Bianchi

linked to the cynarin content, were identified. The proportion of the phenotypic variance explained by each QTL-marker was 12.8–34.4% for chlorogenic acid and 13.1–32.7% for cynarin content. In recent years, this approach to identify genomic regions linked to polyphenols content has expanded to include some flavonoids (apigenin, luteolin) and the evaluation of the genotypes under different environments conditions.

8.6.3.4 Biomass Production

As previously mentioned, cultivated cardoons are the most suitable taxa to be considered as an energy crop, as well as the remnant biomass of globe artichoke after harvesting the capitula. In this context, some efforts have been made to identify and localized QTLs related to biomass productions. Martin et al. (2016b), using the same mapping population previously described, studied five morphological traits associated with biomass production: diameter and height of the plant, dry weight of the leaves, capitula and stalks. The correlation among traits showed that the diameter of the plant has a strong positive association with height of the plant (0.70) and dry weight of the leaves (0.66); whereas dry weight of the capitula showed a highly positive correlation with dry weight of the stalks (0.92). Transgressive individuals were identified, mostly for diameter and plant height. Applying standard interval mapping, except for dry weight of stalks, 16 QTLs were observed for all remaining evaluated traits. Moreover, correlated traits were frequently co-localized at the same genomic region (Martin et al. 2016b). A second study was reported by Portis et al. (2018). They evaluated 11 traits related to biomass over two growing season, in a mapping population originated by the cross of a globe artichoke genotype and a cultivated cardoon from the Mediterranean Region. Correlations between traits were in accord with those of Martin et al. (2016b); applying a multiple QTL mapping strategy, a total of 27 genomic regions for biomass production were identified. The localization of some QTL in the linkage maps was consistent with reports by Martin et al. (2016b), in particular at LG 3 and LG9.

8.7 Genome Sequencing

Recent advances in new DNA sequencing technologies have simplified the acquisition of information about the genomes of more than 100 plant species, and numerous plant genomes are in the process of sequencing and assembly. Construction of a reference genome in an outbreeding species, with high heterozygous levels as globe artichoke, is laborious. Nevertheless, a reference genome of globe artichoke was developed using a clone obtained by three cycles of selfing, with a residual heterozygosity level of about 10% (Scaglione et al. 2016). The reference genome was generated by assembly 133.7 Gb sequencing data into ~13,000 scaffolds obtained using a Illumina HiSeq2000 platform, covering 725 Mb of the genome (67%). The re-sequencing of the genome parents of the mapping population (Portis et al. 2009) and genotyping by sequencing of the F_1 individuals allowed anchoring the scaffolds onto the 17 chromosomes of the species. The reference genome and all data information are available in the public domain database (http://www.artichokegenome.unito.it/cymsatdb/) (Portis et al. 2016). The information on the genome sequences provides the possibility of new genetic assays, assessment of the genetic diversity, gene isolation and marker-assisted breeding.

8.8 Genetic Engineering

Most of the production and consumption area of the artichoke is within the boundary of the European Union, because of this, research aimed at the use of genetic engineering to improve a given clone or cultivar has been discouraged. Also an overlap of distribution exists between the crop and the compatible wild cardoon, not only in the Mediterranean Region, but also in the USA, Argentina and Chile, which could facilitate gene escape, thus hindering any attempt to approve genetic engineering of cultivars.

8.9 Conclusions and Prospects

Globe artichoke is a vegetable crop with a limited breeding history; most attempts in the past were aimed at the purging of given heirloom-cloned varieties already adapted and accepted in local markets. Research on floral biology and advances in sexual reproduction turned the crop into a seed-propagated plant, suitable for annual rotations, which in turn can prevent the spread of soil-borne pathogens and viruses. Sexual reproduction facilitated the study of the inheritance of important traits (head color, presence of spines, male sterility), developed seed and nursery industries, and over the past 20 years a great array of molecular markers became available and positioned in linkage maps. Recently a reference sequenced genome was developed. Nevertheless, in contrast to other vegetables, the number of newly-released cultivars is limited, and a great proportion of the planted area is still covered with traditional cloned varieties. One of the possible reasons for this situation is the failure to obtain, by the classical breeding methods, seed-propagated materials of the early Mediterranean types, with autumn and spring production. The prospect for the next few years should be to attain these types of cultivars. Aiding in the pursuit of this goal may be the clustered regularly interspaced palindromic repeats (CRISPR)/CRISPR-associated 9 (Cas9)-mediated genome editing technology, which has been proven effective in many horticultural crops (Xu et al. 2019), coupled with genomic selection, in order to eliminate exogenous DNA of the editing tools (Voss-Fels et al. 2019).

Appendices

Appendix I: Research Institutes Relevant to Globe Artichoke

Institute/Organization	Specialization research activities	Contact information and website
Università degli Studi della Tuscia, Tuscia University	Genetic diversity, management	Prof. Mario-A. Pagnotta Va S.Maria in Gradi 4 01100 Viterbo, Italy pagnotta@unitus.it
National Agency for the New Technologies, Energy and the Environment (ENEA)	Genetics resources, genetics, in vitro culture	Paola Crinò Lungotevere Grande Ammiraglio Thaon de Revel 76 00196 Rome, Italy
Institute of Science of Food Production – Institute of Biosciences and Bioresources – National Council of Research, (IGV-CNR)	Crop management, genetics, genetic resources	Gabriella Sonnante / Nicola Calabrese Via Amendola 165/a 70,126 Bari, Italy gabriella.sonnante@ibbr.cnr.it
DISAFA Plant Genetics and Breeding, University of Torino	Genetics, breeding, marker development, sequencing	Prof. Sergio Lanteri / Alberto Acquadro Largo P. Braccini 2, 10,095 Grugliasco, Torino, Italy sergio.lanteri@unito.it alberto.acquadro@unito.it
University of Catania	Crop management	Giovanni Mauromicale Via Valdisavoia 5 95123 Catania Italy g.mauromicale@unict.it
Bretagne Biotechnologie Végétale (BBV)	Biotechnology	Christophe Bazinet Pen ar Prat, 29,250 Saint Pol de Léon, France
Universidad Politécnica de Cartagena (UPCT)	Field management, stress	Prof. Juan Fernández Plaza Cronista Isidoro Valverde 30,202 Cartagena, Spain Juan.fernandez@upct.es
Universidad Miguel Hernández	Bioactive compounds	Dr. Daniel Valero Department Food Technology Ctra. Beniel KM 3.2 03312 Alicante Orihuela, Spain daniel.valero@umh.es

(continued)

Institute/Organization	Specialization research activities	Contact information and website
Instiuto Técnico y de Géstion Agricola (ITGA)	Crop management	Juan Igniacio Macua Avda. Serapio Huici 20–22 31,610 Villava. Spain
Groupe d'Etudes et de Contrôle des Variétés et des Semences (GEVES)	Seeds, clonal varieties quality	Chrystelle Jouy La Minière 78,285 Guyancourt, France
Instituto de Investigaciones en Ciencias Agrarias de Rosario (IICAR, CONICET-UNR)	Breeding, genetics	Prof Vanina Cravero Campo Experimental Villarino (S2125ZAA), Zavalla, Santa Fe, Argentina vcravero@unr.edu.ar
Big Heart Seed Company	Breeding	Nestor Rey 1280 Main Street, Brawley, CA 92227 USA rey@bigheartseed.com
Texas A&M AgriLife – Research and Extension Center at Uvalde	Crop management, organic systems, plant stress physiology	Prof. Daniel Leskovar 1619 Garner Fielf Rd., Uvalde, TX 78801, USA d-leskovar@tamu.edu

Appendix II: Genetics Resources of Globe Artichoke

Cultivar	Important traits	Cultivation location
Vegetative propagated		
Spinoso Sardo	Spiny capitula, early production	Sardinia, Liguria
Spinoso di Palermo	Spiny, average early	Palermo, Trapani, Agrigento
Violetto di Toscana	Violet capitula, spring production	Tuscany
Moretto	Violet capitula, spring production	Liguria
Castellammare	Green variegated capitula, spring production	Lazio
Catanese	Variagated capitula, early production	Sicily, Toscany, Puglia
Masedu	Variegated capitula, early production	Sardinia
Sakiz	Green variegated capitula, early production	Turkey
Bianco Tarantino	Green capitula, spring production	Puglia
Blanco de Tudela	Green capitula, early production	Spain, Argentina
Violeto de Provenza	Variegated capitula, early production	France, Italy, Algeria, Egypt

(continued)

Cultivar	Important traits	Cultivation location
Precoce di Jesi	Variegated, violet capitula, spring production	Marche
Empolese	Green variegated capitula, spring production	Tuscany
Romanesco	Violet variegated, spring production	Lazio, Argentina
Camus de Bretagne	Green capitula, spring production	France
Seed-propagated		
Imperial Star	Green capitula, spring production	USA
Opal	Variegated capitula, spring production, hybrid	Italy, Spain, Argentina, Chile
Madrigal	Green capitula, spring production, hybrid	Italy, Spain, Argentina, Chile
Deserto	Variegated capitula, spring production, hybrid	USA
Romolo	Variegated capitula, spring production, hybrid	USA

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Chapter 9
Breeding Strategies of Garden Pea (*Pisum sativum* L.)

Amal M. E. Abdel-Hamid and Khaled F. M. Salem

Abstract Garden pea (*Pisum sativum* L.), a member of the Fabaceae family, is one of the most important self-pollinating legume crops. Globally, the pea is an economic crop, utilized as food, feed and industrial uses. Garden pea is an annual winter-season crop grown around the world from winter to early summer depending on the country. Gene banks have conserved a large genetic resource collection of pea germplasm. *Pisum* harbors significant diversity based on biological status, geographical regions and morpho-agronomic traits. Introgression of novel alleles through crossing between various pea genetic resources, e.g. modern varieties with locally adapted varieties, enhances genetic diversity and preselection for traits of interest, which is required to ensure meaningful natural variation at the phenotypic level. Improving pea for biotic and abiotic stress tolerance traits, quality traits and yield attributes are the main objectives of breeders and geneticists. These can be achieved with genomics tools to augment traditional breeding programs. In this chapter, we will provide an overview of the origin of the pea, distribution, genetic resources, conservation, cultivation practices, recent developments in biotechnology and molecular genetics to improve traditional breeding methods.

Keywords Biodiversity · Biotechnology · Breeding · Genetic improvement · Modern pea breeding · *Pisum sativum* · Traditional breeding

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9.1 Introduction

9.1.1 Origin and Distribution

Ben-Ze'ev and Zohary (1973) reported that pea (*Pisum sativum* L.) originated in the Mediterranean area, western and central Asia and Ethiopia. FAO designates Ethiopia and western Asia as centers of genetic diversity, with secondary centers in southern Asia and the Mediterranean Region (Singh et al. 2019). The first cultivation of pea was in western Asia and it spread to Europe, China and India (Ljuština and Mikić 2010). India is the largest vegetable pea producer worldwide (Vijay et al. 2018). Pea was already well known in Central and East Africa and was established in Uganda and Rwanda by 1860 as an important food crop. The first consumption of edible pods was recorded in the Netherlands and France during the sixteenth century (Blixt 1970).

Peas are found in most tropical countries (Mikić et al. 2007). They are grown in the highlands of East and Central Africa, Ethiopia and southern Africa but are hardly grown in West Africa. In Africa, the pea has a great deal of importance, it is found in French and English-speaking countries. Pea was grown in the United Kingdom in the Middle Ages and was introduced into the Americas after Columbus (Davies et al. 1985). Vavilov (1992) recorded the first centers of origin and diversity of crops, which are presented in Table 9.1.

Pea was the key experimental plant for the first genetic studies, performed by Gregor Mendel (Father of Genetics) in 1850 (Smýkal 2014). Mendel chose *Pisum sativum* because it has several advantages for research in genetics. Pea plants have many varieties with distinct heritable characters, grow quickly and can self-pollinate or be cross-pollinated. Mendel studied the inheritance patterns of seven traits in *P. sativum* plants. Ever since, Mendel's work has been widely analyzed and discussed (Fisher 1936) and became the foundation of the new discipline of genetics (Bateson 1902; Weldon 1902). Despite the tremendous progress in genetics and modern plant breeding in recent years, it will forever rely on the basic principles formulated by Mendel on the garden pea. Genetics has a great role in crop breeding and similarly genomic knowledge is gradually being translated to molecular breeding and genome-wide or genomic selection for the development of improved breeding lines (Smýkal et al. 2016).

About 98,000 pea accessions are preserved worldwide, only 2% are wild pea relatives, approximately 34% commercial varieties, 13% breeding lines, 38% landraces and 2% mutant stocks. In the case of true wild *Pisum* species, there are only 0.46% *P. fulvum*, 0.42% *P.* ssp. *elatius*, 1.2% *P. sativum* ssp. *sativum* (syn. *P. humile/syriacum*) and 0.36% *P. abyssinicum* of accessions Fig. 9.1.

Peas (*Pisum sativum* L., 2n = 14) are consumed as dry seeds or fresh vegetables throughout the world. According to Abbo et al. (2017), pea (*P. sativum*) varieties belong to one of the following groups: a) *P. sativum* L. ssp. *sativum* (field pea, garden pea, spring pea, English pea, common pea, green pea, b) *P. sativum* var.

Table 9.1 Centers of origin and diversity of crops around the world

Center name	Number of species	Crops
Chinese Center	138	Cereals, buckwheat, legumes
Indian Center	117	Rice, millets, legumes
Indo-Malayan Center (Indonesia, Philippines)	55	Root crops, fruit crops, sugarcane, spices
Inner Asiatic Center (Tadjikistan, Uzbekistan)	42	Wheat, rye, many herbaceous legumes, as well as seed-sown root crops, fruits
Asia Minor (Transcaucasia, Iran and Turkmenistan)	83	Wheat, rye, oats, seed, forage legumes, fruits
Mediterranean Center	84	Wheat, barley, forage plants, vegetables, fruits-especially, spices, ethereal oil plants
Abyssinian Center (Ethiopian)	38	Wheat, barley, local grains
South Mexican and Central American Centers	49	*Phaseolus*, maize, fiber plants, spices, cucurbitaceous, fruits
South America Andes region (Bolivia, Peru, Ecuador)	45	Root crops, grain, potatoes, vegetables, fruits, drugs tobacco, quinine, coca
Chilean Center	4	*Solarium tuberosum*
Brazilian-Paraguayan Center	13	*Manihot esculenta* (cassava), *Arachis hypogaea* (peanut), *Ananas comosus* (pineapple), *Hevea brasiliensis* (rubber), *Theobroma cacao* (cocoa)

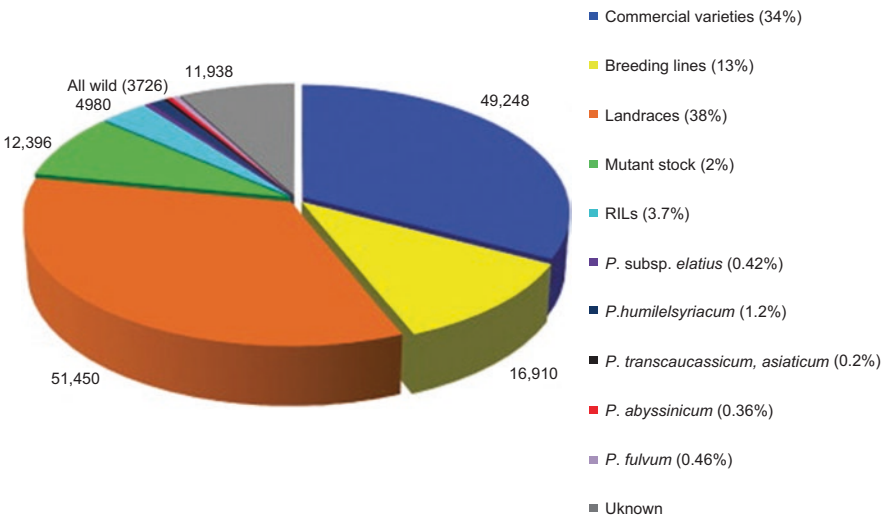

Fig. 9.1 Pea germplasm stratification. (Source: Smýkal et al. 2013)

Table 9.2 Nutritional value of garden pea

Content	Concentration (%)
Protein	21.3–32.9
Starch	36.9–49.0
Resesitant starch	2.1–6-3
Soluble sugars	5.3–8.7
Dietary fiber	14–26
Insoluble fiber	10–15
Soluble fiber	2–9
Amylose	20.7–33.7
Lipids	1.2–2.4
Ashe	2.3–3.4

Source: Dahl et al. (2012)

Fig. 9.2 Different varieties of *Pisum sativum*. (Source: www.flickr.com/photos)

saccharatum (snow pea) and c) *P. sativum* var. *macrocarpon* (snap pea or sugar snap pea) (Table 9.2; Fig. 9.2).

Holdsworth et al. (2017) assembled the USDA Pea Single Plant Plus Collection (PSPPC), which contains 431 *Pisum sativum* accessions. The collection was characterized genetically in order to maximize its value for trait mapping and genomics-assisted breeding (Fig. 9.3).

9.1.2 Economic Importance and Health Benefits

Pisum sativum plants are commonly used in several ways: fresh, canned or frozen. Peas have great nutritional value because they contain protein, carbohydrates, fiber, minerals, vitamins and antioxidant compounds (Amarakoon 2012; Hall et al. 2017; Hedley 2001; Nilsson et al. 2004; Paul and Southgate 1988). Young shoots are used

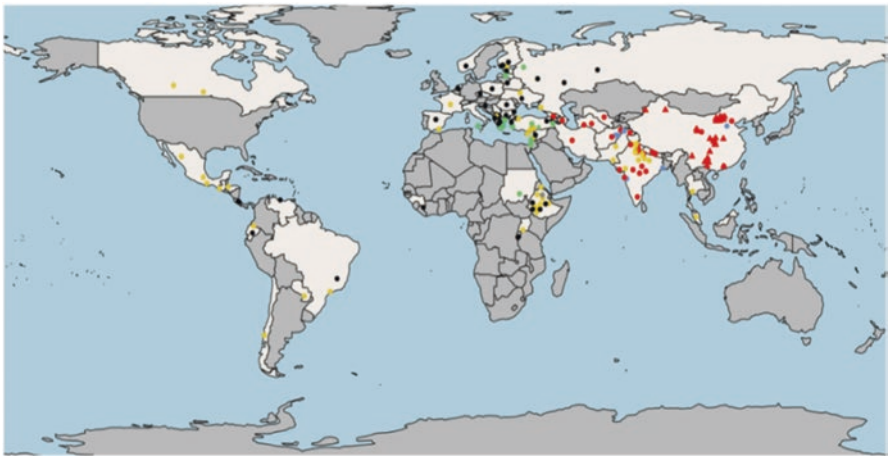

Fig. 9.3 Map of *Pisum sativum* accessions around the world. Circles indicate accessions in the original Pea Single Plant Collection (PSPPC) and triangles indicate accessions from the Chinese core collection *P. sativum* ssp. *elatius* (green); *P. sativum* ssp. *abyssinicum* (gray); *P. sativum* ssp. *sativum* – Primary (gold); *P. sativum* – Central Asia (dark blue) and *P. sativum* ssp. *sativum* -non-Mediterranean Asia (red)

as a leafy vegetable in Malawi and some Asian countries. Dry pea seeds are used for animal feed (Hedley 2001) and pea straw is used as forage, hay, silage or green manure. Importantly, peas play a key role in soil fertility by fixing atmospheric nitrogen (Messiaen et al. 2006).

Pea seeds are reputed to have beneficial effects on skin conditions in the form of face masks used to treat wrinkled skin (Aburjai and Natsheh 2003). Worldwide, peas are one of the major food legumes grown in various regions especially in Europe (Ljuština and Mikić 2010; Rana et al. 2017). Pea production has increased rapidly; production now occupies fourth place among world food legumes production after soybeans, peanuts and dry beans (Adsule and Kadam 1989). Peas are highly nutritive (Table 9.2) for both human diet (Dahl et al. 2012) and animal feed as an alternative to soybeans (Cruz-Suarez et al. 2001; Hedley 2001). Altogether, these factors position peas at a similar economic level to cereals.

Peas are of great interest as a crop in Europe, due to their capacity to produce a higher yield compared to local cultivars (Annicchiarico 2008). High yield and its stability, tolerance for biotic and abiotic stresses, in addition to high protein content, are important traits for pea development as a feed crop (Khodapanahi et al. 2012).

The increasing load of environmental pollutants, particularly heavy metal ions in soil, water and air during the last decades, due to the extensive and/or uncontrolled human activities, are reported to impose a drastic environmental stress on growth, morphogenesis and yield on higher plants, particularly those of nutritive value for humans and certain livestock (Lyanguzova 1999; Mishra and Choudhuri 1999; Nyarai-Horvath et al. 1997; Obroucheva et al. 1998). Certain vascular plants such as

legumes can respond to heavy metal ions at concentrations much lower than those required to elicit a response in animals and human beings. These plants can be utilized as indicators for pollution in the environment and to monitor their concentrations as biomonitors. In this regard, Abdel-Hamid (2000) revealed that *Pisum sativum* tends to be one of these bio-monitors. Aissani et al. (2019) found that peas can be irrigated with yeast industrial liquid effluent and give good germination and growth.

9.1.3 Domestication, Selection and Early Improvements

Harlan (1992) stated that the family Fabaceae has the greatest number of domesticated crops of any plant family. Fabaceae members have an excellent system to study as to the extent parallel variations in morphology are determined by similar mutations.

The earliest archaeological and hereditary investigation shows that the pea was domesticated in the Near East and the Mediterranean Basin (Zohary and Hopf 2000). Also, peas were found in the late Neolithic era of present-day Greece, Syria, Turkey and Jordan. In Egypt, early finds date from 4800 to 4400 BC in the Nile Delta and from 3800 to 3600 BC in Upper Egypt. Peas were present in Pakistan and western and northwestern India in 2250–1750 BC. The pea was also present in the Republic of Georgia, the Ganges Basin and southern India as a legume crop in the fifth millennium BC (Chimwamurombe and Khulbe 2011).

Pisum sativum was domesticated from the wild *P. humile* which is common in northern Iran, Iraq, Jordan, Turkey, Syria and Palestine. *Pisum sativum* arrived in India and China via the Himalayan trade routes and the Greeks. *Pisum elatius* is another wild species which is found in North Africa, southern Italy and throughout the Near East (Harlan et al. 1976; Yamashita 1980; Zeven and De Wet 1983).

The *Pisum sativum* group is cultivated around the world including in tropical Africa. Both *P. fulvum* and *P. sativum* were domesticated in the Near East about 12,000 years ago, likely from *P. humile* (otherwise called *P. sativum* ssp. *elatius*). *Pisum abyssinian* is cultivated in the northern and southeastern regions of Ethiopia; it originated from *P. sativum* independently in the Old or Middle kingdoms of Egypt around 5000 years ago. It is also grown in Yemen (Weeden 2018). Other cultivar groups, varieties or subspecies occur in southern Europe and western Asia. Subsequent breeding and developments have resulted in the production of thousands of pea genotypes today (Govorov 1937; Smýkal 2014; Vershinin et al. 2003) (Figs. 9.4 and 9.5).

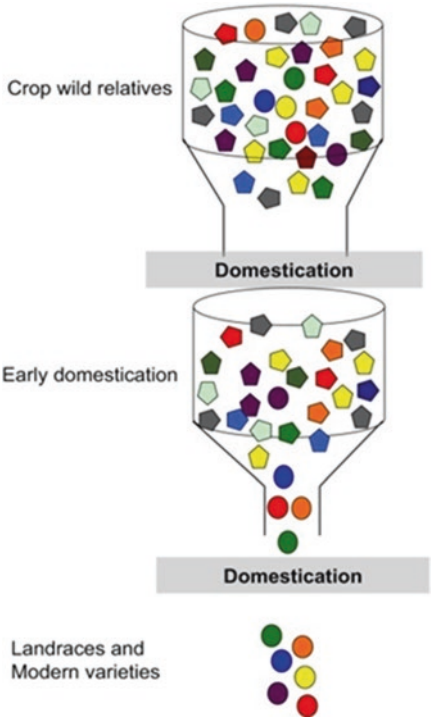

Fig. 9.4 Domestication of *Pisum sativum.* (Source: Zhang et al. 2016)

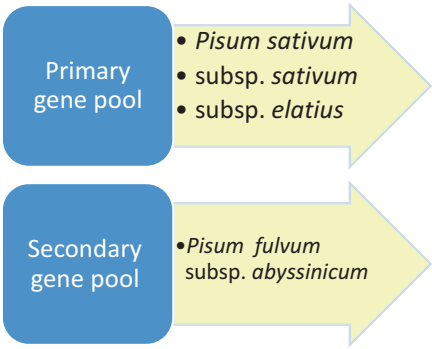

Fig. 9.5 Gene pools of *Pisum sativum*. (Prepared by Amal M.E. Abdel-Hamid)

9.2 Current Cultivation Practices and Challenges

9.2.1 Current Cultivation Practices

Garden pea (*Pisum sativum*) is one of the most common and important winter vegetable crops grown on a global scale and consumed either fresh or in processed form. It improves soil fertility due to the fixation nitrogen by *Rhizobium* bacteria (Messiaen et al. 2006; Phillips 1980). Peas are mixed with other vegetables or used

alone; they are also processed for canning and freezing to meet consumer requirements during the off-season. Important quality attributes of peas are good flavor, high chlorophyll content, the dark green color of the pods, high glucose and fructose content and good texture. There are also different quality standards required for various manufactured products. For canning, extruded peas should have a light green color and resist washing out of chlorophyll by the salty liquid in the can. However, seed freezing varieties should be dark. The color intensity of the seeds is positively associated with color pods. Peas for dryness should be large in size, wrinkled, dark green with high dry material content. Peas improve soil fertility by providing nitrogen for successive crops in rotation schemes, without the need for supplementary fertilizer (Bobille et al. 2019).

Pea cultivation requires a temperature of 18–22 °C to obtain a good germination rate. They can germinate under a starting temperature of 4–5 °C and tolerate moderate frost. As the temperature rises to 25 °C and above, the percentage of germination decreases. Pea can be grown in all soil types except heavy clays. The pea gives the best growth in acidic soils with soil pH ranging from 6.0 to 7.5. It is preferable to add organic matter or compost before planting where it serves to improve soil properties, fertility and structure. Soil service varies depending on the previous crop and plot status. If cultivated after a crop that left behind organic matter it must be tilled in and disc plowed twice perpendicular and then disked twice to create the desired soil structure. Sowing is carried out in two ways. One, by sprouting, which is double sowing if planted toward the end of September to the beginning of October, in the Northern Hemisphere. Planting seed manually or mechanically the distances between lines and other plants should be 60 cm and 25 cm, respectively, and irrigation continued until germination and the appearance of shoots above the soil surface and two, planting where there is the presence of soil moisture and irrigation of the land before planting for a sufficient period or as a result of rain, the moisture allows germination without damage to the seed shell. In the case of heavy soils, given the hard seed shell, there is less aeration and absorption of plant nutrients, which can lead to plant yellowing and death.

In fields where peas have not been grown before, seeds should be treated with nitrogen-fixing *Rhizobium* bacteria. This ensures the formation of bacterial nodes, good growth and crop quality (Messiaen et al. 2006). Manual or chemical weed control can be used. In the latter case, appropriate pesticides and specialized spraying of plants and soil must contain enough moisture to obtain high efficiency of the pesticide.

9.2.2 Current Agricultural Challenges

Vegetable crops face many abiotic and biotic stresses, which affect growth and yield due to global warming and related climate changes (Atkinson et al. 2013; Mahalingam 2015; Mittler 2006; Narsai et al. 2013; Pandey et al. 2015; Prasad et al. 2011; Prasch and Sonnewald 2013; Ramegowda and Senthil-Kumar 2015; Suzuki

et al. 2014). Salinity, drought, heat and other types of abiotic stress together are more destructive to the growth and production of vegetable crops than a stress factor occurring separately at different stages of vegetative growth of a crop (Mittler 2006; Prasad et al. 2011).

Abiotic stresses affect the spread of pathogens, insects and weeds (Coakley et al. 1999; McDonald et al. 2009; Peters et al. 2014; Scherm and Coakley 2003; Ziska et al. 2010). Also, in the future, pests may become a greater threat to the growth and production of crops (Duveiller et al. 2007). Environmental stress conditions play a direct role in plant pest interactions by altering plant organ functions and resistance (Scherm and Coakley 2003). Similarly, abiotic stress conditions such as drought increase the competitiveness of weeds for water use compared to crops (Patterson 1995; Valerio et al. 2013; Ziska et al. 2010).

9.2.3 Genetic Improvement Objectives

Early maturity and high productivity are the main objectives of pea breeding. Early-maturing crops have an important comparative advantage for farmers because of higher prices at the beginning of the production season. Also, pod attributes such as pod size and seed size are the most important qualities as they are qualities that affect the market price of peas. Reproduction for disease resistance and the development of new genotypes are the main targets of the breeding programs in some areas such as those related to *Fusarium* wilt (Shubha et al. 2019), crushed mold, rust, pea-borne mosaic virus, structural mosaic virus and yellow mosaic virus. As well is breeding for pest resistance and the development of genotypes resistant to leaf miner, weevils and aphids. Moreover, peas are frost sensitive and resistance to it is among the breeding targets to ameliorate environmental stresses. Also, peas are grown for fresh consumption, processing, canning, and freezing (Hedley 2001; Paul and Southgate 1988).

9.3 Germplasm Biodiversity and Conservation

Germplasm is the crude raw material that pea breeders use to create new genotypes. It is comprised of different types of genetic accumulations, for example, natural hybrids, primitive cultivars, wild species, obsolete varieties, breeding lines, elite lines and mutants (Haussmann et al. 2004).

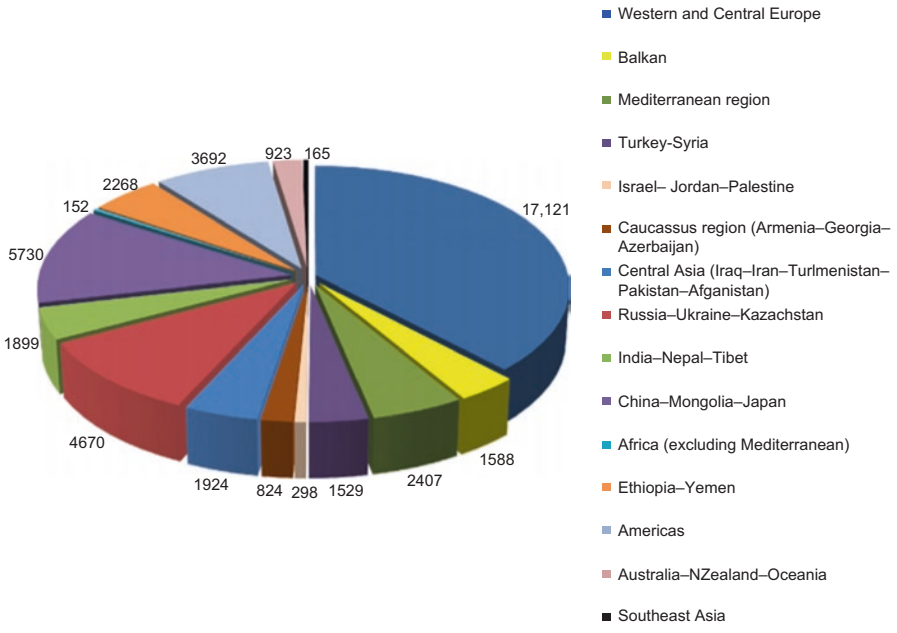

Fig. 9.6 Pea germplasm collections around the world. (Source: Smýkal et al. 2013)

9.3.1 Germplasm Diversity

A large amount of genetic diversity of *Pisum sativum* has been found in Africa and Asia. Many germplasm collections of *P. sativum* cultivars are held around the world as detailed in Fig. 9.6, and Appendix II-A. The collections contain wild and primitive varieties, cultivars with multiple disease resistance, lines carrying structural mutations, breeding lines and cultivars of specific interest (Zong et al. 2008).

Morphological and agronomical traits that are resistant to biotic and abiotic stresses, identified to individual genotypes, increases the importance of the germplasm (Ceyhan and Avci 2015; Ghafoor et al. 2005). The economic importance of a population is associated with morphology, agronomic traits, seed nutritional and quality traits. The efficient utilization of indigenous germplasm requires data on the genetic diversity of economic interest (Singh et al. 2019).

9.3.2 Cultivars Characterization and Phylogeny

Morphological traits can help breeders to develop better maintenance strategies and economic utilization of pea genetic resources. Morphological traits are influenced by environmental factors (Ceyhan and Avci 2015); therefore, breeders need stable characters to characterize different germplasm accessions. A classical method of

estimating diversity in a population is the use of molecular markers in pea (Hanci 2019).

McClendon et al. (2002) identified 8 AFLP and 15 RAPD markers associated with *Fusarium* wilt race 1 resistance in pea. These DNA markers are suitable for marker-assisted selection in pea breeding programs. Marker-assisted selection (MAS) is now being integrated into on-going conventional pea breeding. MAS is useful to speed-up selection for those traits that express lateness in plant development. Such target traits include resistance to diseases, and even lodging and seed characters. Isozyme marker alcohol dehydrogenase (*Adh1*) has been shown to be linked with resistance to pea enation virus (En.). Two new examples associated with disease resistance are the development of PCR markers designed from cDNA-AFLP fragments giving close linkage to genes (*subm-1*, *mo*) presenting resistance to pea seed-borne mosaic virus and SSR marker suitable for resistance to powdery mildew of peas, as mentioned by Ambrose (2008). QTLs for lodging resistance have been reported.

The primary example of genetic linkage in pea was described by Vilmorin and Bateson (1911) and the first genetic map was developed by Wellensiek (1925). In the twentieth century, whole genetic maps consist of 7 linkage groups (LGs) based on the pea karyotype; RAPD and RFLP markers were constructed and shown in Table 9.3 (Aubert et al. 2006; Bordat et al. 2011). Lately, the availability of pea EST databases has resulted in the design and mapping of numerous gene-based molecular markers in *Pisum sativum*. Advancement of next-generation sequencing (NGS) allowed distinguishing a great many single nucleotide polymorphism sites (SNPs) (Duarte et al. 2014; Kaur et al. 2012; Leonforte et al. 2013; Ma et al. 2017; Sindhu et al. 2014; Tayeh et al. 2015; Yang et al. 2015; Zhernakov et al. 2017). Guindon et al. 2016 used the SRAP (sequence-related amplified polymorphism) technique for linkage mapping in *P. sativum* (Fig. 9.7). Many studies (Ellis 2011; Jing et al. 2010; Smýkal et al. 2011) of *P. abyssinicum* placed it between *P. fulvum* and *P. sativum* ssp. *elatius* and additional branches were found within the cultivated pea (Fig. 9.8).

9.3.3 Genetic Resources Conservation Approaches

Conservation of *Pisum sativum* genetic resources is becoming increasingly important because of the loss of traditional varieties adapted to a specific region being substituted by foreign-origin varieties (Khoury et al. 2016), emerging new crop diseases, environmental pollution and developments in crop processing.

Table 9.3 List of different types of markers tagged for disease resistance in pea

Trait	Gene	Marker	References
Bean yellow mosaic virus resistance	*mo*	Pgm-p (isozyme), P252 (RFLP)	Weeden et al. (1984)
Pea seed borne mosaic virus	*sbm-1*	GS185 (RFLP)	Timmerman-Vaughan et al. (1993)
Ascochyta blight resistance	*QTL*	Af& I (linkage group I);p227, p105 (RFLP Linkage group IV; p236 RFLP LG VI)	Dirlewanger et al. (1994)
Fusarium wilt resistance	*Fw*	H19, Y14, Y15 (RAPD) p254, p248, p227, $p10_{\mu}$ (RFLP)	Dirlewanger et al. (1994)
Powdery mildew resistance	*er-1*	p236 (RFLP) $PD10_{650}$(RAPD to SCAR)	Dirlewanger et al. (1994)
Powdery mildew resistance	*er-2*	(SCAR) 3 AFLP primers	Tiwari et al. (1998)
Powdery mildew resistance	*er-1*	Sc-OPO-18_{1200}, Sc-OPE-16_{1600}	Frew et al. (2002)
Mycosphaerella pinodes resistance	*mp*	ccta2 (SSR), cccc1 (SSR), *acct1* (SSR)	Dita et al. (2006)
Powdery mildew resistance	*er1 er2 er3*	MAS	Ghafoor and McPhee (2012)
Pea enation mosaic virus (PEMV)	*en*	EST, MAS, RAPD, SSR, STS, TRAP,	Jain et al. (2013)
Ascochyta blight resistance	*abI-IV-2.1*	SNP	Jha et al. (2017)
Mycosphaerella blight resistance	*QTLs*	SNP	Gali et al. (2018)
Ascochyta blight resistance	*QTLs*	SNP	Carpenter et al. (2018)
Fusarium root rot resistance	*Fsp-Ps 2.1*	Ps900203	Coyne et al. (2019)

9.3.3.1 Ex Situ Conservation

The ex situ conservation of plant genetic resources began in the twentieth century as a response to the rapid loss of biodiversity and the replacement of local varieties with developed genotypes (Gepts 2006; Khoury et al. 2014; Van de Wouw et al. 2009). This replacement was done with the introduction of advanced machinery, herbicides, pesticides, fertilizers into agrarian systems that allowed the cultivation of improved varieties everywhere (Khoury et al. 2016).

A large amount of ex situ *Pisum sativum* germplasm has been collected and preserved around the world in numerous agricultural centers. These centers and the international consortium for pea genetic resources (Pea GRIC) collaborate to link key collections in Europe, the USA, Africa, Asia and Australia. In India, about 2000 pea germplasm accessions are conserved at the National Bureau of Plant Genetic Resources (NBGPGR), Indian Institute of Vegetable Research (IIVR) and Indian

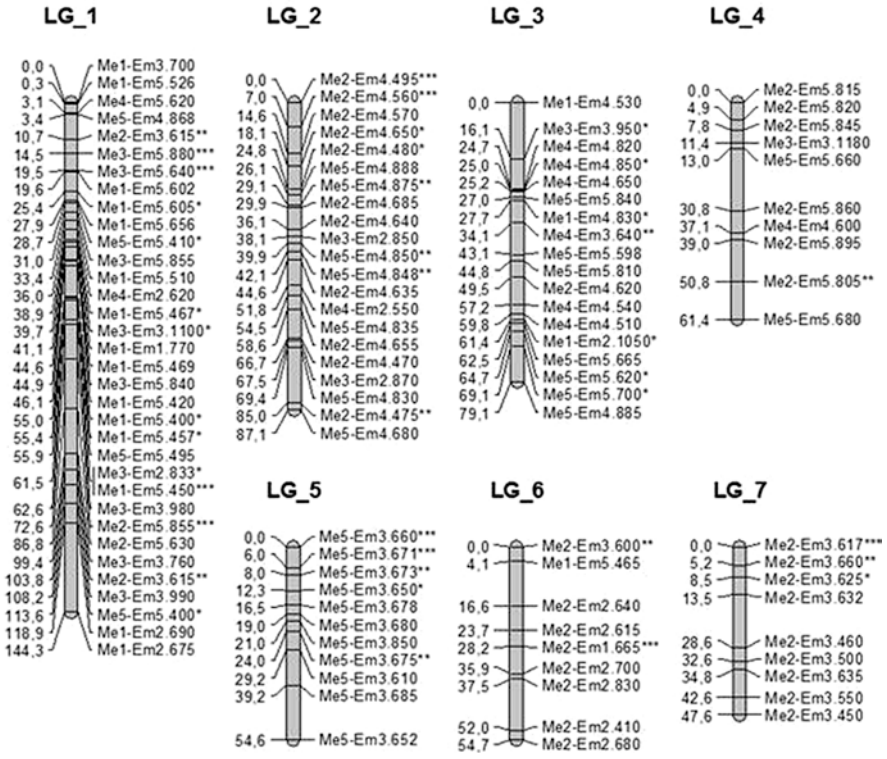

Fig. 9.7 *Pisum sativum* consensus functional map. (Source: Guindon et al. 2016)

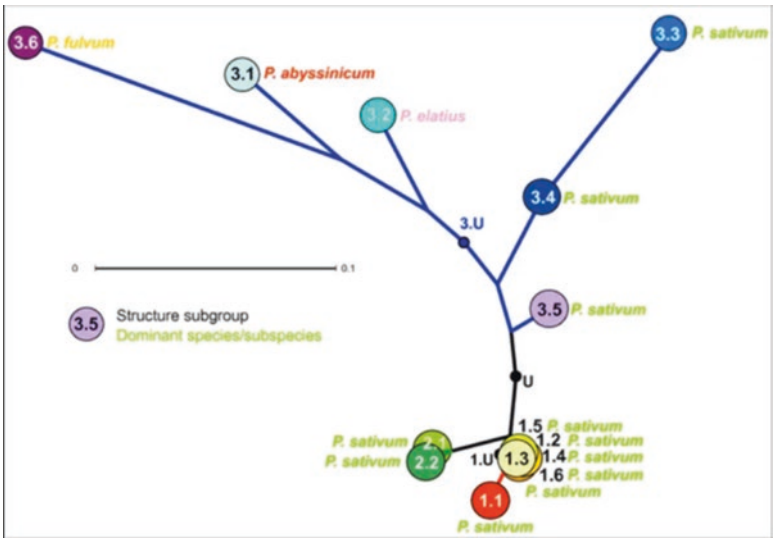

Fig. 9.8 *Pisum* genus diversity and phylogeny. (Source: Jing et al. 2010)

Table 9.4 Number of *Pisum* accessions conserved ex-situ in major collections

Species	AARI	ATFC	ICARDA	IPK	JI	NGB	W-6	VIR
P. sativum var. *sativum*	10	3683	882	2384	1680	1150	3718	6509
P. sativum var. *arvense*	15	13	ND	ND	ND	ND	58	ND
P. sativum var. *elatius*	8	17	10	15	31	8	51	3
P. sativum var. *brevipedunculatum*	1	ND	ND	ND	ND	ND	ND	ND
P. sativum var. *pumilio*	2	7	1	0	4	2	24	0
P. abyssinicum	4	16	6	41	33	4	17	4
P. fulvum	2	53	31	4	55	10	48	2
P. formosa	0	0	0	0	0	1	0	5
Total	42	3789	930	2444	1803	1175	3916	6523

AARI Aegean Agricultural Research institute, Turkey, *ATFC* Australian Temperate Field Crop, Australia, *ICARDA* International Center for Agricultural Research in the Dry Areas, Syria, *IPK* Leibniz-Institut für Pflanzengenetik und Kulturpflanzenforschung, Gatersleben, Germany, *JI* John Innes Centre, Department of Applied Genetic, UK, *NGB* Nordic Gene Bank, Sweden, *W-6* The Western Regional Plant Introduction Station, USA, *VIR* Vavilov Research Institute of Plant Industry, St. Petersburg, Russia, *ND* not differentiated

Institute of Pulses Research (IIPR), Kanpur. Besides, a few state agricultural universities are rich in vegetable pea germplasm such as Punjab Agricultural University.

Details about the status and the composition of the conserved *Pisum* species is documented by the World Information and Early Warning System on Plant Genetic Resources (WIEWS), which contains information on national PGR holdings (www.fao.org/ag/agp/pgr/'"iews/) and System-wide Information Network for Genetic Resources (SINGER) which contains information on CGIAR holdings (http://www.cgiar.org/singcr) (Maxted and Ambrose 2001) (Table 9.4).

9.3.3.2 Cryopreservation

Cryopreservation is described as preserving biological samples and storing them at especially low temperatures by using liquid nitrogen (Berjak et al. 1995; Haskins and Kartha 1980; Kartha and Engelmann 1994; McAdams et al. 1991). Low temperature preserves samples by slowing their metabolic processes and prevent deterioration of tissue (Jang et al. 2017; Kartha 1981). At present, there is no international center for pea breeding and genetic conservation (Flavell et al. 2011). No released collection is of great size or diversity. Data have been published on diverse groups of *Pisum* containing more than 2000 accessions in national gene banks of various countries (Table 9.5) (Ambrose et al. 2011; Ford-Lloyd et al. 2010; Miles et al. 2011). There is a high level of duplication among collections, which creates a deceptive impression of the true level of diversity (Ambrose et al. 2011; Miles et al. 2011). A list of important world gene banks for pea genetic resources conservation is given in Table 9.5.

Table 9.5 List of important gene banks for pea genetic resources

Gene Bank	Country	Website
Institute of Botany NAS RA, Yerevan	Armenia	http://www.botany.sci.am/
Winter Cereal Collection Gene Bank	Australia	www.dpi.nsw.gov.au/about-us/research-development/centres/
National Center for Vegetable Crops Research (CNPH) EMBRAPA	Brazil	http://www.cnph.embrapa.br
Institute for Plant Genetic Resources	Bulgaria	https://www.genesys-pgr.org/ar/wiews/BGR001
Institute of Crop Science, Chinese Academy of Agricultural Sciences	China	http://www.cgris.net/icgr/icgr_english.html
Czech Republic Gene Bank	Czech Republic	http://genbank.vurv.cz/genetic/resources/asp2/default_a.htm
Plant Gene Resources of Canada, Saskatchewan,	Canada	http://www.agr.gc.ca/pgrc-rpc
Ethiopian Biodiversity Institute (EBI)	Ethiopia	http://www.ebi.gov.et/
Centre de RessourcesGénétiques des Légumineuses, INRA-UMR LEG Dijon	France	http://www.reseau-graines.org/platform.htm
Leibniz-InstitutfürPflanzengenetik und Kulturpflanzenforschung (IPK)	Germany	https://www.ipk-gatersleben.de/en/gbisipk-gaterslebendegbis-i/
Institute for Agrobotany, Tapioszele	Hungary	http://www.nodik.org/english/
ICAR-National Bureau of Plant Genetic Resources	India	http://www.nbpgr.ernet.in
Institute of Biosciences and Bioresources	Italy	http://www.igv.cnr.it
Genetics Resources for Wheat Sciences Gene Bank	Japan	https://shigen.nig.ac.jp/wheat/komugi/
Centre for Genetic Resources, Wageningen	Netherlands	https://www.wur.nl/en/Research-Results/Statutory-research-tasks/Centre-for-Genetic-Resources-the-Netherlands-1/Expertise-areas/Plant-Genetic-Resources.htm
Plant Breeding and Acclimatization Institute Blonie Radzikow	Poland	http://www.igr.poznan.pl/
N.I. Vavilov Research Institute of Plant Industry, St. Petersburg	Russia	http://www.vir.nw.ru
Instituto Tecnológico Agrario de Castilla y León	Spain	http://www.itacyl.es

(continued)

Table 9.5 (continued)

Gene Bank	Country	Website
International Center for Agricultural Research in the Dry Areas (ICARDA), Gene Bank	Syria	http://www.icarda.org/
Nordic Genetic Resource Centre, NordGen	Sweden	http://www.nordgen.org/sesto
Yurjev Institute of Plant Breeding, Kharkov	Ukraine	http://www.bionet.nsc.ru
John Innes Centre, Norwich	UK	http://www.jic.ac.uk
USA Plant Germplasm Introduction and Testing Research Station, Pullman	USA	https://www.ars-grin.gov

9.3.3.3 In Vitro Conservation

In vitro conservation of *Pisum sativum* can be accomplished by somatic embryogenesis or organogenesis from callus cultures (Bala et al. 2010). Using organogenesis to induce shoot, root, and callus production was achieved in an Egyptian genotype of P. *sativum*. Calli were initiated from hypocotyl, leaf, root and mature embryo explants then cultured on MS medium Murashige and Skoog (1962) with some supplementation (Ghanem et al. 1996). Durieu and Ochatt (2000) tested protoplast fusion and regeneration of *P. sativum*.

9.3.3.4 DNA and Seed Banks

Gene banks were established in the mid twentieth century to preserve agricultural biodiversity when landraces began to be replaced by improved varieties (Díez et al. 2018). The major objective was to conserve biodiversity for future breeding programs (Fowler and Hodgkin 2004). Gene banks are a means of long-term preservation of genetic resources by extraction and preservation of DNA from wild and cultivated plants, and even from endangered or fossilized plant specimens (Rogers and Bendich 1985). Some important DNA gene banks for plant genetic resources conservation are listed in Table 9.6.

The most common method for storing DNA is by dissolving it in a TE buffer and storing at −80 °C or in alcohol (Mandal 1995; Mandal et al. 2000). The Svalbard global seed vault project is in an area of permafrost 1300 km north of the Arctic Circle and is the world's largest secure seed storage facility. Seeds of many of the world's legume crops are kept in this and other gene banks (Foyer et al. 2016).

9.3.3.5 Cytogenetics

All taxa of *Pisum* are diploid (2x, 2n = 14) (Smýkal et al. 2012). Samatadze et al. (2008) mention that C-banding patterns of some *P. sativum* varieties showed differences in chromosome size, the appearance of satellites and polymorphisms in heterochromatin bands located near the nucleolus-organizing regions (Fig. 9.9).

Some pea chromosomes have secondary constriction (satellites), which give valuable cytogenetic markers, facilitating differentiation between various species (Navrátilová et al. 2005; Neumann et al. 2002). The standard pea karyotype consists of seven chromosomes, five acrocentric chromosomes and two (4 and 7) with satellites (Neumann et al. 2002) (Fig. 9.10).

Samatadze et al. (2018) studied the peculiarities of meiosis, the distribution of C-heterochromatin (C-HC), and the activity of nuclei-regulating regions (NORs) of chromosomes. It was noted that meiosis analysis did not reveal any significant violations in *space* plant cells and that the total amount of C-HC did not differ significantly from control, despite the multiple-scale chromosome patterns.

Table 9.6 List of important DNA Gene banks for plant genetic resources conservation

DNA bank	Website
Australian Plant DNA Bank (APDB), Centre for Plant Conservation Genetics, Southern Cross University, Lismore, NSW, Australia	http://www.dnabank.com.au
Botanic Garden and Botanic Museum (BGBM) DNA Bank, Berlin, Germany	http://www.bgbm.org/bgbm/research/dna/
DNA Bank Brazilian Flora Species, Rio de Janeiro Botanic Garden, Brazil	http://www.jbrj.gov.br/pesquisa/div_molecular/bancodna/index.htm
DNA Bank at Kirstenbosch, South African National Biodiversity Institute, Kirstenbosch, South Africa	http://www.nbi.ac.za/research/dnabank.htm
International Rice Research Institute (IRRI), DNA Bank, Philippines	http://www.irri.org/GRC/GRChome/Home.htm
Missouri Botanic Garden DNA Bank, (MBGDB) St Louis, MO, USA	http://www.mobot.org/MOBOT/research/diversity/dna_banking.htm
National Bureau of Plant Genetic Resources (NBPGR). New Delhi, India	http://www.nbpgr.ernet.in/
National Herbarium Netherlands DNA Bank (NHNDB), The Netherlands	http://www.nationalherbarioum.nl/taskforcemolecular/dna_bank.htm
National Institute of Agrobiological Science (NIAS) DNA Bank, Tsukuba, Ibaraki, Japan	http://www.dna.affre.go.jp/
Plant DNA Bank Korea (PDBK), Graduate School of Biotechnology, Korea University, Seoul, Korea	http://www.pdbk.korea.ac.kr/index.asp
Royal Botanic Garden Edinburgh DNA Bank, Edinburgh, Scotland	http://www.rbge.org.uk/rbge/web/science/research/
Royal Botanic Garden Kew DNA bank, Richmond, England	http://www.rbgkew.org.uk/data/dnaBank/
TCD DNA Bank, Department of Botany, School of Natural Sciences, Trinity College, Ireland	http://www.dnabank.bot.ted.ie
Tropical Plant DNA Bank, Fairchild Tropical Botanical Garden and Florida International University, FL, USA	http://www.ftg.org/research

9.4 Traditional Breeding

Traditional breeding of *Pisum sativum* needs to be improved because of the high cost and effort needed for its application in terms of soil preparation, use of pesticides and fertilizer, weed control and the selection of seeds that can give good quality and yield.

9.4.1 Improvement Strategies

New pea cultivars are needed to provide lodging resistance, powdery mildew resistance, and to contend with yield quality and consistency (Warkentin et al. 2015). The dissection of these into the attributes of their component characters is needed,

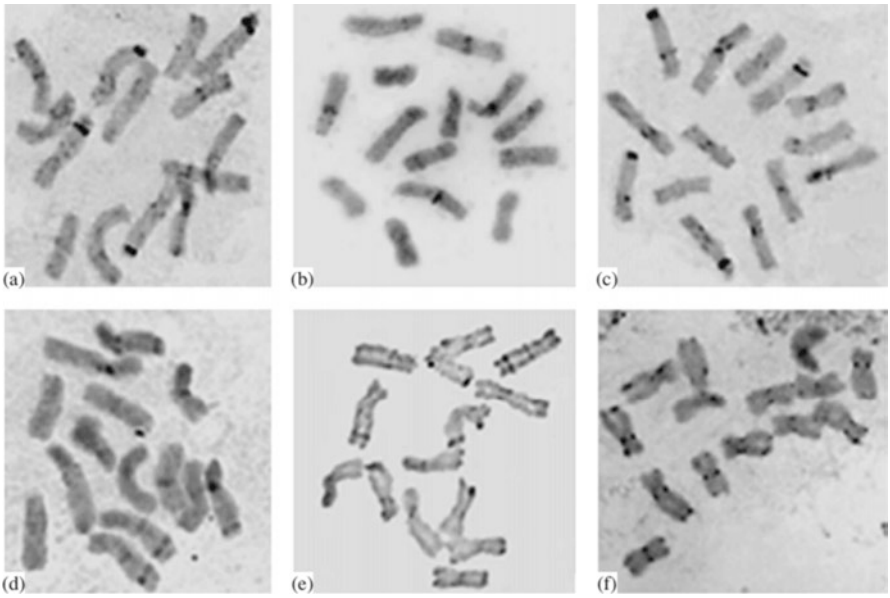

Fig. 9.9 C-metaphase banding patterns in different varieties of *Pisum sativum.* (Source: Samatadze et al. 2008)

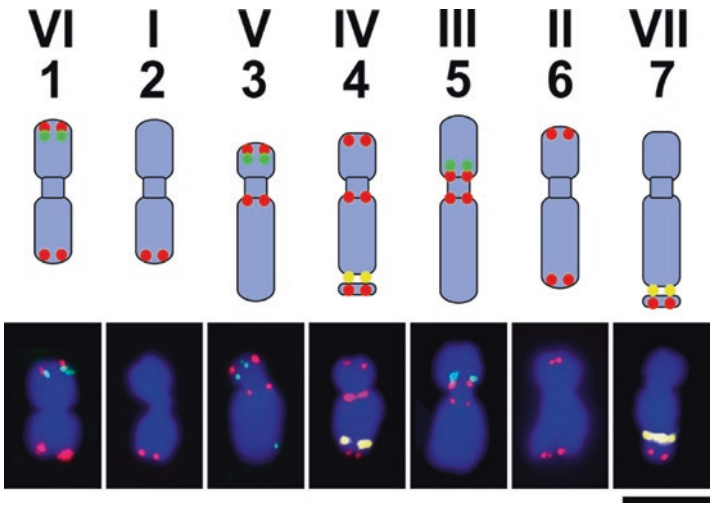

Fig. 9.10 The pea karyotype. (Source: Neumann et al. 2002)

and a continuous review of new information and resources with the point of view of their application or integration into hybridization programs. The ever-present challenges of biotic and non-biotic stresses take high precedent for action, along with changing climatic patterns in many regions of the world; together these factors increase the challenges of pea crop management (Warkentin et al. 2015).

9.4.2 Methodologies and Limitations

The most common breeding strategies used for pea improvement are selection (Mital and Verma 1991; Qasim et al. 2002; Vikas et al. 1996), pedigree, backcross (Aryamanesh et al. 2012; Clement et al. 2009) bulk selection (Kuo 1999) and single seed descent. These strategies aim to maintain desirable characters, high green pod yield, good quality attributes, sweetness, tolerance to abiotic stresses, suitability for canning and freezing and disease resistant cultivars (Gritton 1986; Kumar et al. 2015; Simakov 1989).

Genetic transformation in *Pisum* is not easy (Warkentin et al. 2015) and plant regeneration is difficult (Švábová and Griga 2008). Therefore, to discover the molecular bases underlying agriculturally evaluated characters, information is needed about the gene sequences of genomic regions which control traits of interest.

The use of next-generation sequencing technologies such as genotyping, transcriptome, and gene and genome mapping genetic resources, will contribute to pea breeding (Tayeh et al. 2015).

9.4.3 Role of Biotechnology

Biotechnology has developed rapidly in recent years and one of the benefits is the production of genetically-modified organisms (GMOs). GMO crops are tastier and healthier when grown without using pesticides or fertilizers, give high yield, have tolerance to abiotic stresses and resistance to many diseases (Kirakosyan and Kaufman 2009; Nielsen 2005) and have improved mycorrhizal and root nodule symbioses (Leppyanen et al. 2019). Metabolic engineering is another important application of biotechnology. Cells can achieve higher growth activity by a growth-based selection process (Fong et al. 2005; Jantama et al. 2008).

9.5 Molecular Breeding

9.5.1 Molecular Marker-Assisted Breeding

In recent years, pea breeding programs have begun to use PCR-based markers to reveal polymorphisms, facilitating the development of molecular maps for pea traits. Marker-assisted selection can utilize favorable gene combinations for desirable traits (Bohra et al. 2014; Collard and Mackill 2008).

Pea linkage maps containing molecular markers have been published (Dirlewanger et al. 1994; Ellis et al. 1992; Weeden et al. 1998), which help researchers characterize quantitative trait loci (QTLs) for seed weight and green seed color (Timmerman-Vaughan et al. 1997). Molecular markers have been used, including random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) (Sreedevi et al. 2009), sequence tagged sites (STS) and simple sequence repeats (SSR) (Hanci 2019).

DNA markers linked to recessive genes for resistance to *Pisum sativum* diseases have been identified like seed-borne mosaic virus (PSbMV) pathotype P-1 (Timmerman-Vaughan et al. 1993) and powdery mildew fungus (Timmerman-Vaughan et al. 1994). Current advances in molecular markers and marker-assisted selection (MAS), together with the advances in powerful new *omic* technologies, show excellent potential to develop new breeding varieties (Vignesh et al. 2011).

The rapid development of next-generation sequencing (NGS) technologies helped in the study of pea genetics. Whole-genome sequencing of *Pisum* is incomplete, but transcriptome research provides important information to create gene-based molecular markers and in building high-resolution genetic maps (Duarte et al. 2014; Gali et al. 2019; Leonforte et al. 2013; Sindhu et al. 2014; Tayeh et al. 2015).

9.5.2 Functional Genomics

Functional genomics describes the genomic and transcriptomics of an organism. It focuses on gene transcription, translation, gene expression and protein-protein interactions (Gibson and Muse 2009; Pevsner 2009). Development of NGS technologies allowed designing and mapping numerous gene-based molecular markers in pea (Aubert et al. 2006; Bordat et al. 2011) and in identifying many single nucleotide polymorphism sites (SNPs) across *Pisum* species and construction of its genetic maps (Boutet et al. 2016; Duarte et al. 2014; Kaur et al. 2012; Leonforte et al. 2013; Ma et al. 2017; Sindhu et al. 2014; Tayeh et al. 2015; Yang et al. 2015; Zhernakov et al. 2017).

ESTs (expressed sequence tags) is a valuable technique used to discover new genes and also provide a resource to develop markers in *Pisum sativum* (Davey et al. 2011; Gong et al. 2010; Zhuang et al. 2013) proving that NGS is an efficient tool to rapidly improve EST-derived SSR markers. These new EST-SSR markers will be

important tools for marker-assisted breeding, development of genetic linkage maps and the comparative mapping of *P. sativum*.

9.5.3 Bioinformatics

Gathering abundant genetics and genomics data about important crops provides a reliable source for using marker-assisted selection (MAS) and genomics-assisted breeding (GAB) for crop improvement (Bohra et al. 2014; Collard and Mackill 2008). Therefore, the convenience of genetic and genomic data is very dependent on the possibility to merge several sorts of these resource data (Appendix I-B). Despite the existence of many marker databases, they are not able of providing large quantities of data in an easy and suitable method.

The Pea Marker Database (PMD) has facilitated marker development and gene mapping by gathering data for pea gene-based markers into one database with a clear and easy-to-use interface (Kulaeva et al. 2017; Tayeh et al. 2015). It comprises two versions, (i) PMD1 contains about 2484 genetic markers, their positions in linkage groups, the sequences of corresponding pea transcripts and (ii) PMD2 an updated version including 15,944 pea markers in a similar format with numerous advanced features.

9.6 Tissue Culture

9.6.1 Micropropagation

Plant cell structure has been used in plant pathology (Braun 1974), plant morphogenesis, plant micropropagation, cytogenetics and plant breeding. Protoplast culture has been used in investigations of cell wall biosynthesis, somatic cell hybridization and genome manipulation (Power et al. 1970).

Plant biotechnology has furthered research by plant physiologists, plant breeders, botanists, agronomists, biochemists and pharmacists. The principal reasons for using biotechnology are to develop new genotypes that are resistant to biotic and abiotic stresses, have improved field-crop yield, enhanced seed germination for plant propagation and to advance the use of natural products produced by plants to satisfy human needs (Grant and Cooper 2006; Grant et al. 2003). Grant and Cooper (2006) used MS medium supplemented with 1.3 mg/L BA, 30 g/L sucrose, 8 g/L agar (Bacto), pH 5.8 and 200 mg/L timentin for pea multiplication.

9.6.2 Embryo Rescue

To create homozygous pea populations, Surma et al. (2013) examined the suitable conditions for the culture of pea embryo as the initial step to generate an in-vitro assisted single seed descent. Embryos separated from mature green seeds and cultured in vitro on modified MS media (Murashige and Skoog 1962) at 20–22 °C (day/night) to achieve shoot and root development (Fig. 9.11).

Transformation of peas was successfully achieved by Grant and Cooper (2006). An *Agrobacterium tumefaciens* strain AGL1 containing the desired construct was used. Separating immature embryos from cotyledons and cultured on B5 supplement with 1.3 mg/L 6-benzylaminopurine (BA), 30 g/L sucrose, 8 g/L agar (Bacto), pH 5.5 and 20 mg/L acetosyringone, BAP and NAA until reaching plantlet regeneration, as shown in Fig. 9.12. B5 medium supplemented with 1.3 mg/L BA, 30 g/L sucrose, 8 g/L agar (Bacto), pH 5.8, 200 mg/L timentin and 75 mg/L kanamycin sulfate were used for regeneration. B5 medium supplemented with 30 g/L sucrose, 8 g/L agar (Bacto), pH 5.8, 15 mg/L indole acetic acid and 200 mg/L timentin were used for rooting. B5 medium supplemented with 30 g/L sucrose, 8 g/L agar (Bacto), pH 5.8, 50 mg/L kanamycin sulfate, and 200 mg/L timentin were used for root selection. B5 medium supplemented with 30 g/L sucrose, 8 g/L agar (Bacto), pH 5.8, and 200 mg/L timentin were used for root elongation. However, MS medium supplemented with 1.3 mg/L BA, 30 g/L sucrose, 8 g/L agar (Bacto), pH 5.8, and

Fig. 9.11 Embryo-regenerated pea plants. (Source: Surma et al. 2013)

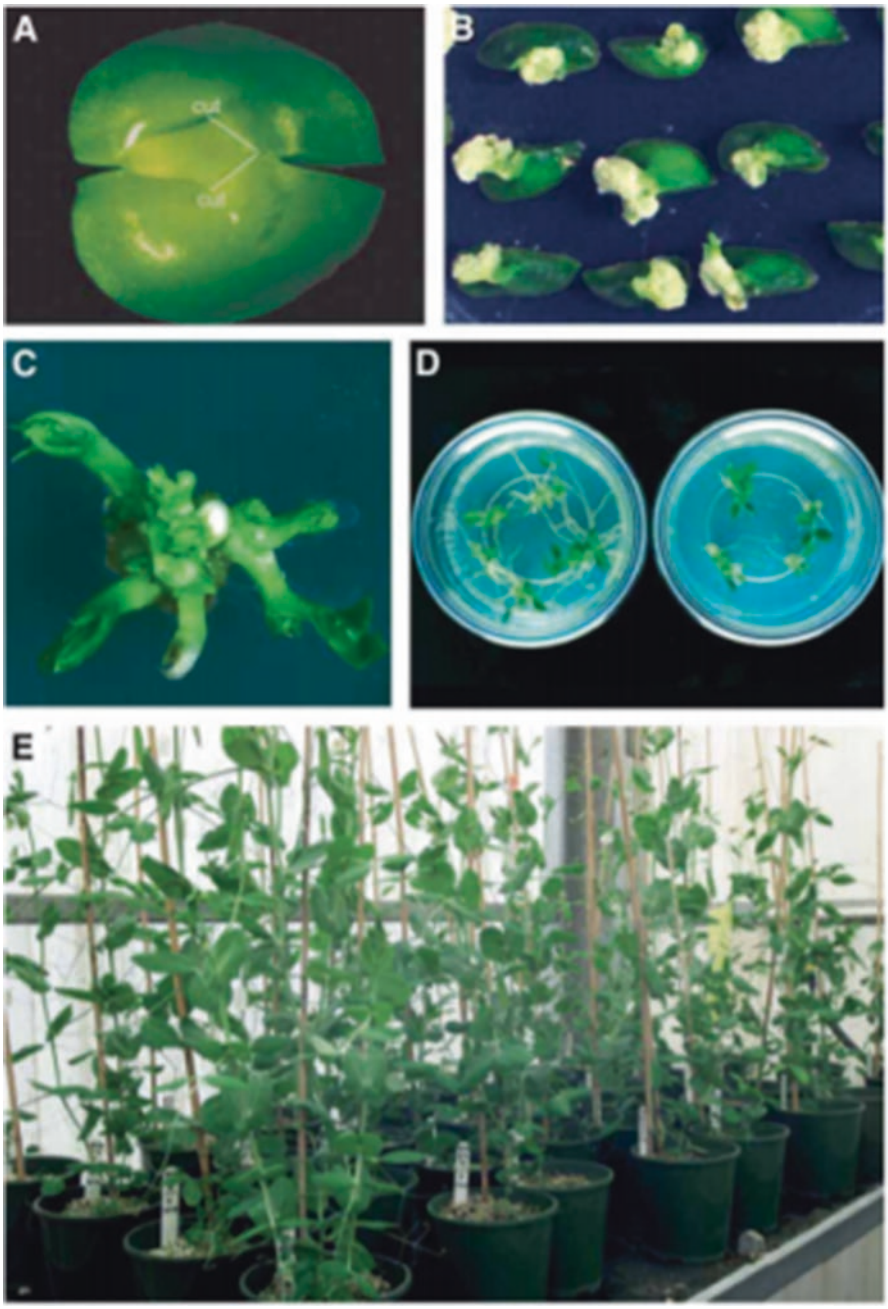

Fig. 9.12 Steps for transformation of pea. (Source: Grant and Cooper 2006)

200 mg/L timentin were used for multiplication. The frequency of regeneration depended on the genotype. Natali and Cavallini (1987a, b) demonstrated de novo origin of the shoots via organogenesis in pea, the chromosome number of regenerated plantlets showed a range of 12–16.

9.7 Genetic Engineering and Gene Editing

Genetic engineering is playing an important role in the development of plant biotechnology. Plant biotechnology aims to improve productivity, the growth of many crops and developing new crops through gene editing and metabolic engineering.

EST-derived simple sequence repeat (eSSR) markers have become an important tool for pea gene discovery and comparative mapping studies (Decarie et al. 2012). The proteome of mitochondria in mature leaves and stems of pea were analyzed by Schiltz et al. (2004).

Most genes and QTLs that account for the domestication of pea and responsible for the modifications of plant form and function have been identified (Weeden 2018). Significant contributions to the qualitative genetics of peas have been made by several scientists. Blixt (1974) has given a list of 324 qualitative genes. Vignesh et al. (2011) listed QTLs identified for different polygenic traits in pea, as shown in Table 9.7.

There is an urgent need to increase agricultural production through innovative breeding technology to increase the supply of nutritious foods worldwide. Recent advances in genome editing in CRISPR/Cas technology offer effective targeted modification in most crops (Shimatani et al. 2017), and promise to accelerate crop improvement (Chen et al. 2019). Basic editing tools that allow targeted nucleotide alternatives and describe different delivery systems, especially DNA-free methods,

Table 9.7 List of QTLs identified for different traits in *Pisum sativum*

Trait QTL	References
Green seed color	McCallum et al. (1997)
Seed weight	Timmerman-Vaughan et al. (1996)
Leaf shape	Villani and DeMason (2000)
Grain yield, seed protein and maturity	Taran et al. (2004)
Lodging resistance	Zhang et al. (2006)
Winter hardiness	Weller et al. (2012)
Frost tolerance	Dumont et al. (2009)
Ascochyta blight resistance	Hamon et al. (2011)
Salinity tolerance	Leonforte et al. (2013)
Seed mineral	Ma et al. (2017)
Agronomic traits (flowering, maturity, lodging) and seed quality traits (seed weight, grain yield)	Gali et al. (2018)

have been linked to genome editing in crop breeding (Komor et al. 2016). Genome editing applications are used to improve properties, develop gene control regulation, virus reproduction strategies and use highly productive mutant libraries (Huang et al. 2018). The future perspective is one of genome editing in plant synthetic biology and domestication, advances in delivery systems, the specificity of editing, symmetric repair and gene engines. Finally, there are challenges and opportunities for raising smaller plants and their bright future in agriculture.

9.7.1 Methodologies and Enhanced Traits

Recently, several studies have been published on pea systematics, seed quality and breeding (Arnoldi et al. 2015; Bohra et al. 2014; Dahl et al. 2012; Smýkal et al. 2015; Varshney et al. 2015). Genetics in peas develops rapidly from regular to larger methods with the help of molecular-assisted approaches to detect the molecular bases of important traits and enhance breeding. The single seed descent (SSD) technique, in combination with immature embryo culture, is applied to shorten the breeding time. Currently, SSD populations are commonly used to replace other techniques in genetic and genomic investigations (Kuchel et al. 2006; Marza et al. 2005).

9.7.2 Transgenic Cultivars

In recent years, transgenics has played an important role in improving traits that help pea plants tolerate abiotic stresses. A reproducible transgenic *Pisum sativum* plant was developed by using explants from the embryonic axis of immature seeds (Schroeder et al. 1993). Discovering genes and their functions have helped to understand the mechanisms of genes in plants under stress, which improved the productivity of pea crops under different abiotic stresses (Ali et al. 2018). Timmerman-Vaughan et al. (2001) demonstrated that transgenic pea plants can partially resist AMV (alfalfa mosaic virus). Gene editing will permit using data in gene banks more effectively and rapidly and contribute to a better explanation of their functioning (Díez et al. 2018). Transgenic *P. sativum* will need to be productive enough to justify the high costs and time involved in bringing genetically-modified peas to the market (Kahlon et al. 2018).

9.8 Mutation Breeding

Programs to increase the genetic improvement of peas using mutations were initiated in the 1940s by Gelin (1954, 1955). Methods of pea mutation are the same as those used in other annual crops (Anonymous 1980, 1982, 1983, 1984). Seeds are usually treated either with X-rays, gamma rays or chemical mutagens (Aney 2013). The highest rate of mutations was obtained using ethyl methanesulphonate (EMS), ethylene amine (EI), methyl nitroso urea (MNU), N-nitroso-N-methyl urea (NMU) and ethyl nitroso urea (ENU). Mendelian pea breeding is dedicated to inheriting seven evolutionary mutations. All the models Mendel used for crosses were available on the market; he used development anomalies to improve crops long before the concepts of genes or mutation emerged. Recently, only four of the seven Mendelian mutations have been discovered at the molecular level (Reid and Ross 2011).

9.8.1 Mutagenesis

Worldwide, there are about 3500 mutations of many morphological traits in the pea, such as stem, root, flowers and other traits, found in Sweden, Germany, Poland, Italy, India, Russia and the Netherlands (Hofer et al. 2009; Sagan et al. 1994; gan and Duc 1996; Wang et al. 2008). These mutations affect many morphological traits. Mutations have strong effects on germination, growth inhibition and infertility. Several environmental factors modulate radiosensitivity, such as seed moisture, oxygen and temperature before, during and after irradiation, as well as growth conditions, especially during the germination of treated seeds (Anonymous 1977; Badr et al. 1975; Blixt 1972; Hussein et al. 1974; Sharma and Kharkwal 1983). Seeds may be soaked in an aqueous solution for 12–16 h. Uncoated seeds are easily mutated in tissues and cells and shorten the treatment time are considered optimal. The temperature to induce mutations is 21–24 °C. The time required for radiation exposure may vary from 0.5 to 24 h, but 2 to 4 h have been shown to be enough to cause mutations. The recommended concentrations in aqueous solutions range from 0.05 to 0.3% FAO/IAEA (2018).

A clear change in the growth habit observed in mutants is age or laziness. Ageotropic or *lazy* mutations were found in peas in the late 1930s and described in the review by Howard et al. (2014).

9.8.2 *In Vitro Mutagenesis and Selection*

Sharma et al. (2009) reported that mutation induction is effective to improve the yield of pea seeds to produce the M_1 generation. A broad range of mutations in chlorophyll content and agronomic mutations were found in M_2 generations (The rate of chlorophyll mutation in generations M_2 has been increased by increased gamma radiation dose). As well as the chlorophyll mutations, the rate of xantha type (pale yellow seedlings) was greater followed by chlorina (yellowing of leaves) and albino type (exactly chlorophyll-free) mutants. In general, 0.3% EMS treatment was the most efficient in producing desired mutations at the highest rate. Over the years, desirable mutations have been separated from long and dark green pods, three or more flowers or pods on the stem, branching abundance and pod, short internal, dark green pods and male infertility of various treatments. Fatal or biotic injury was exhibited in low germination, increases with a higher dose of gamma rays and EMS. The efficiency of the mutation's effectiveness is normally increased by a rising EMS dose. Mutation breeding can play a role in improving peas to cause a positive variation of needed qualities to develop promising genotypes.

Sinjushin (2013) reported that studies on some forms in peas seem promising to reveal this unique and interesting aspect of plant development. Certainly, bridging this gap can use genomic and post genomic approaches. Targeted mutations and correct gene expression based on knowledge of the structure of some genes can serve to further improve this valuable crop culture (Sinjushin 2013).

9.8.3 *Molecular Analysis*

The development of genetically-representative collections of single or limited groups of characters is a recent activity dating back to the end of the nineteenth century. Lists were compiled of older collections of 21 pairs of pea lines cultivated for contradictory traits including plant shape, leaves, flowers and seeds that were genetically researched in a set greater than 550 genotypes (Hofer et al. 2009; Sagan and Duc 1996; Sagan et al. 1994; De Vilmorin 1911; Wang et al. 2008). Induced mutations have become widespread as a means of promoting mutation rates for developing new genetic variance for selection and the importance of using induced mutants. In legumes, development programs are still recognized (Blixt 1972; Dalmais et al. 2008; Duc and Messager 1989; Kharkwal et al. 2010; Sagan et al. 1994). The main mutant pea groups include: (i) John Innes Collection, Norwich, UK (575 accessions); (ii) IPGR group, Plovdiv, Bulgaria (122 accessions); (iii) TILLING-induced localized lesions with 4817 lines (1840 described by phenotype) and (iv) 93 symbiotic mutations for 26 genes participate in nitrogen fixation (Hofer et al. 2009; McAdam et al. 2018; Sagan and Duc 1996; Sagan et al. 1994; Wang et al. 2008).

9.8.4 Enhanced Traits and Improved Cultivars

Induced mutations were used to obtain direct mutations or in hybridization (Ahloowalia et al. 2004) to overcome yield plateaus and generate the required morphological traits. Mutation breeding programs have significantly increased plant development, leading to the release of at least 2250 genotypes of various crops. For example, in India, at least 300 genotypes have been improved in at least 55 plant species (Kharkwal et al. 2004). The effectiveness of mutagenesis used in mutagenesis programs needs to be explained. The success of the mutation breeding program depends on improved testing procedures to isolate desired mutations, which appear at very low rates, among many other mutations of small reproductive importance (Solanki and Sharma 2002). Analysis of induced variance of chlorophyll and potential morphological mutations in the M_2 generation was the most reliable tool for using valuable mutations for effective crop development (Kumar et al. 2007). The number of known and characterized mutations is strongly disproportional for different categories. For example, 66 mutations influencing the leaf development are listed in the PGene database (Zelenov et al. 2008).

Over the past 20 years, traditional breeding programs have made important contributions in developing pea varieties. A yield increase of approximately 2% was achieved annually (Warkentin et al. 2015). Lodging resistance was improved by selection for stem strength (Banniza et al. 2005). Varieties adapted to winter sowing have been developed and disseminated in Europe and the northwestern USA, providing the possibility to achieve better yields due to the length of the growing season, higher biomass production and early maturation, to avoid late-season drought and heat stress (Hanocq et al. 2009). Quantitative inheritance, transgressive segregation and heritability have been moderately high for seed color, shape and dimpling (Ubayasena et al. 2011) allowing good progress in breeding programs. For example, the seed protein concentration was maintained in pea varieties (Jha et al. 2013).

9.9 Hybridization

9.9.1 Conventional Hybridization

In general, landrace collections are protectors of genetic variability and sources of many valuable genes, especially those for adaptation (Chahal and Gosal 2002). They are used either for release after selection for high yield and wide or specific adaptation or crossed with exotic materials. In the absence of the desired variability from existing materials, hybridization is the best method to create variability (Lakić et al. 2019). In most cases, the exotic materials, with desirable characters (large seed size, white/green seed color, erect plant stature) but not adaptable, will be crossed with the local adapted materials, but lack some useful characters. Based on the

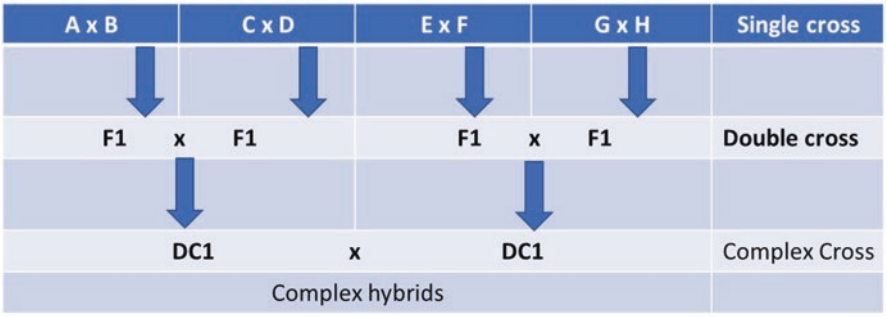

Fig. 9.13 Type of hybrids in pea breeding program for high yield and its component traits (A, B, C, D, E, F, G, H are different pure line parents). (Prepared by Khaled F.M. Salem)

taxonomic relationship between the two parents, hybridization may be intraspecific or interspecific. When the parents involved belong to the same species, it is referred to as intraspecific or intervarietal hybridization. Contrarily, when the parents involved belong to different species within a genus, it is referred to as interspecific or distant hybridization. Crosses can be single (A x B), when only two parents are involved, or multiple, when more than two parents are involved (Fig. 9.13). If three parents are involved, it is referred to as a three-way cross [(A × B) × C]. Similarly, if four parents are involved, then it is referred to as a double-cross [(A × B) × (C × D)], and so on. When the desirable characters are distributed among several parents, to bring all the desired traits into a single genotype, multiple crossing needs to be employed (Fig. 9.13). For instance, a series of crosses are required to bring the desired traits distributed among eight separate parents into a single genotype (Fig. 9.13). Trials to produce F_1 hybrids have been successful. Thus, hybridization relies on three principles: (i) pedigree method, (ii) backcross (Aryamanesh et al. 2012; Clement et al. 2009) and (iii) single-plant or mass selection (Mital and Verma 1991; Qasim et al. 2002; Vikas et al. 1996). To ensure the flowering of all plants, different parents are staggered (Acquaah 2012).

On sunny and warm days, flowers open in the morning; on cloudy and cool days it is delayed. Crossing can be carried out according to Jarso et al. (2009). F_2 seeds can be produced by the selfing of a single F_1 plant. F_2 seeds are sown and 200 seedlings are randomly selected, transplanted and genotyped. The plants are self-harvesting and the resulting F_3 seeds are harvested for each F_2 plant.

9.9.2 Somatic Cell Hybridization

Since methods have been established to incorporate protoplasts and assimilate DNA and external organelles by protoplasts, emphasis has been on physical hybridization in higher plants. Polyethylene glycol (PEG) is an effective catalyst for fusion (Constabel 1984). Protoplast treatment with PEG produces especially

heterogeneous fusion products of 5–30%. Protoplasts of various species, races, and even families were matching at fusion. A number of protoplast sets (soybeans/corn, soybeans/peas, soybeans/tobacco, carrots/barley) initiated fusion products that exhibit constant cell division and callus formation. Fusion products showed heterogeneity at first. In heterogeneous cell division, it was observed that the random distribution of the mitotic nucleus is accompanied by a multi-wall formation and is caused by cerebral callus. The nucleus of juxtaposition proposed the division of nuclear fusion and hybrid formation (Arcioni et al. 1997). The fusion of heterogeneous interphase nuclei is manifested in soybeans and peas and carrots and heteromalt bristles. Provided that parental protoplasts carry appropriate markings, fusion products can be identified.

For successful isolation and cloning of hybrid cells, integration must be completed with the selection system. Complementing two non-allelic genes that stop or inhibit growth under special cultural conditions is a principle based on the selection of somatic hybrids. Since some species are initiated to regenerate entire plants, the improvement of hybrid plants from primitive fusion products is now possible and has already been introduced into tobacco.

In the achievement of transgenic development, the pea genotype regeneration system independency and replicability are the primary prerequisites. In peas, a protocol for regeneration has been described by several researchers. Embryogenesis or organogenesis has been described for different pea explants such as immature leaflets (Fujioka et al. 2000; Mroginski and Kartha 1981; Rubluo et al. 1984), cotyledonary nodes (Grant et al. 1995; Jackson and Hobbs 1990; Rajput and Singh 2010), hypocotyl regions (Nielsen et al. 1991), embryos (Kysely et al. 1987; Natali and Cavallini 1987b; Sanchez and Mosquera 2006; Surma et al. 2013; Tetu et al. 1990), various organs of seedlings (Aslam et al. 2006; Ezhova et al. 1985; Hussey and Gunn 1984; Malmberg 1979; Pniewski and Kapusta 2005; Sharma and Kaushal 2004; Tzitzikas et al. 2004), mature seeds (Zhihui et al. 2009), cotyledons (Pniewsky et al. 2003) and protoplast cultures (Jacobsen and Kysely 1984; Lehminger-Mertens and Jacobsen 1989; Puonti-Kaerlas et al. 1990; Tapingkae et al. 2012).

9.9.3 Hybrid Cultivars

The most expensive seed genotypes in agricultural markets are often classified as F_1 hybrid seeds. In the hybridization of two pure lines with each other, the result is known as an F_1 hybrid. The following year, hybrid seeds are planted. As a result of this mutual fertilization, genetic development occurs. There are difficulties, of course, where it takes 7–8 years to develop a pure line through conventional breeding. Sometimes, a pure line consists of several previous crossings to begin creating and developing the required correct features before they are used in hybridization.

The F_1 hybrid is the result of crossing two pure lines to achieve the desired traits. Strict scientific breeding programs have helped not only to focus the outstanding qualities of native plants, but in most cases, these qualities have been improved and

new desirable traits added to hybrids. In addition to qualities such as vigor, quality, high yield and components and the standardization of hybrid plants, other characteristics such as resistance to diseases, tolerance to drought, salt and early maturing are integrated into most F_1 hybrids.

The uniformity and maturity of plants, along with the uniformity in maturity, shape or size, make hybrids in general very suitable for mechanical harvesting. Since the development of pure lines to produce F_1 hybrids requires several years, these pure lines must be maintained continuously, so that the F_1 hybrid seed can be obtained every year. Hybrid seed is expensive because of the high cost of production annually by manual methods. Sagar and Chandra (1977) reported that the appearance of heterosis in legume crops may be applied to the selection of possible hybrids in legumes for their genetic improvement. Also, Singh et al. (2017) reported that the appearance of heterosis in pea may be applied to the selection of possible hybrids for yield and its related traits. The difficulty is exacerbated because to certify that self-pollination does not occur, sometimes all hybridization of the two pure lines must be performed manually. The seeds are often collected manually to make sure that every plant is as productive as possible. Not only do growers benefit, but there are benefits for breeders as well. With regular genotypes, anybody can plant them and harvest seeds that can be replanted in the field or sold. Therefore, plant breeders who do a lot of work in creating a non-F_1 genotype can find someone else who sells seed and gets a share of the financial reward. But the seeds collected from F_1 hybrids will not produce plants like those they are collected from (yield decreases by 50% at least in F_2 generation). Only by crossing pure lines can the variety be made, and only the original breeder has the pure lines necessary.

9.10 Conclusion and Prospects

In view of the great economic importance of peas due to their uses as food, feed, seed, and industrial uses, considerable research has already been carried worldwide. Since classical breeding methods are laborious and time-consuming, the introgression of novel alleles through crossing plants from various plant genetic resources e.g. modern varieties with locally adapted varieties enhances the pea genetic diversity and pre-selection for traits of interest which is required to ensure that meaningful natural variation at the phenotype level. Although new pea biotechnology approaches that use DNA sequences and molecular methods have attracted pea breeders and geneticists, traditional pea breeding methods are still the key and initial point to develop new pea cultivars with desirable traits. Many promising varieties adapted to climate change and biotic and abiotic stress conditions have been developed. For that, breeding approaches to develop new varieties are needed in pea for high yield and resistance to biotic and abiotic stresses.

Overpopulation and associated increased food demands, along with biotic and abiotic stresses, are the most important challenges in pea breeding. Pre- and post-flowering stresses are key obstacles affecting pea growing. Global climate change

such as frost or high temperature are the major important climatic changes attracting considerable pea breeder attention worldwide. Also, diseases such as seed-borne mosaic virus (SbmV), bean yellow mosaic virus (BYMV), pea enation mosaic virus (PEMV) and pests such as pea aphids, spiny pod borer, which are present in some regions, are predicted to spread fast and affect food security in the involved countries and worldwide. Therefore, there is a need for more investment in breeding programs and training of new pea pathologists and breeders. Also, more efforts must be made to breed new varieties with wide adaptability to extend pea cultivation under abiotic stress such as drought and saline soils, to reduce the effect of global warming. Recent biotechnology tools have been applied to develop promising new pea cultivars with desirable traits. Also, the new pea genome has been sequenced and molecular methods have attracted breeders and geneticists to develop new pea cultivars with desirable agronomic traits and tolerance to biotic and abiotic stresses.

Because there are no genetically distinct pure lines in peas of the most economically-important crop traits, it is necessary to obtain pure lines through the production of haploids and doubled haploids (DH) lines which can be exploited in breeding programs. As well, DNA markers closely linked to important biotic and abiotic stresses, physiological, yield and related traits must be developed. Genes or QTLs should be identified for qualitative and quantitative attributes to improve these traits. Furthermore, germplasm and biotechnology should be improved to speed up and facilitate the improvement of promising new lines with high yield and quality. Optimization of pea interactions with microorganisms like mycorrhiza and rhizobia to give crops the ability to better tolerate stresses. Additionally, there is the need for adaptation of seed composition and plant morphology and phenology into novel breeding efforts.

Appendices

Appendix I-A: Major World Institutions Holding Pisum Germplasm

Country/ Continent	FAO Inst. code	Institute	Number of accessions
Africa	IBCR	Institute of Biodiversity Conservation, Addis Ababa, Ethiopia	1600
Australia	AFTC	Australian Temperate Field Crop Collection, Horsham, England	6567
Bulgaria	SAD	Institute of Plant Introduction and Genetic Resources, Sadovo, Bulgaria	2787
China	ICAR-CAAS	Institute of Crop Sciences, CAAS, China	3837

(continued)

Country/ Continent	FAO Inst. code	Institute	Number of accessions
Czech Republic	CZE	AGRITEC, Research, Breeding and Services Ltd., Sumperk, Czech Republic	1284
France	INRA	INRA CRG Légumineuse à grosses graines, Dijon, France	1891
Germany	GAT	Leibniz Institute of Plant Genetics and Crop Plant Research, Gaterleben, Germany	5336
Hungary	HUN	Institute for Agrobotany, Tapioszel, Hungary	1188
Italy	BAR	Istituto del Germoplasma, Bari, CNR – Istituto di GeneticaVegetale, Italy	4297
Netherlands	CGN	Centre for Genetic Resources, Wageningen, Netherlands	1008
Poland	WTD	Plant Breeding and Acclimatization Institute Blonie, Radzikow, Poland	2899
Russia	VIR	N.I. Vavilov Research Institute of Plant Industry, St. Petersburg, Russia	6790
Sweden	NGB	Nordic Gene Bank, Nordic Genetic Resource Centre, Alnarp, Sweden	2724
Syria	ICARDA	International Center for Agricultural Research in the Dry Areas, Aleppo, Syria	6105
Ukraine	UKR	Yurjev Institute of Plant Breeding, Kharkov, Ukraine	1671
United Kingdom	JIC	John Innes Centre, Norwich, UK	3557
United States	USDA; NYSAES	Plant Germplasm Introduction and Testing Research Station, Pullman; NY State Agricultural Experiment Station, USA	5400; 2500

Source: Smýkal et al. (2012)

Appendix I-B: List of Web Databases Providing Links to Pea Related Information

Database	Website
Bioinformatics gateway towards integrative legume biology	http://www.legoo.org/
Cool Season Food Legume Genome Database	http://www.gabcsfl.org/
INRA Dijon Legume genetic and genomic resources	http://www.thelegumeportal.net
INRA Legume Base	http://195.220.91.17/legumbase/index.php?mode=0&id=
International Legume Database & Information Service (ILDIS)	http://www.ildis.org/
Know Pulse	http://knowpulse2.usask.ca

(continued)

Database	Website
Legume Information System (LIS)	http://www.comparative-legumes.org/
Legume IP	http://plantgrn.noble.org/LegumeIP
Legume phylo-informatics database	http://www.public.asu.edu/~mfwojci/legumephylo_dBase.html
Legume proteomes	http://iant.toulouse.inra.fr/plants/legumes/cgi/legumes.cgi
Medicago truncatula HapMap Project	http://www.medicagohapmap.org/cgi-bin/gbrowse/mthapmap/
Phytozome -Soybean Gbrowser	http://www.phytozome.net/cgi-bin/gbrowse/soybean/
UTILLdb: URGV TILLING pea database	http://urgv.evry.inra.fr/UTILLdb

Source: Smýkal et al. (2012)

Appendix II-A: List of Recommended Varieties of Peas in India

State	Recommended varieties
Bihar	DDR-23 (Pusa Prabhat), V L Matar -42
Chhattisgarth	Shubhra (IM-9101), Vikas (IPFD-99-13), Paras
Gujarat	JP-885, IPFD 10–12, Indra, Prakash
Haryana	Uttra (HFP-8909), DDR-27 (Pusa panna), Hariyal (HFP-9907 B), HFP-9426, Alankar, Jayanti (HFP-8712), Aman(IPF5-19)
Jharkhand	PL Matar-42, V L Matar -42
Madya Pradesh	Prakash (IPFD 1–10), Vikas (IPFD-99-13)
Maharashtra	JP-885, Ambika, Indra (KPMR-400), Adarsh (IPF 99-25), IPFD 10–12
Punjab	Jay (KPMR-522), Pant pea-42, KFP-103 (Shikha), Uttra (HFP8909), Aman (IPF5-19)
Rajasthan	DMR-7 (Alankar), Pant Pea-42
Uttar Pradesh	Swati (KFPD-24), Malviya Matar-15 (HUDP-15), Vikas, Sapna (KPMR-1441), IPF 4-9
Uttarakhand	Pant Pea-14, Pant Pea-25, V L Matar -47

Source: Seednet GOI, Min of Agri & FW & ICAR-IIPR, Kanpur, Dhall (2017)

Appendix II-B: World List of Recommended Varieties of Pisum sativum in some Producing Countries

Country	Recommended varieties
Czech Republic	Adept, Alan, Baryton, Bohatyr, CanisCarrera, Catania, Garde, Gotik, Grana, Hardy, Harnas, Herold, Jackpot, Janus, Kamelot, Komet, Lantra, Madonna, Menhir, Merkur, Olivin, Pegas, Power, Primus, Profi, Romeo, Sonet, Sponzor, Tempra, Terno, Tyrkys, Zekon
Egypt	Master B, Little Marvel, Lincoln, Luxer, Sugary, Sohag 1, Sohag 2, Ambassador, Hurst Greenshaft, Senator, Sugar Snap, Delikett, Victory Freezer
Ethiopia	Burkitu, Adet-1, Sefinesh, Gume, Tegegnech, Wolmera, Hassabe
Pakistan	Climax, Matar, Meteor, Climax, Greenfeast and Rondo
UK	Manager, Cascade, Capulet, Deity, Croft, Pastoral Swift, Venture, Madras, Salamanca

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Chapter 10
Genetic Improvement of Yardlong Bean (*Vigna unguiculata* (L.) Walp. ssp. *sesquipedalis* (L.) Verdc.)

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Abstract *Vigna unguiculata* (L.) Walp. ssp. *sesquipedalis* (L.) Verdc. is commonly known as asparagus bean, Chinese long bean, long-podded cowpea, pea bean, snake bean or yardlong bean, and is an underexploited crop with rich nutritional value. Yardlong bean has evolved and was domesticated from cowpea but differs phenotypically as a result of divergent selection. This crop has its origin in Southeast Asia and its habitat in Asia, Europe, Oceania and North America. It is a warm season crop and besides being a food source, it is used as an ornamental plant and for fodder. Ample germplasm diversity exists in this crop. Major commercial varieties were developed by pure line selection. At present, other breeding methods and principles have limited scope. The presence of genetic diversity in this crop needs to be studied with molecular tools. DNA marker analysis with RAPDs, SSRs and SNPs has begun in yardlong bean. However, genomic studies need to be further developed for the construction of high-density genetic maps and application of modern biotechnology tools. This chapter is an overview of yardlong bean origin, evolution and domestication, general cultivation practices, germplasm biodiversity, characterization, conservation strategies, world and Indian gene banks, biotic and abiotic stress tolerance and crop improvement strategies including conventional breeding, biotechnology, tissue culture and micropropagation.

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10.1 Introduction

Yardlong bean, *Vigna unguiculata* (L.) Walp. ssp. *sesquipedalis* (L.) Verdc., (2n = 22), is an annual legume, considered as one of the important vegetable crops of the tropics and subtropics, widely grown in Southeast Asia, South China and West Africa under annual temperatures of 25–35 °C. The crop is versatile in utilization as livestock feed and food supplement to humans (it can be eaten raw or cooked) with moderately high nutritive value. It is considered as a good source of plant protein (23.5–26.3%), and has been given the nickname *meat of the poor* (Heiser 1990). The yardlong bean crop is an annual dwarf to climbing twining plant bearing trifoliate leaves, and grows in tropical climates. It is one of the most popular vegetables of Southeast Asia (Kuo 2002), with ~7% of total vegetable production (Ali et al. 2002). Even though yardlong bean is a self-pollinated crop, cross-pollination is feasible by insects. The stem is usually shallowly lobed with extra flora nectaries. The extra floral nectaries present on yardlong bean share a great similarity with cowpeas, mung beans and adzuki beans (Headings et al. 2008). Flower clusters develop into pods that appear inflated at young age, but constricted at maturity. It can be grown through seed in a wide range of soils, preferably pH ranges of 5.5–7.5. First flowering usually occurs within 30 days and the plants reach a height of 3–4 m, usually with the support of a trellis. The first harvest of beans starts at around 50 days and may continue for 25–30 days. The crop is genetically adapted to harsh environments viz., extreme temperatures, soil infertility and water limiting conditions, inferring low input farming systems in yardlong bean (Sivakumar et al. 2018). This chapter gives an insight into the morphological and molecular diversity of yardlong bean, which will be useful to plant breeders as well as biotechnologists to exploit and improve this crop for wider utilization.

This chapter aims to give expansive depiction to research on yardlong bean, through introducing the crop, followed by botanical classification, distribution and its importance. In addition, current crop cultivation practices, genetic resources and crop improvement strategies (conventional and modern approaches), as well as abiotic and biotic stress tolerances are discussed.

10.1.1 Botanical Classification and Distribution

Cowpea (*Vigna unguiculata* (L.) Walp.), is one of the important crops based on planting area (Singh 2005). It is botanically classified as class Dicotyledonae, order Fabales, family Fabaceae, subfamily Papilionoideae, tribe Phaseoleae, subtribe

Phaseolinae and genus *Vigna* (Padulosi and Ng 1997). Cultivated cowpeas were categorized into 20 bionomials before the nineteenth century, but in the latter half of that century, these bionomials were ranked at infraspecific levels (Pasquet 1999). In the present system of classification, the cultivated cowpea varieties were pooled under *Vigna unguiculata* (L) Walp. ssp. *unguiculata* var. *unguiculata* and the wild counterparts were grouped under ssp. *unguiculata* var. *spontanea* (Schweinf.) Pasquet. Cultivated cowpea was divided into four cultivars, namely Unguiculata, Biflora, Sesquipedalis and Textilis (Ng and Maréchal 1985). This classification has been followed over the past decade. Currently, two major groups of cowpea, viz., *V. unguiculata* (L.) Walp. ssp. *unguiculata* cv.-gr. *unguiculata* and *V. unguiculata* (L.) Walp. ssp. *unguiculata* cv.-gr. *sesquipedalis* are popularly grown based on their uses, growing area and domestication (Smartt 1990).

Yardlong bean is cultivated for its edible tender green pods, stem tips and young leaves. The crop is widely distributed in Southeast Asia (Fery 2002), Malaysia, Philippines, Indonesia, Thailand, South China, India and Pakistan (Benchasri et al. 2012), with germplasm of about 1570 accessions maintained by the Asian Vegetable Research and Development Center (AVRDC), The World Vegetable Center. The germplasm includes several important varieties (Appendix I). In India, research on yardlong bean was undertaken at Kerala Agricultural University due to its favorable climate, resulted in development of various high-yielding varieties (Lola, KMV-1, Mallika, Sharika, Vellayani, Vyjayanthi) with a pod length range of 40–60 cm (Kamala et al. 2014). Some of the popular varieties/cultivars of yardlong bean under cultivation in Australia, South and Southeast Asia and Africa are given in Appendix I.

Yardlong bean cultivation area was estimated at 18,560–20,160 ha annually in Thailand (Sarutayophat et al. 2007), whereas cultivation in China exceeds 250,000 ha annually (Rubatzky and Yamaguchi 1997). In India, it is considered an underutilized vegetable crop, cultivated in the peninsular region mainly in Andhra Pradesh, Odisha, Kerala, Tamil Nadu, West Bengal, Assam and other regions of the northeastern hills (Kamala et al. 2014), with the overall area coverage of about 7.7 million ha (Yadav et al. 2004). The cultivable area for this crop has been expanding due to an increase in awareness about the nutritional importance of the crop (Manjesh et al. 2019) (Fig. 10.1).

10.1.2 Nutritive Value and Importance of Yardlong Bean

Yardlong bean is an ancient legume and a low calorie vegetable crop (young immature pods, 100 g, generate only 47 calories). It contains good amounts of soluble and insoluble fibers besides being an excellent source of vitamin A and C, accounting for 17 and 13% of the daily dietary allowance, respectively. Nutrient status of mature seeds (100 g edible portion) is given in Table 10.1 (Chanapan et al. 2017).

Fig. 10.1 Global distribution of domesticated yardlong bean species. (Source: The figure is a slight modification of world map from royalty free image of bjdesign/mapsfordesign (https://www.bjdesign.com/; https://www.mapsfordesign.com/))

Table 10.1 Nutritional status of the yardlong bean per 100 g edible portion

Component	Quantity	Component	Quantity
Carbohydrates	8.35 g	Sodium	4 mg
Protein	2.8 g	Potassium	240 mg
Total fat	0.4 g	Calcium	50 mg
Folates	62µg	Copper	0.05 µg
Niacin	0.41 mg	Iron	0.47 mg
Pantothenic acid	0.06 mg	Magnesium	44 mg
Pyridoxine	0.02 mg	Manganese	0.21 mg
Riboflavin	0.11 mg	Phosphorus	59 mg
Thiamin	0.11 mg	Selenium	1.5 µg
Vitamin C	18.8 mg	Zinc	0.37 mg
Vitamin A	865 IU		

Source: Chanapan et al. (2017)

Due to economic recession and acute shortage of animal protein, a majority of Nigerians depended on beans for protein source, as a source of animal protein is beyond their means (Henshaw and Sanni 1995; Olawuni et al. 2013), signifying a good protein source under low economic production costs.

As a *meat for the poor*, yardlong bean can be commercially produced for immediate sale or can be processed for packaging. The nutrition value of yardlong bean is influenced by the type of thermal processing methods (boiling, roasting); perhaps roasting of beans is advised for commercial production and storage of yardlong bean (Nzewi and Egbuonu 2011). The yardlong bean can also be grown as an ornamental plant. The emergence of yardlong bean flowers (large violet-blue in color) and development of drooping pods can be attractive in parks, offices and homes.

10.2 Origin, Evolution and Domestication

Although the origin of this crop is West Africa, it has been domesticated in warmer parts like Southeast Asia, Southern China and India (Manjesh et al. 2018); its greatest diversity occurs in Southern China. The genus *Vigna* is pantropical with ~100 species, among which 2 *Vigna* species were domesticated in Africa and 7 in Asia, constituting a total of 9 domesticated *Vigna* species (Kongjaimun et al. 2012; Maríchal et al. 1978). The details of the 9 species of *Vigna* species from African and Asian continents are given in Table 10.2 (Tomooka et al. 2002).

Domestication is a complex evolutionary process, where the variation in genetic architecture of wild species is brought through careful repetitive selection of species and adapted to man-made environments (Harlan 1966). The performance strength of the adapted species gradually shifting to the new environments from the natural habitat (Kaga et al. 2008). Archeological evidence suggests typical morphological and physiological differences between domesticated and wild ancestors, which in turn depend on the target trait of domestication (Purugganan and Fuller 2009). Complete variation is arrived at in the domesticated species from the wild progenitors due to either natural selection or human selection, collectively called the *domestication syndrome* (Kaga et al. 2008). The name *vegetable cowpea* applied to

Table 10.2 Domesticated *Vigna* species

Region	Species Name
African species	Cowpea [*V. unguiculata* (L.) Walp.]
African species	Bambara groundnut [*V. subterranea* (L.) Verdc.]
Asian species	Mungbean [*V. radiata* (L.) Wilczek]
Asian species	Blackgram [*V. mungo* (L.) Hepper]
Asian species	Moth bean [*V. aconitifolia* (Jacq.) Maréchal]
Asian species	Azuki bean [*V. angularis* (L.) Ohwi & Ohashi]
Asian species	Rice bean [*V. umbellata* (Thunb.) Ohwi & Ohashi]
Asian species	Jungli bean [*V. trilobata* (L.) Verdc.]
Asian species	Creole bean [*V. reflexo-pilosa* Hayata]

yardlong bean is apt, perhaps due to its origin and evolution from cowpea through phenotypically-divergent selection (Kongjaimun et al. 2012). The flow of genetic information among individuals of the crop contributes to domestication and evolution. An initial report on the genomic regions identification related to domestication traits by Kongjaimun et al. (2012) revealed the presence of conserved regions between yardlong bean and azuki bean (seed dormancy, pod dehiscence, seed and pod size) and genomic regions between yardlong bean and cowpea (seed size). Determination of precise origin and domestication of the bean is quite complicated as described by many authors (Ng 1995; Pasquet 2000; Rawal 1975). To gain a full insight of the evolution of yardlong bean, further studies related to domestication traits are needed.

10.3 Cultivation Practices of Yardlong Bean Production

Seeds and seedlings are used to propagate yardlong bean, warm soil (20–30 °C) is recommended for good seed germination, as low soil temperatures will lead to poor germination and rotting. Seed quality is vital in crop production, because ~25% of the yield is dependent on seed material quality (Isaac and Mathew 2016; KAU 1991). Seed germination commences within a week and plantlets will emerge depending on the atmospheric temperature. Even though it grows in various soil types (varying from sandy loam to clay), loam and sandy loam soils are well suited for yardlong bean production, with a pH range of 6.2–7.0 (Bounnhong 1997). Loose and friable soil with optimum content of nitrogen is well suited for yardlong bean production; higher soil nitrogen results in vigorous vegetative growth rather than pod development. The success of crop production in yardlong bean is dependent on sunshine days, since cloudy and rainy weather cause dropping of flowers and young pods, leading to poor yield. Based on the plant growing habit of yardlong bean, two types viz., bush type or pole type (Pidigam et al. 2019), where the support of a trellis leads to the best for production. The yardlong bean plant with flower growth stages and stages of fruit development are shown (Figs. 10.2 and 10.3)

Pole sitao (yardlong bean) in the Philippines is cultivated mainly for its young pods and beans in home gardens, near paddy fields, under shaded areas and as a companion or commercial crop. The strategies employed in cultivation of pole sitao are given in Fig. 10.4.

10.4 Challenges of Yardlong Bean Production

In the present situation of world agricultural production, increased concern is being paid to food production and nutrient-rich foods with low input cultivation, especially in the developing countries. Yardlong bean, being one of such crops, is a nutrition-rich and income-generating crop for farmers. Despite the nutritional

Fig. 10.2 (**a**) Yardlong bean plants grown at Sri Konda Laxman Telangana State Horticultural University, (**b**) Different growth stages in flower development

Fig. 10.3 Stages of yardlong bean pod development

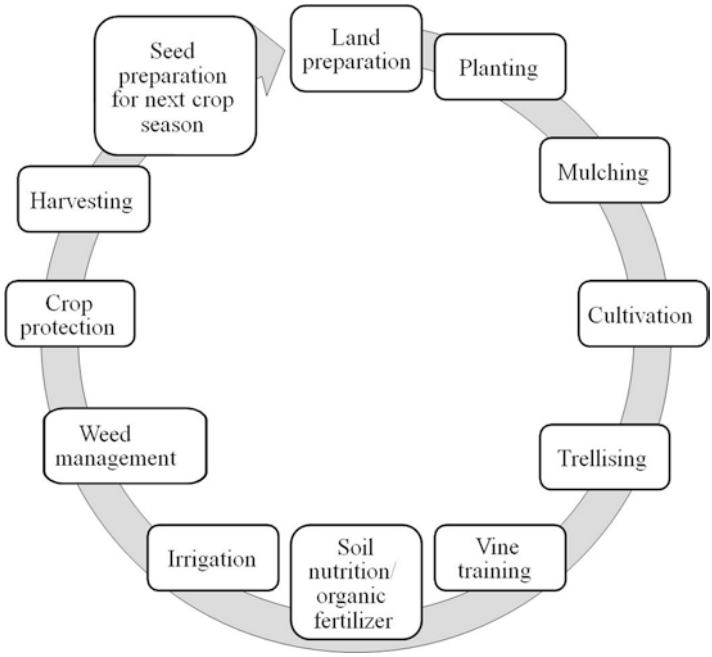

Fig. 10.4 Culture practices in pole sitao. (Source: Adapted from: Production guide-pole Sitao (2013))

importance of yardlong bean, it is considered as risky for large-scale cultivation in Thailand due to its sensitivity to unfavorable environment, low yield stability and pest/disease incidence (Sarutayophat et al. 2007). Susceptibility to disease and pests is reported in yardlong beans for powdery mildew and aphids (Bland and Knausenberger 1985). Reduction in seed yield and pod production was reported because of root knot nematode (*Meloidogyne* spp.) infection (Rhoden et al. 1990). Compared to other vegetable legumes, there is a dearth of availability of yardlong bean commercial varieties with high yield and good pod quality. Hence, research focusing on yardlong bean production and improvement is needed for further improvement and valorization of the crop.

10.5 Germplasm Biodiversity

The term biodiversity refers to the abundance in range of life forms that exist on planet earth. Knowledge of genetic divergence and similarity of genotypes can aid in selections for crop improvement (Chowdhury et al. 2002). Plant morphology is a visual depiction of genotypic performance, which is useful in characterization of germplasm against high heritability and stable traits. Phenotypic characterization (qualitative and quantitative traits) in 41 yardlong bean accessions revealed

significant variation in the measured traits (Rambabu et al. 2016a) and based on the D^2 statistical analysis, the accessions were grouped into 7 clusters with a considerable amount genetic diversity in the materials (Rambabu et al. 2016b), indicating wide scope in the selection of desirable combination of genotypes for further exploitation and improvement of desirable traits. Previous studies of genetic variation in yardlong bean were based exclusively on qualitative and quantitative traits (Asoontha and Abraham 2017; Huque et al. 2012; Ullah et al. 2011; Vavilapalli et al. 2014).

Preliminarily, variations in qualitative and quantitative agronomic traits alone were considered to assess diversity; but the expression of these traits is under strong environmental influence (Kameswara 2004). Hence, a complete perception of genetic variation among species and populations became an essential prerequisite for proper exploitation of plant genetic resources toward crop improvement. Since genes are the underlying construction for plant phenotypic performance, studying the diversity at the genomic level became reliable, with minimal impact from the environment. Progress made in DNA marker technology in the late nineteenth century revealed novel ways to study genetic diversity through overcoming the limitations of environmental influence. Among all the DNA markers, random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR's), inter-simple sequence repeats (ISSRs) and start codon targeted (SCoT) are frequently used in diversity studies. Genetic diversity in yardlong bean was studied with RAPD markers (Pidigam et al. 2019; Sarutayophat et al. 2007), STS/SSR (Phansak et al. 2005), SSR/ISSR (Saxena and Tomar Rukam 2020; Tantasawat et al. 2010). Genetic diversity studies in yardlong bean with IRAP (inter retrotransposon amplified polymorphism) markers was reported recently by Widyawan et al. (2020).

In the Indian context, genetic diversity with RAPD markers in yardlong bean accessions belonging to different growth habitats, distributed in South India, was studied by Pidigam et al. (2019). A study by Kamala et al. (2014) revealed significant diversity in the germplasm collection of coastal Andhra Pradesh and Odisha with respect to pod and seed size, color, etc. In addition to the existing information of yardlong-bean diversity, generation of genome sequence data through next-generation sequencing will of great help in the characterization of yardlong bean for enhanced utilization.

10.6 Categorization of *Vigna* Species and Characterization of Yardlong Bean

Originally cowpea was domesticated from wild cowpea in parts of Africa and yardlong bean subsequently evolved from it. Hence, the implication of descriptors for identification and grouping of accessions, parents and germplasm has potential applications in crop improvement. Minimal descriptors/characteristics for grouping of three *Vigna unguiculata* varieties/ species (Table 10.3) and specifically for

Table 10.3 Minimal characters for grouping of *Vigna* species (grain, vegetable, fodder cowpea)

Character	Grain Cowpea	Vegetable Cowpea (Yardlong bean)	Fodder Cowpea
Days to 10% flowering			✓
Days to 50% flowering	✓	✓	
Growth habit			✓
Number of primary branches per plant			✓
Plant growth habit	✓	✓	
Plant height			✓
Plant twining tendency	✓		
Plant type		✓	
Pod color		✓	
Pod length	✓	✓	
Pod stringiness		✓	
Pod thickness		✓	
Seed color	✓		
Seed eye color	✓		

yardlong bean (Fig. 10.5) are described (Guidelines, Protection of Plant Varieties and Farmers Rights Act (PPVFRA), India).

10.7 Conservation

Gathering and conserving genetic resources of a cultivated crop is significantly important for plant breeders; the main objective in conservation of plant genetic resources (PGR) is to preserve the existing genetic diversity. Preservation and maintenance of genetic diversity of crops could aid in the replacement of similar crops with different genetic architecture whenever needed (Crisp and Astley 1985). PGR of crops are comprised of wild varieties, land races, released varieties, breeding lines, weedy races and genetic stocks (Polegri and Negri 2010). Introduction of new cultivars in vegetables with a narrow genetic base encounters the risk towards genetic erosion (Crisp and Astley 1985). Hence, adapting various conservation strategies will be of great advantage.

10.7.1 In Situ Conservation

In situ conservation is a crucial component in conservation and management of genomic sources, and is complementary to ex situ conservation. Wild genetic resources are repositories of importance genes often employed in crop improvement

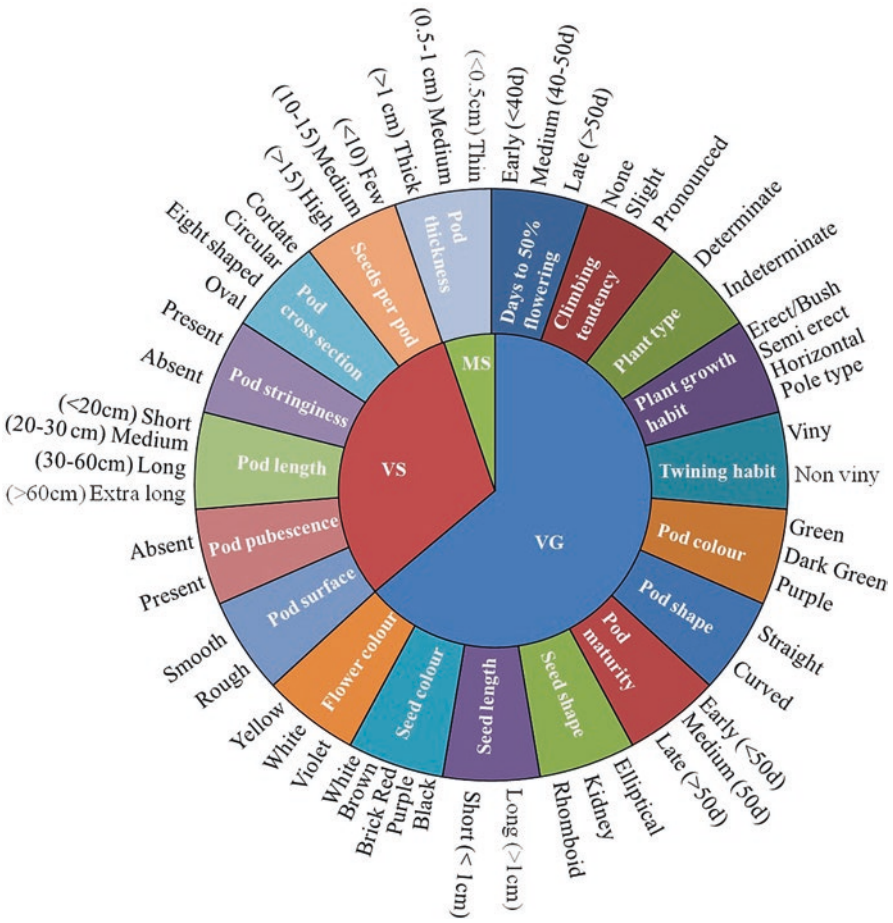

Fig. 10.5 Overview of descriptors employed in vegetable cowpea crop; *VG* Visual assessment by a single observation of a group of plants or parts of plants, *VS* Visual assessment by observation of individual plants or parts of plants, *MS* Measurement of a number of individual plants or parts of plants

program, particularly towards biotic, abiotic stress, improvement of favourable traits in established crops. Conservation of vegetable species through in situ methods has dynamic benefits of allowing for new species evolution besides preservation of novel genomic sources of wild and weedy species (Maxted et al. 1997). Strategies employed for in situ conservation of wild genetic resources depend on the distribution of crop genera (Al-Atawneh et al. 2008; Maxted et al. 2004, 2008; Ramírez-Villegas et al. 2010).

Conservation regions of wild relatives of African *Vigna unguiculata* were identified over 100 established areas with multiple priority taxa (Moray et al. 2014). Initial cost-effective strategy for in situ conservation of *Vigna* wild relatives can be attained

through identification and exploitation with the existing resources (Moray et al. 2014). Along with the identification of regions for in situ conservation, an on-farm conservation strategy by farmers in the agro-ecosystems can also be employed, which is associated with local infrastructural development and increased livelihood for farmers (Jarvis et al. 2000). India is considered a rich source of biodiversity; established biosphere reserves in India are given in Fig. 10.6 (Anonymous 1983, 1987).

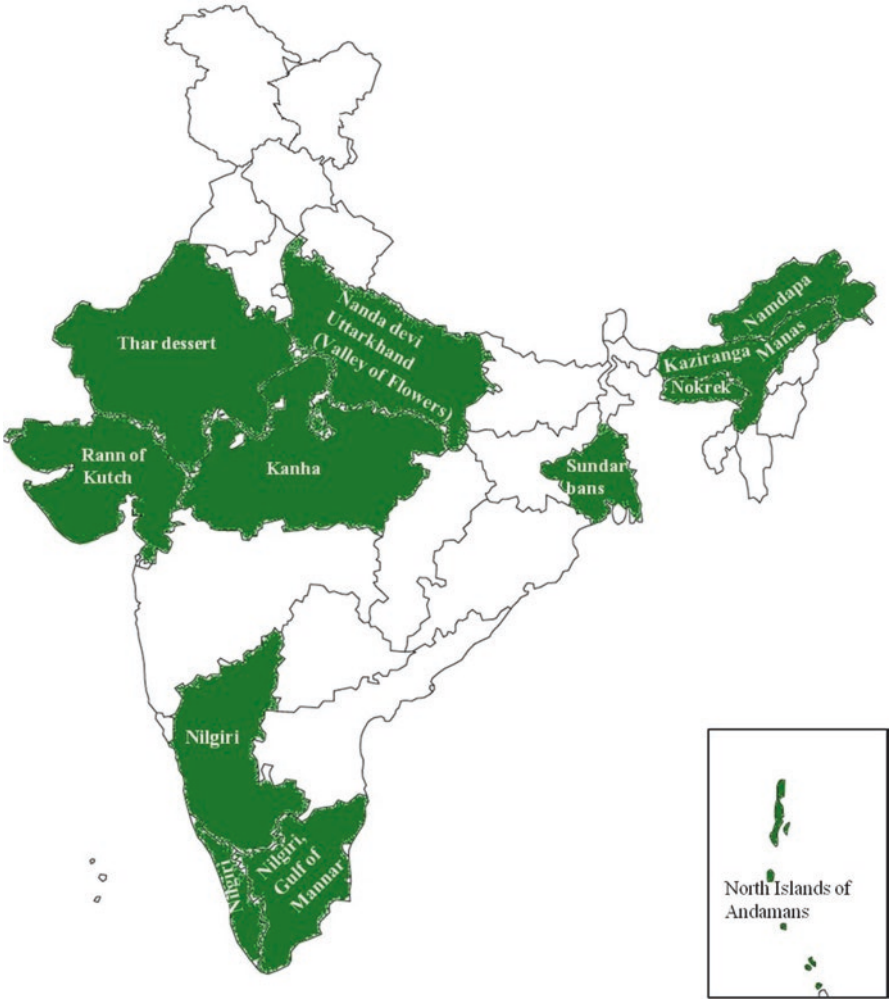

Fig. 10.6 Established biosphere reserves in India. (Source: Modified source map from https://d-maps.com/)

10.7.2 Ex Situ Conservation

Preservation of plant genetic resources to combat emerging pests, diseases and the production of new varieties with stable yields having adaptation efficiency to changing climatic conditions is of prime concern for conservation (FAO 2010). The ex situ method constitutes genetic material conservation outside their natural habitat with eventual release into the ecosystem, ensuring easy accessibility of germplasm for assessment and exploitation. In ex situ conservation of seeds, preservation has been done for more than 50 years without any loss of viability (Usberti and Gomes 1998). Several countries have made indigenous collections of yardlong bean from diversity sites and ex situ collections worldwide and in India (Fig. 10.7a, b). Its vast germplasm diversity is evidenced in India due to agro-climatic differences.

Ex situ conservation of horticultural crops is generally executed through maintenance in botanical gardens, gene banks (seeds, field, in vitro) and cryogene banks. Selection of the appropriate method of conservation depends on the biology of the plant, affordability of technologies and availability of infrastructure to gene bank managers. Depending on the methodology of conservation and type of species, in vitro techniques helps in the storage of germplasm from months to 2–3 years without needing subculture (Agrawal et al. 2019; Benson et al. 2011; Pandey et al. 2015). In vitro conservation is executed through different approaches viz., conservation through slow growth, and long term conservation through cryopreservation.

Incubating plant organs/cultures under suboptimal room temperatures typically in the range of 4–20 °C has been found to be effective in reduction of the plant growth rate in several species. However, the storage temperature needs to be altered for tropical species. In addition to cold preservation, utilization of growth retardation properties of various chemicals such as hydrazide (MH), abscisic acid (ABA), cycocel (CCC) and n-dimethyl amino succinamic acid (DSA) are employed in the concentration of 5–50 mg/l for conservation of germplasm. Different kinds of osmotic regulators are also deployed for the storage of the germplasm, which needs to be validated for the yardlong bean accessions for the actual process. The type of enclosure for conservation plays an important role in the success of in vitro conservation. A study of the assessing of appropriate storage device for the cowpea seeds, conducted by Sarma et al. (2014) revealed that tin containers were suitable for the long term storage of cowpea seeds. With the advent of the liquid nitrogen (−196 °C), cryopreservation of living tissue is made feasible either through classical cryopreservation techniques (controlled freezing) or new vitrification-based techniques. There were reports of successful cryopreservation of fruits, tubers, ornamental plants, spices, medicinal and aromatic plants.

Fig. 10.7 Distribution of yardlong bean. (**a**) Worldwide collections and conservation of yardlong bean genetic resources (red squares), (**b**) Yardlong bean germplasm collection sites in India (red triangles)

Table 10.4 Yardlong bean germplasm holdings by various countries

Country	Number of Accessions
Belgium	7
Austria	1
Brazil	8
Germany	39
Hungary	145
India*	173
Portugal	14
Russia	135
Spain	11
Taiwan	617
USA	194
Other	2
Total	1346

Source: Genesys database www.nbpgr.ernet.in

10.7.3 Gene Banks

An effective gene bank requires collection, assembly, maintenance and conservation, characterization, documentation, and distribution of germplasm. India is considered as the second most important center for diversity of cowpea, accounting for the conservation of >3000 accessions at the National Bureau of Plant Genetic Resources, Delhi, but few of them are identified as yardlong bean (Kamala et al. 2014). Country-wide yardlong bean accessions maintained at gene banks are provided in Table 10.4. The biological status of the yardlong bean germplasm are categorized into advanced/improved cultivar, breeder lines (breeding/research material), landrace, wild and others. The details of the same are provided in Table 10.5.

The yardlong germplasm storage worldwide is depicted in Table 10.6. A list of germplasm collected from various states of India and conserved in the National Gene Bank is provided in Table 10.7. A list of gene banks maintained internationally for conservation of biodiversity is given in Table 10.8.

10.8 Stress Tolerance

10.8.1 Climate Change and Abiotic Stress

In recent decades, world climate factors such as temperature, precipitation, relative humidity and gaseous composition are rapidly varying and increases in atmospheric carbon dioxide (CO_2) and ozone (O_3) levels have also been ubiquitous. The

Table 10.5 Biological status of the yardlong bean germplasm maintained in world gene banks

Category of the Germplasm	Number of Accessions	Countries
Advanced/ improved cultivar	96	Austria, Belgium, Brazil, Germany, Hungary, India, Portugal, Russia, Spain, Taiwan and United States of America
Breeders Line	19	
Breeding/Research Material	7	
Not specified	410	
Traditional cultivar/Landrace	748	
Wild	2	
Others	64	
Total	1346	

Source: Genesys database www.nbpgr.ernet.in

Table 10.6 Storage types of yardlong bean germplasm

Type of Storage	Number of Accessions	Countries
Long term seed collection	233	Austria, Belgium, Brazil, Germany, Hungary, India, Portugal, Russia, Spain, Taiwan and United States of America
Medium term seed collection	170	
Short term seed collection	7	
Seed collection	330	
Not specified	606	
Total	1346	

Source: Genesys database www.nbpgr.ernet.in

consequence associated with climate change is a reduction in quality and quantity of agricultural produce through crop failures, diminished yields and disease incidence. According to the Intergovernmental Panel on Climate Change (IPCC), stress associated with the variations in climate is one of the major hurdles for crop production in a majority of the developing countries (Andy 2016).

Plants growing in open environments are often exposed to various types of environmental stress, which subsequently affect crop performance. Abiotic stresses such as flooding, drought, high/low temperatures, salinity, radiation, toxic/heavy metals and excessive macro/micro nutrients are known to cause significant losses to world agricultural production.

Drought is one of the major stresses with increasing area under its influence, causing difficulty in crop production through increasing temperature and reduced relative humidity, mainly in tropical and subtropical regions (Muñoz-Perea et al. 2006). Lestari et al. (2019) studied 8 Indonesian varieties of yardlong bean (BU-1, BU-2, BU-3, BU-4, BU-5, BU-6, Br-4, Bg-6) for drought stress tolerance (50 and

Table 10.7 Yardlong bean germplasm collected and conserved in National Gene Bank, India

State and Total Accession Numbers	Accession
Arunachal Pradesh (15)	IC622557, IC622558, IC622559, IC622560, IC622561, IC622562, IC622563, IC622564, IC622565, IC622566, IC622567, IC622568, IC622569, IC626165, IC626166,
Kerala (38)	IC622570, IC622571, IC622572, IC622573, IC622574, IC622601, IC626136, IC626137, IC626138, IC626139, IC626140, IC626141, IC626142, IC626143, IC626144, IC626145, IC626146, IC626147, IC626148, IC626149, IC626150, IC626151, IC626152, IC626153, IC626154, IC626155, IC626156, IC626157, IC626158, IC626159, IC626160, IC626161, IC626162, IC626163, IC626170, IC626171, IC626172, IC626173
Gujarat (1)	IC622602
Mizoram (24)	IC590798, IC590799, IC590800, IC590801, IC590802, IC590803, IC590804, IC590805, IC590806, IC590807, IC622587, IC622588, IC622589, IC622590, IC622591, IC622592, IC622593, IC622594, IC622595, IC622596, IC622597, IC622598, IC622599, IC622600
Nagaland (8)	IC614769, IC622575, IC622577, IC622578, IC622579, IC622580, IC622585, IC622586
Odisha (1)	IC582850

Source: www.nbpgr.ernet.in

100% of the field capacity) and found an increase in the activities of superoxide dismutase (SOD) and peroxidase (POX) enzyme activities. The results indicated drought-tolerance capacity through increasing scavenging reactive oxygen species in the cells, which include the escape mechanisms. Drought stress switches various morphological (reduced leaf area), physiological (stomatal closure), biochemical (increasing antioxidant enzymes) and molecular responses (differential gene expressions) in the plant to adapt to the adverse conditions.

Saline soils are one of the major factors limiting crop production and the affected soils cover ~10% of the earth land surface and 50% of the irrigated cropland (Shabala 2013). Salinity has a repressive effect on plant productivity through inhibiting photosynthesis, metabolic dysfunction and damaging cellular structure. Transcriptional studies for salinity stress tolerance in yardlong bean using 17 SNPs related to 6 salt-stress induced differentially expressed genes (Pan et al. 2019). QTL (quantitative trait loci) studies for salt tolerance in yardlong bean revealed 4 major QTLs on chromosome 9 for traits associated with salt tolerance (Zhang et al. 2020).

Temperature is a crucial factor affecting cellular functions and low temperature is one of the growth-limiting environmental factors leading to significant yield losses (Heidarvand and Amiri 2010; Thomashow 1999). Tan et al. (2016) identified differentially-expressed genes for chilling stress in susceptible and tolerant yardlong bean accessions. Germination studies under low and high temperature stress in yardlong bean cultivars revealed a significant increase in malondealdehyde content, inferring significant membrane damage under temperature fluctuation (Wan et al. 2007). Several traits related to abiotic stress tolerance are regulated by multiple

Table 10.8 International gene banks maintained for conservation of diversity

Name	Samples Maintained	User Access
Biodiversity International group	Major crop species collections	https://www.bioversityinternational.org/
AVRDC	Vegetable germplasm	https://avrdc.org/avrdc-genebank/
ICRISAT	Accessions of chickpea, groundnut, various millets, sorghum and pigeon pea	http://genebank.icrisat.org
Genebank Africa Rice Center	World's largest collection of African rice	http://www.africarice.org/warda/genebank.asp
International Musa Germplasm Transit Centre	Collection of about 1500 banana and plantain accessions	https://www.bioversityinternational.org/banana-genebank/
International Center for Tropical Agriculture	Large and diverse collections of beans (38,000 accessions), tropical forages (23,000) and cassava (6600) in Colombia	https://ciat.cgiar.org/
International Maize and Wheat Improvement Center	Houses outstanding wheat (153,000 accessions) and maize (28,000) collections, near Mexico City	https://www.cimmyt.org/
International Potato Center	Potato, sweet potato and Andean root and tuber crops, located in Lima, Peru	https://cipotato.org/
World Agroforestry Centre	Domesticated, partially domesticated and wild tree species used for fruit, timber, medicines and other products	http://www.worldagroforestry.org/
International Center for Agricultural Research in the Dry Areas	Barley, chickpea, grass pea, lentil and wheat; and Lebanon for crop wild relatives of cereals and legumes	https://www.icarda.org/
International Institute of Tropical Agriculture	Banana, cassava, yam, maize, soybean and crops such as bambara groundnut, cowpea and African yam, bean, less well known but nonetheless vital for local food security. Based in Nigeria	http://www.iita.org/
International Livestock Research Institute	Tropical forage species	https://www.ilri.org/
International Rice Research Institute	Accessions of rice varieties and wild relatives	https://www.irri.org/

genes; for instance, 19 genes were identified from cowpea drought tolerance studies (Agbicodo et al. 2009; Mickelbart et al. 2015). Clearly, in-depth studies on the complex regulatory network of stress tolerance in yardlong beans is required to understand the mechanism of tolerance.

10.8.2 Biotic Stress

Biotic stress is another major constraint on yardlong bean production, e.g., pest infestation (aphids, jassids, white fly, green hopper, beetles, jute hairy caterpillar, red spider mite, shoot fly, pod fly), nematodes (root knot nematode), fungal (bean anthracnose), bacterial (blight) and viral agents (cowpea mosaic, broom virus).

Aphids are group of sucking insects of the Arthropoda phylum which feed on yardlong bean, from seedling to pod maturity stages causing significant yield reduction (Begum et al. 1991; Pedigo 2002). Besides different groups of compounds controlling the incidence of aphids, extracts from tobacco and neem can possibly control pests in yardlong bean (Bahar et al. 2007). Another group of pests known as *greasy cut worms* (*Agrotis ipsilon*) are potential destructors of plants after the emergence of branches (Grubben 1993).

Root knot nematodes (*Meloidogyne incognita*) are harmful pests, causing significant losses to yardlong bean crop production, exhibiting symptoms of patches of stunted and yellow plants, root galls and reduced root systems despite excessive branching. Infection at the seedling stage results in reduced germination and death (Mishra 1992).

Among these biotitic constraints, rust disease caused by *Uromyces vignae* is one of the fungal diseases that adversely affects crop production and quality. The resistance gene for this disease has been identified in a few accessions. Rust resistance was controlled by single dominant gene designated *Rr1*, which was identified in the cross of ZN016 and Zhijiang 282 (Li et al. 2007). Bulked segregant analysis was applied to an F_2 population derived from these parents, and an AFLP marker (E-AAG/M-CTG), 150 bp in size, was detected in the resistant bulk, which was converted to a SCAR marker ($ABRS_{AAG/CTG98}$), and the genetic distance between the marker and the *Rr1* gene was found to be 5.4 cM. This identified SCAR marker for screening yardlong bean germplasm for rust resistance (*Rr1*) through marker-assisted selection (MAS) towards development of rust-resistant asparagus bean cultivars.

10.9 Crop Improvement Strategies of Yardlong Bean

The genetic architecture of a plant and the nature of the environment it grows in are key factors in crop performance. Crop improvement is achieved thorough an understanding of morpho-physiological, agronomic and genetic potential of germplasm, followed by selection of suitable genotypes and genes pertaining to the trait of interest. Because of very low crossing incidence between plants in a row (< 1%) (Sitathani 1977), outcrossing is low and varies with the environment. The improvement of varieties is mainly through selection from pure lines, mass selections and breeding strategies (Boukar et al. 2016).

10.9.1 Conventional Breeding

Conventional/traditional breeding is one of the widely followed methods for the development of yardlong bean varieties using older tools and natural processes (Jain and Kharkwal 2012), through conservative manipulation of the plant genetic architecture within the natural boundaries of genome. In traditional breeding, the desirable traits from closely-related traits are incorporated into the plant through crossing. Criteria considered for adaptation of breeding strategy depend on mode of reproduction of the species (self-pollinating or cross-pollinating). Breeding for qualitative traits are easy compared to quantitative traits, since the number of genes controlling qualitative traits are fewer compared to quantitative traits. It is necessary to breed crop plants to retain and sustain the genetic purity in the natural mating system. The core of plant breeding consists of the selection of better types among the available variants.

Variability among self-pollinated populations has been targeted for selection of germplasm through pure-line selection, which yielded several commercial varieties, especially with respect to yield improvement. A list of released varieties in India for yardlong bean are presented in Appendix III. A schematic representation of traditional breeding is outlined in Fig. 10.8.

The selection procedure for succeeding generations plays a key role in any breeding program. The efficiency of the selection method depends on the heritability of the desired character. There are any number of methods for selection viz., pedigree selection (PS), single seed descendent (SSD) and bulk selection (BS) are the most commonly used methods. However, the PS method was reported to be more effective for grain yield in cowpea (Padi and Ehlers 2008), the same was confirmed by DePauw and Shebeski (1973) in wheat and by Hanson et al. (1979) in barley. In yardlong bean, PS and SSD selection procedures were studied in two populations and results showed that both methods were effective for yield improvement (Sarutayophat and Nualsri 2010). Despite the success in conventional breeding of yardlong bean, more precise and accurate methods of developing new varieties are possible with the advent of molecular markers in plant breeding.

10.9.2 Application of Biotechnology in Plant Breeding

The development of new plant varieties by conventional breeding takes a long duration, even up to 12 years. Therefore, to overcome the barrier of pre-fertilization and post-fertilization techniques, there is an urgent need to develop modern nonconventional breeding methods to assist plant breeders. Biotechnological approaches like transgene transfer, micropropagation and use of molecular markers make plant breeding more efficient.

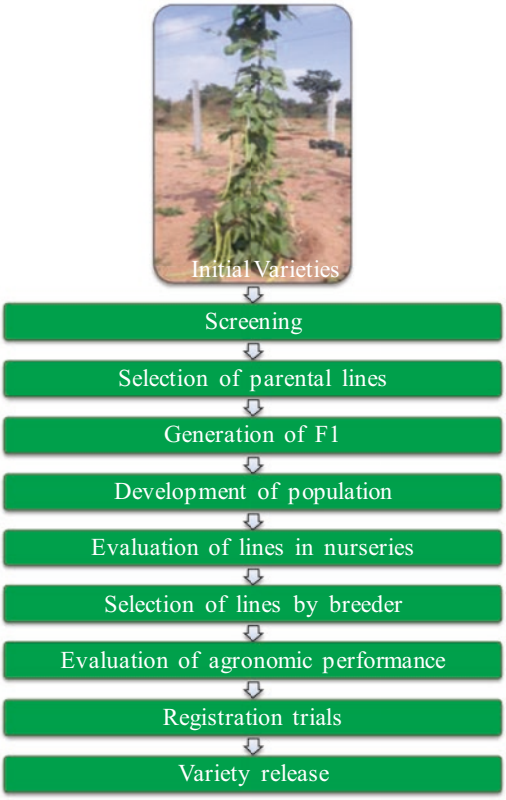

Fig. 10.8 Schematic representation of traditional breeding

10.9.2.1 Molecular Breeding

Molecular breeding is defined as the use of genetic manipulation performed at the genomic level to improve characters of interest; this includes genetic engineering or gene manipulation, molecular-marker assisted selection and genomic selection. Molecular breeding, also known as molecular-marker assisted breeding (MAB), in the modern sense is defined as including marker-assisted selection (MAS), marker-assisted backcrossing (MABC) and marker-assisted recurrent selection (MARS) (Ribaut et al. 2010).

Many genomic tools have been applied to breeding programs in cowpea as the genome sequence has been characterized by high-density genetic mapping (Muchero et al. 2009), but very little is known about yardlong bean. The first genetic map of yardlong bean was reported by Xu et al. (2011), based on SNP and SSR markers. The map consists of 375 loci mapped onto 11 linkage groups (LGs), with 191 loci detected by SNP markers and 184 loci by SSR markers. The overall map length is 745 cM, with an average marker distance of 1.98 cM. There are 4 high marker-density blocks distributed on 3 LGs and 3 regions of segregation distortion (SDRs) identified on 2 other LGs, 2 of which co-locate in chromosomal regions syntenic to

the SDRs in soybean. The genetic basis of horticulturally-important traits of asparagus bean is still poorly understood, hindering the utilization of targeted, DNA marker-assisted breeding in this crop. QTLs for 24 domestication-related traits were mapped using 2 temporal segregation populations derived from an asparagus bean × wild cowpea cross (Kongjaimun et al. 2012). In a study by Xu et al. (2013), major, minor and epistatic QTLs were found to contribute to the inheritance of the days to first flowering (FLD), nodes to first flower (NFF), leaf senescence (LS) and pods per plant (PN). Positions of many of these QTLs are conserved among closely-related legume species, indicating the common mechanisms they share. To the best of our knowledge, this is the first QTL mapping report on a asparagus bean × asparagus bean intervarietal population and provides marker-trait associations for marker-assisted selection. The first NGS-based genetic nap of this crop was constructed by Huang et al. (2018). The map consisted of 5,225 SNP markers in 11 LGs, spanning a total distance of 1,850.81 cM, with an average distance between markers of 0.35 cM. Some previous studies of markers in yardlong bean breeding are given in Table 10.9.

The understanding associated with genetic diversity and molecular markers can be applied in genome editing for plant trait improvements. The emergence and applications of genome editing technologies such as site-specific recombinase (SSR) or a site-specific nuclease (SSN) system in field crops has brought considerable anticipation with a precise clear-cut targeted approach towards development of improved crop varieties either through addition or removal of traits under consideration (Abdallah et al. 2015). Different systems were employed in SSR technology (Cre/loxP, Flp/FRT) and SSN technology (mega-nucleases, ZFNs, TALENs, CRISPER/Cas). CRISPER holds promising results for loss of function, gain of function mutants and gene-expression analysis with versatile applications to produce high-quality agricultural products and sustainability (Arora and Narula 2017). The applicability of CRISPER/Cas9 system to cowpea (*Vigna unguiculata*) was successfully verified with a symbiotic nitrogen-fixation gene by Ji et al. (2019).

Table 10.9 Details of molecular markers used in various yardlong bean studies

Accessions Studied	Marker Used	References
24 accessions from Thailand	5 RAPD markers	Sarutayophat et al. (2007)
28 accessions from India	48 RAPD markers	Pidigam et al. (2019)
23 accessions	SSR and microsatellite markers isolated from soy bean	Tantasawat et al. (2010)
50 accessions from India	16 SSR markers	Ogunkanmi et al. (2007)
15 accessions from China, Laos, Philippines, Taiwan and Thailand	16 Cowpea STMS primers	Phansak et al. (2005)
99 accessions	1127 SNP markers	XU et al. (2012)
10 accessions	51,128 SNP markers	Carvalho et al. (2017)

Further studies exploiting gene-editing tools for improvement of *V. unguiculata* ssp. *sesquipedalis* need to be undertaken.

10.9.2.2 Mutation Breeding

Introducing genetic manipulation into the genome through induced mutations dates back to the beginning of twentieth century; the process consists of exposing cells to chemicals or radiation to generate mutations with desirable characters. With the discovery of mutagenic actions by x-rays on fruit fly, the technology was adapted for application to crop improvement by Stadler (1928) in maize, barley and wheat crops during late 1920s. Based on the importance of mutation breeding, a division (FAO-IAEA Division of Atomic Energy in Agriculture) was established in Vienna during 1964 and started supporting training activities from 1970. China, Japan and India are the top three countries involved in mutant production (Fig. 10.9) with global shares of 24, 14 and 10%, respectively (FAO-IAEA; https://www.iaea.org/).

Nanda et al. (1997) induced mutations in yardlong bean by using ethyl methanesulfonate (EMS), which resulted in the decrease in the survival rate, days to 50% flowering, height of the plant and also days of first picking in M_1 generation; whereas, the M_2 generation exhibited an increase in the number of branches, pods, pod size, pod weight and yield of the plant. It was also reported that in this generation an increase in the dosage of EMS resulted in the increase of foliar mutations. In a recent study on *Vigna unguiculata*, mutations were induced in seed with a combination of gamma rays and sodium azide (Raina et al. 2020).

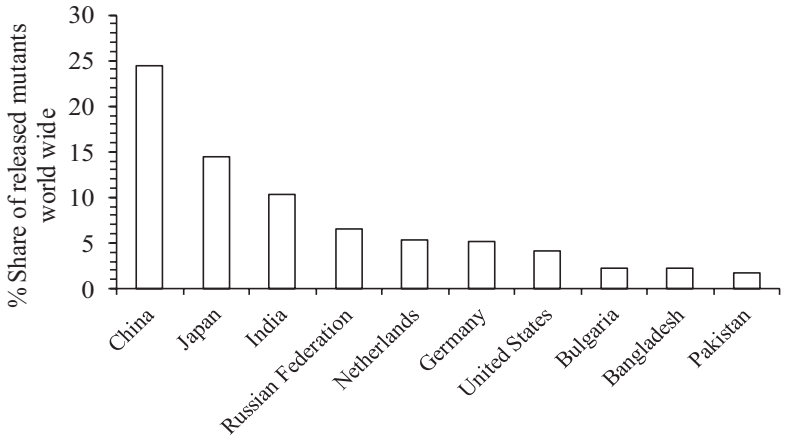

Fig. 10.9 Released mutants; statistics of top ten countries. (Source: FAO-IAEA (2019); https://www.iaea.org/)

10.9.2.3 Tissue Culture and Micropropagation

Cell and Tissue Culture Despite the molecular-marker applications in yardlong bean improvement, plant biotechnology facilitates an alternative approach to it in the form of plant tissue culture. In vitro culture of explant tissue from vegetable crops includes isolation, culture of different cells/tissues (protoplasts, organs, embryos, ovules, anthers, microspores) and their regeneration into complete plantlets. Tissue culture is one of the key aspects of plant biotechnology to improve agronomic performance, quality and yield in cowpea cultivars (Popelka et al. 2004; Zaidi et al. 2005). The potential developments in plant tissue culture through rDNA technology depend on the availability of a reproducible transformation system for the generation of transgenic plants (Aragão and Campos 2007). In vitro manipulation of legumes is challenging due to their genetically recalcitrant nature (Bakshi and Sahoo 2013; Somers et al. 2003). However, reports of tissue culture of cowpea were reported for somatic embryogenesis from suspension culture (Ramakrishnan et al. 2005), photoautotrophic tissue culture (Aliyu et al. 2016) and multiple shoot induction (Odutayo et al. 2005). Bett et al. (2019) devised an improved transformation method for cowpea through *Agrobacterium*-mediated gene delivery and selections based on kanamycin and geneticin, alternatively. Previously reported tissue culture research work on cowpea is shown in Table 10.10.

Vigna species are targeted by many pests, insects, and fungal, bacterial and viral diseases. Wild species of *Vigna* were found to harbor resistance genes for these pathogens. But, lack of cross-compatibility of wild species with the cultivated ones represents one of the major hurdles to the transfer of resistant genes. Genetic engineering methods offer great solutions to overcome the problem through gene transformation. However, there are reports of failure to produce mature transgenic *Vigna* plants for calli because of embryo imbibition (Kononowicz et al. 1997) and kanamycin-resistant callus (Garcia et al. 1986, 1987). Muthukumar et al. (1996) used de-embryonated cotyledons to express hygromycin marker gene by cocultivating with *Agrobactetrium tumifaciens,* but they failed to germinate. Lingaraj et al. (2000) succeeded in producing transgenic plants with *Agrobacterium tumifaciens*, but the stability of transgene integration was a failure.

The transformation approach was strongly supported with various gene delivery methods for plant cells viz., chemical procedure, electroporation, microprojectile bombardment, *Agrobacterium*-mediated transfer, biolistic methods, etc. Stable integration of transgene through particle bombardment was achieved in *Vigna unguiculata* and the gene was introduced through T_1, T_2 and T_3 progenies (Ikea et al. 2003). *Vigna unguiculata* transformation with an improved *Agrobacterium*-mediated transformation system by employing sonication and vacuum infiltration was developed by Bakshi et al. (2011) with an 88.4% increment in transformation efficiency. These approaches can be applied to yardlong bean to transfer genes related to abiotic and biotic stress which are not achievable through a breeding approach.

Micropropagation A technique used to rapidly multiply plants by in vitro culture to create true-to-type plants of selected genotypes (Singh 2015). Different types of

nutrient media and plant growth hormones were used to micropropagate *V. unguiculata* (Table 10.10). Jahan et al. (2015) developed an in vitro protocol by using shoot tip, leaf segment and nodal explants obtained from a cross between two selected parental lines for the propagation of yardlong bean, where a high frequency of shoot regeneration was obtained on MS medium supplemented with 0.5 mg/l BAP and 0.2 mg/l NAA. In the current scenario of yardlong bean research, development of micropropagation protocols with varied plant tissues is a prerequisite for further improvement of this crop towards incorporation of resistance against pests, as well as bacterial and viral infestations.

10.9.2.4 Doubled Haploids and Embryo Rescue

Doubled haploids produce the level of homozygosity in one generation, in contrast to the conventional breeding program, which require minimum of four generations to reach the level of near-homozygosity (Lulsdorf et al. 2011). However, production of doubled haploids in cowpea and yardlong bean is sparse, even though reports of callus production are available (Table 10.10).

Embryos are the specialized structures that develop into whole organisms under normal conditions of desirable parentage. But under special circumstances, the development of embryos will be aborted, and need to be rescued through in vitro support, failure leads to cessation of development, ultimately leading to death. The earliest embryo culturing dates back to eighteenth century, with *Phaseolus* and *Fagopyrum* embryos by Charles Bonnet (Sharma et al. 1996). Fatokun and Singh (1987) successfully generated hybrids from cultivated and wild cowpea through the embryo rescue technique. Pellegrineschi et al. (1997) studied the factors responsible for development of immature embryos of *V. ungiculata* and proposed deployment of kinetin, 6-benzylaminopurine, zeatin and cytokinins in successful promotion and maturation of embryos.

10.10 Conclusions and Prospects

Considering the great nutritional value and economic affordability for developing countries, research and development in yardlong bean is needed for crop improvement. Development of region-specific and niche-specific yardlong bean varieties through the application of the advancement in conventional and biotechnological approaches made in the past will be beneficial in enhancing cowpea production to improve world food security and nutrition (Singh 2014). Previous research work on yardlong bean involved development of lines with diverse maturity days and plant types, resulting in generation of wide germplasm base in the species. The current released varieties could be further exploited in improvement of yardlong bean yield. Good research advancement has already been initiated in drought, heat and shade

Table 10.10 Details of tissue culture research carried out on *Vigna unguiculata*

Tissue	Growth Medium	Hormones Used in Study											Reference
		BAP	NAA	2,4-D	2,4,5-T	ZE	IAA	ABA	TDZ	GA3	KT	IBA	
Meristem (apical)	MS	0.1–0.005 μM	10 μM									1 μM	Kartha et al. (1981)
Immature cotyledons	MS, MSB	0.5 mg/l		2 mg/l			0.1 mg/l				0.5 mg/l		Xue-bao et al. (1993)
Primary leaves	MS			2 mg/l									Kulothungan et al. (1995)
Primary leaves	B5	5 μM			0.8 μM								Muthukumar et al. (1995)
Hypocotyls and cotyledons	MSB	0.1, 0.5, 1 mg/l	0.1, 0.5, 1 mg/l			0.1, 0.5, 1 mg/l	0.1, 0.5, 1 mg/l				0.1, 0.5, 1 mg/l		Pellegrineschi et al. (1997)
Shoot tip	MS	5 mg/l	0.01 mg/l										Brar et al. (1999a)
Cotyledons	MS	66.6–155.3 μM											Brar et al. (1999b)
Primary leaves	MS			0.05, 4.52 μM				5 μM					Anand et al. (2000)
Cotyledonary thin cell layer explants	MSB								10 μM			1 μM	Le et al. (2002)
Embryo explants	MS	1 μM	1 μM										Odutayo et al. (2005)
Primary leaves	MSB, B5			0.1 mg/l				5 μM					Ramakrishnan et al. (2005)

Shoot apices epicotyls, stem hypocotyls	MSB	0.44–88.8 µM								1.4–14 µM		49 µM	Mao et al. (2006)
Cotyledonary nodes	MS, MSB	10 µM										2.5 µM	Chaudhury et al. (2007)
Cotyledonary nodes	MS, B5	0.1, 0.5, 1, 2, 3 mg/l	0.1, 0.5 mg/l								0.1, 0.5, 1 mg/l		Diallo et al. (2008)
Shoot meristem	MS	8.9 µM											Manoharan et al. (2008)
Shoot meristem	MS								0.25 mg/l			0.50 mg/l	Aasim et al. (2009a)
Cotyledonary nodes	MSB	0.5 µM											Raveendar et al. (2009)
Shoot tip explants (pulse treatment with BAP)	MS	0.25–1.25 mg/l	0.1 mg/l									0.5 mg/l	Aasim et al. (2009b)
Plumular leaf explant (pulse treatment with NAA)	MS	0.25–1 mg/l	1-4 mg/l									0.5 mg/l	Aasim (2010)
Hypocotyls, shoot tips, embryo and embryo with cotyledons	MS			5 mg/l									Dadmal and Navhale (2011)
Cotyledonary nodes	MSB	1.25 mg/l											Tang et al. (2012)
Cotyledonary nodes	MSB	3 mg/l											Tie et al. (2013)

(continued)

Table 10.10 (continued)

Tissue	Growth Medium	Hormones Used in Study										Reference
		BAP	NAA	2,4-D	2,4,5-T	ZE	IAA	ABA	TDZ	GA3	KT	IBA	
Shoot tip, nodal and leaf segment	MS	0.5 mg/l	0.2 mg/l									3 mg/l	Jahan et al. (2015)
Embryo explants	MS	0.1 mg/l	0.1 mg/l										Aliyu et al. (2016)
Cotyledons node explants	MS	1 mg/l										0.5–5 mg/l	Sheikh et al. (2016)
Cotyledonary node	MS	2.5 mg/l											Chekroun and Belkhodja (2017)
Primary leaves	MS, B5	2.22 μM		6.78 μM									Sani (2018)
Seeds	MS									1 mg/l			Vinoth et al. (2019)

MS: Murashige and Skoog medium (Murashige and Skoog 1962), MSB: Modified MSB medium (Robert et al 1987), B5: Gamborg's medium (Gamborg et al. 1968)
Provide the original reference for each of these media

tolerance of cowpea, which can be incorporated into improved yardlong bean varieties for better adaptation to divergent climate.

The level of resistance to pests and pathogens is limited in yardlong bean, but can be improved with adaptation of an integrated pest management strategy. Along with the disease resistance, development of weed resistance in yardlong bean could be targeted to benefit farmers through implementation of biotechnological tools. Recent studies of transfer of herbicide resistance genes into cultivated crops can be implemented in yardlong bean to enhance herbicide resistance. The inherent vigor in the wild accessions of yardlong bean can be dissected for their genetic constitution to improve yield in cultivated varieties. QTLs controlling morpho-physiological, agronomical traits need to be focused for improvement of yardlong bean.

Appendices

Appendix I: Popular Varieties/Cultivars of Yardlong Bean with Traits Under Cultivation in Australia, South and Southeast Asia and Africa

Varietal Identification No.	Country	Trait	Growth Habitat	Pod Color	Flower Color	Seed Color
VI034177	Australia	ILCA O#77253 (REYCALOONA)	Twining	Violet	Pale tan or straw	Tan with brown saddle
VI047698	Bangladesh	BARBARI	Twining	Mauve pink	Pale tan or straw	Brown and tan
VI047713	Bangladesh	BARBARI	Twining	Mauve pink	Pale tan or straw	Brown with whitish color at top end
VI034170	China	Green tail	Twining	Violet	Pale tan or straw	Mixture
VI034176	Ethiopia	VAR TVU 1977-OD	Twining	White	Pale tan or straw	Tan with brown saddle
VI055597	Laos	THUA NAO	Twining	Violet	Pale tan or straw	Black
VI055635	Laos	THEUA NHAG	Twining	Mauve pink	Dark tan	Red with brown mottles

(continued)

Varietal Identification No.	Country	Trait	Growth Habitat	Pod Color	Flower Color	Seed Color
VI034393	Malaysia	MPK-4	Twining	Dark tan	Mauve pink	Brown with whitish color at one end
VI034394	Malaysia	MPK-5	Twining	Dark tan	Mauve pink	Brown with whitish color at one end
VI034408	Malaysia	RED TAIL	Twining	Violet	Dark tan	Red
VI034416	Malaysia	Snake bean	Twining	White	Dark brown	White
VI034426	Malaysia	BALATONG (DUSUN)	Twining	Violet	Dark tan	Mixture
VI034439	Malaysia	EX-S'KAN	Twining	White	Dark brown	Tan with black saddle
VI034440	Malaysia	T.S.3	Twining	Mauve pink	Dark tan	Mixture
VI034416	Malaysia	Snake bean	Twining	White	Dark brown	White
VI034426	Malaysia	BALATONG (DUSUN)	Twining	Violet	Dark tan	Mixture
VI034441	Malaysia	T.S.6	Twining	White	Pale tan or straw	Red with whitish color at one end
VI034442	Malaysia	T.S.9	Twining	Mauve pink	Pale tan or straw	Black
VI034494	Malaysia	MKP4	Twining	Mauve pink	Dark tan	Red with whitish color at one end
VI034495	Malaysia	MKP5	Twining	Mauve pink	Dark tan	Red with whitish color at top end
VI034553	Malaysia	TAIWAN VAR	Twining	White	Dark brown	White
VI034554	Malaysia	BANGKOK VAR	Twining	White	Pale tan or straw	Red with whitish color at one end
VI034184	Mali	MALI#79114	Twining	Violet	Pale tan or straw	Tan
VI057925	Nepal	BODI	Twining	White	Pale tan or straw	Tan

(continued)

Varietal Identification No.	Country	Trait	Growth Habitat	Pod Color	Flower Color	Seed Color
VI034247	Nigeria	IT-82E-18	Twining	White	Pale tan or straw	Mixture
VI034252	Nigeria	N#82237 TVU1476	Twining	Violet	Pale tan or straw	Tan with purple mottles
VI034395	Philippines	CSL-14	Twining	Dark tan	Violet	Brown with whitish color at one end
VI034397	Philippines	CSL-19	Twining	Pale tan or straw	Mauve pink	Brown with whitish color at one end
VI036252	Philippines	BALATONG	Twining	Dark tan	Violet	Brown
VI041733	Philippines	SITAO	Twining	Dark tan	Violet	Mixture
VI041717	Philippines	SITAO	Twining	Violet	Dark tan	Red
VI034396	Philippines	CSL-16	Twining	White	*Pale tan/ straw	Red with whitish color at one end
VI034398	Philippines	SANDIGAN	Twining	Mauve pink	Pale tan/ straw	Red with whitish color at one end
VI034378	Philippines	EGPS 45-0-11	Twining	Violet	Dark tan	Brown
VI045112	Surinam	SIDOREDJO	Twining	Violet	Pale tan or straw	Red with brown mottled
VI040004	Thailand	THUA PEE	Twining	Pale tan or straw	Violet	Black
VI040032	Thailand	THUA NUEA	Twining	Pale tan or straw	Violet	Red
VI040606	Thailand	THUA-FAK-YAAO	Twining	Pale tan or straw	Violet	Red
VI040625	Thailand	THUA-FAK-YAAO	Twining	Pale tan/ straw	Mauve pink	Brown
VI040705	Thailand	THUA-FAK-YAAO	Twining	Pale tan or straw	Mauve pink	Brown with whitish color at one end
VI040726	Thailand	THUA-FAK-YAAO	Twining	Pale tan/ straw	Mauve pink	Brown with whitish color at one end

(continued)

Varietal Identification No.	Country	Trait	Growth Habitat	Pod Color	Flower Color	Seed Color
VI040748	Thailand	THUA-FAK-YAAO	Twining	Pale tan or straw	Mauve pink	Brown with whitish color at one end
VI040777	Thailand	THUA-FAK-YAAO	Twining	Pale tan	Mauve pink	Red with whitish color at one end
VI040695	Thailand	THUA-FAK-YAAO	Twining	Mixture	Mauve pink	Mixture
VI040828	Thailand	THUA-FAK-YAAO	Twining	Dark tan	Mauve pink	Mixture
VI040862	Thailand	THUA-FAK-YAAO	Twining	Dark brown	Mauve pink	Red with whitish color at one end
VI040886	Thailand	THUA-FAK-YAAO	Twining	Dark tan	Mauve pink	Red
VI040938	Thailand	THUA-PAK-YAAO	Twining	Pale tan or straw	Mauve pink	Red with whitish color at one end
VI040997	Thailand	THUA-FAK-YAAO	Twining	Pale tan or straw	Mauve pink	Mixture
VI041016	Thailand	THUA-FAK-YAAO	Twining	Pale tan or straw	Mauve pink	Mixture
VI041057	Thailand	THUA-FAK-YAAO	Twining	Pale tan or straw	Mauve pink	Red with whitish color at one end
VI041070	Thailand	THUA-FAK-YAAO	Twining	Pale tan or straw	Mauve pink	Mixture
VI041074	Thailand	THUA-FAK-YAAO	Twining	Pale tan or straw	Mauve pink	Red with whitish color at one end
VI040004	Thailand	THUA PEE	Twining	Pale tan or straw	Violet	Black
VI040032	Thailand	THUA NUEA	Twining	Pale tan or straw	Violet	Red
VI041098	Thailand	THUA-FAK-YAAO	Twining	Pale tan or Straw	Mauve pink	Mixture
VI045927	Vietnam	DAU HOANG DAO	Twining	Mauve pink	Pale tan or straw	Tan
VI045931	Vietnam	DAU DUA	Twining	Mauve pink	Pale tan or straw	Red

(continued)

Appendix II: List of Yardlong Bean Crop Improvement Research Institutes in India

Name of the Research Institute	Specialization and Research Activities	Accessibility
World Vegetable Center (Formerly AVRDC), Hyderabad, India	Germplasm storage, evaluation and improvement of yardlong bean	https://avrdc.org/
ICAR-Indian Institute of Horticulture Research (ICAR-IIHR), Bengaluru, Karnataka, India	Evaluation and improvement of yardlong bean	https://www.iihr.res.in/
Sri Konda Laxman Telangana State Horticultural University (SKLTSHU), Hyderabad, Telangana, India	Evaluation and improvement of yardlong bean	https://www.skltshu.ac.in
Kerala Agricultural University, Thrissur, Kerala, India	Evaluation and improvement of yardlong bean	http://www.kau.in/
ICAR Research complex for Eastern Region, Patna, India	Evaluation and improvement of yardlong bean	http://www.icarrcer.in
ICAR-National Bureau of Plant Genetic Resources, New Delhi, India	Collection, passport data, conservation and distribution	http://www.nbpgr.ernet.in/

Appendix III: List of Yardlong Bean Released Varieties in India

Variety	Year and Institute of Release	Habitat	Characteristic Features	Fresh Pod Yield and Maturity Days
Swarna Haritha	2008 – ICAR Research complex for Eastern Region, Patna, India	Pole type	Pure line selection recommended for Jharkhand and Bihar	27.21–31.75 mt/ha, 50–55 days
			Pods are dark green, very long (50–60 cm), straight, round and fleshy	
			Cooking quality is excellent	
			Seeds are light brown, elongated kidney shaped	
			Tolerant to mosaic viruses and rust under field conditions	

(continued)

Variety	Year and Institute of Release	Habitat	Characteristic Features	Fresh Pod Yield and Maturity Days
Swarna Sweta	2004 – ICAR Research complex for Eastern Region, Patna, India	Pole type	Pure line selection recommended for Jharkhand and Bihar	22.6–27.21 mt/ha, 50–55 days
			Pods are white in color, medium long (30–35 cm), straight round and fleshy with good cocking quality	
			Resistant to mosaic viruses and rust, Tolerant to pod borer	
Swarna Suphala	2006 – ICAR Research complex for Eastern Region, Patna, India	Pole type	Pods are light green in color, medium long (30–35 cm) with bulges at seed positions	22.6–27.21 mt/ha, 50–55 days
			Seeds are bicolored (sandalwood color with brown mottling)	
			Field resistant to cowpea mosaic viruses and field tolerant to pod borer during summer	
			Recommended for Jharkhand, Bihar, Karnataka and Kerala	
Arka Mangala	ICAR- Indian Institute of Horticultural Research, Bangalore, India	Pole type	Pure line selection for yield	22.6 mt/ha, 60 days
			Pods are very long (70–80 cm), string less, crisp, light green in color, tender and easy to snap without parchment	
			Number of pods per plant are 42	
Lola	2001 – Kerala Agricultural University, Thrissur, Kerala, India	Pole type	High yielder, pods are long with pale green color	18.14 mt/ha
			Seeds are black in color	
Vijayanthi	1998 – Kerala Agricultural University, Thrissur, Kerala, India	Pole type	A selection from Perumpadavam local (PS) for yield.	11.43 mt/ha
			Pods are long with pink color	
Vellayani Jyothika	2006 – Kerala Agricultural University, Thrissur, Kerala, India	Pole type	A selection from Sreekaryam local yield	17.53 mt/ha
			Pods are long with light green color	

(continued)

Variety	Year and Institute of Release	Habitat	Characteristic Features	Fresh Pod Yield and Maturity Days
Githika	2015 – Kerala Agricultural University, Thrissur, Kerala, India	Pole type	High yielder with mosaic virus resistance	25.03 mt/ha
			Pods are long, thick, and fleshy with light green color	
			Pods are long (53.4 cm) with reddish-brown seeds	
Mithra	2018 – Kerala Agricultural University, Thrissur, Kerala, India	Trailing growth habit	High yielder suitable for riverine alluvium of Central Travancore	18.77 mt/ha
			Pods are attractive, long (78.6 cm), light green color	
			Seeds are brown with white speck at one end	
Telangana Podugu Bobbarlu	2019 -Sri Konda Laxman Telangana State Horticultural University, Hyderabad, India	Pole type	Pure line selection for good yield	19.95 mt/ha, 76 days
			Pods are long, light green color, fleshy with good cocking quality	
			Tolerant to aphids	

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Part III
Mushrooms

Chapter 11
Enoki Mushroom (*Flammulina velutipes* (Curtis) Singer) Breeding

Ved P. Sharma, Anupam Barh, Rakesh Kumar Bairwa, Sudheer K. Annepu, Babita Kumari, and Shwet Kamal

Abstract Enoki is a popular edible mushroom well recognized for its culinary, nutritional and medicinal properties. Owing to its popularity, enoki cultivation has been rapidly expanded in the recent years in China and Japan. Bioactive compounds such as flammulinolide, enokipodin, proflamin and other polysaccharides extracted from both mycelium and fruiting bodies have demonstrated possible antitumor, anti-hypertension, anti-hypercholesterolemia and other therapeutic benefits. The life cycle of enoki mushroom is similar to that of other basidiomycetes and exhibits dedikaryotization which can produce both monokaryotic and dikaryotic fruiting bodies. Morphological variations in cultivated edible strains are clearly distinguished as Jinhua and Jinzhen types. Based on the available literature, it was observed that the yellow strains (Jinhua) may be domesticated directly from the wild strains while the cultivated white (Jinzhen) strains may have evolved through hybridization between wild cultivars. Genetic diversity studies indicate that the cultivated strains have a relatively narrow genetic base. With the increased pace in production activity, the need for genetic improvement of enoki has attained significance. This chapter reviews the available literature relating to the biology, sexuality, breeding behavior, conventional and molecular breeding approaches aimed at the genetic improvement of enoki mushroom.

Keywords Breeding · Edible mushroom · Genetic diversity · Genetic improvement · Hybridization · Winter mushroom

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11.1 Introduction

Flammulina velutipes (Curtis) Singer, popularly known as enoki or winter mushroom, is a delicious edible mushroom cultivated in China, Japan and other Asian countries. It also has several other vernacular names such as enokitake, golden needle mushroom, velvet stem and velvet foot mushroom. This mushroom grows naturally on the stumps of the Chinese hackberry tree, known as enoki in Japanese. Morphologically, there is a clear distinction between the naturally grown and domesticated strains. The cultivated strains of enoki have white, thin, slender stems and the wild ones have yellowish to brown colored basidiocarps. The yellow strain is known as *Jinhua* mushroom in Taiwan (Hu et al. 2019) and the white stipe strain as *Jinzhen* mushroom (Fig. 11.1). Historical records showed that the enoki mushroom was cultivated for the first time in China during the eighth century and then spread to Japan. For over 300 years, enoki was cultivated on wood logs under semi-wild conditions. Recent bottle cultivation technology used in enoki cultivation has gained wide popularity. Until the 1990s, Japan was the leading world producer of enoki mushroom. Since then China has replaced Japan in production. At present the majority of the enoki production units in China are fully mechanized with an annual production capacity of 2.4 million mt (Li and Li 2014).

Enoki is a common element of Japanese cuisine, salads and in gourmet restaurants. This mushroom gives a pleasing aroma to a dish. Like all other edible mushrooms, enoki contains essential nutrients including proteins, unsaturated fatty acids, minerals and fiber required by the human body. *Flammulina* is a good source of several bioactive proteins. FIP-fve (fungal immunomodulatory protein) is extracted from the fruiting bodies and has been studied extensively for its immunomodulatory and antitumor properties (Chang et al. 2013; Lee et al. 2013). Flammin, velin, velutin and flammulin are other ribosome inactivating proteins extracted from enoki with similar bioactive properties. Advances in medical mycology have shown many purported therapeutic benefits of enoki mushroom. Among them, antitumor (Gu and Leonard 2006), anti-hypercholesterolemia (Rahman et al. 2015) and

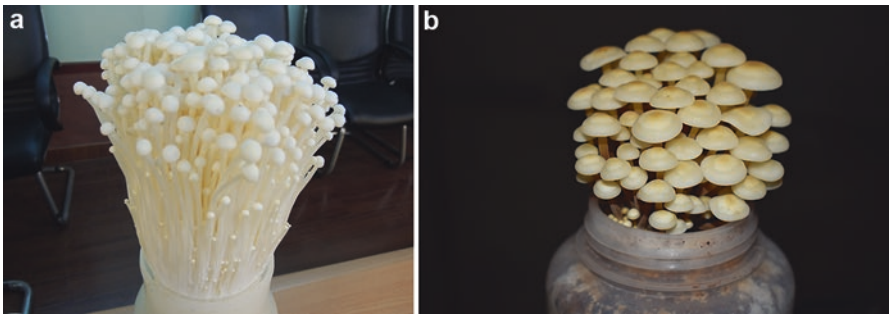

Fig. 11.1 (**a**) Cultivated Jinzhen enoki mushroom with creamy white colored fruiting bodies, (**b**) Cultivated Jinhua enoki mushrooms with yellowish brown fruiting bodies. (Photo **a** by Sudheer Kumar Annepu. Photo **b** by ICAR-DMR, Solan)

neurotransmitters restoration (Yang et al. 2015) associated with the human ability to learn and memorize have attracted the attention of both pharmaceutical and dietary supplement industries. A few recent studies of the biosynthesis of gold nanoparticles from enoki is an important research area for pharmacology (Rabeea et al. 2020).

Consumption of both the mycelium and fruiting bodies has potential health benefits. Because they possess several nutritional and therapeutic properties, the cultivation of golden needle mushrooms has obviously expanded in recent years. In view of the increased pace in its cultivation, the need for genetic improvement has also gained significance. The ultimate outcome of crop breeding is mainly superior cultivars. To develop the improved cultivars through a systematic breeding plan, it is essential to identify the desirable traits available in different individuals of the species and bring them together to develop a better individual stock. To achieve this, the breeder must have a thorough knowledge of the basic life cycle and mating systems of the targeted mushroom species. The biology and sexual behavior of enoki mushrooms is explained below.

11.2 Biology

11.2.1 Habitat and Distribution

Flammulina velutipes grows in caespitose, gregarious, clusters on wooden logs, stumps and roots of living deciduous trees in subtropical to temperate regions. Hughes et al. (1999) proposed that the Northern Hemisphere *Flammulina* species consisted of *F. velutipes* along with *F. mexicana* Estrada & R.H. Petersen, *F. populicola* Redhead & R.H. Petersen, *F. rossica populicola* Redhead & R.H. Petersen, *F. ononidis* (Arnolds), *F.elastica* (Sacc.) Redhead & R.H. Petersen and *F. fennae* Bas. Bas (1983) classified *F. velutipes* into two varieties, *F. velutipes* var. *velutipes* and *F. velutipes* var. *ladea*, a pale form with white to cream pileus. Variety *velutipes* was further subdivided into formae, *F. velutipes* and *F. longispora.*

11.2.2 Life Cycle

Flammulina velutipes shows heterothallism with a tetra-polar mating system. The mature fruiting bodies produce monokaryons, but sometimes a few of these develop into fruiting bodies that are sterile. The fruiting bodies developed by secondary mycelium are fertile and develop basidiospores (Xu et al. 2015) (Fig. 11.2).

The fruiting mechanism in *Flammulina* is unclear at the molecular level. Various studies have been carried out at the genomic and proteomic levels to understand clearly the mechanism of *Flammulina* fruiting. Ando et al. (2001) showed that the *fvh1* gene was expressed in mycelia after the induction of the fruiting and primodia

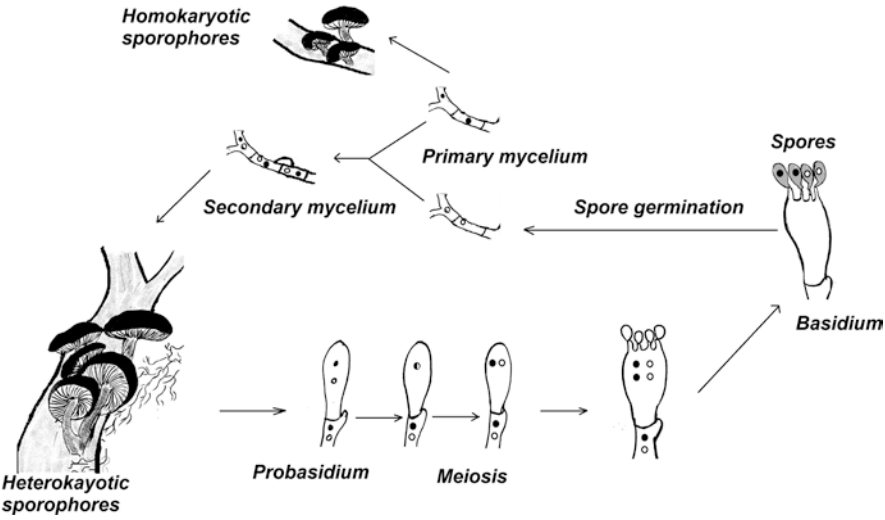

Fig. 11.2 Life cycle of *Flammulina velutipes*. (Drawing by Babita Kumari and Anupam Barh)

stage. The gene encodes the hydrophobin protein. Yamada et al. (2006) isolated cDNAs from pinhead and fruiting bodies of *Flammulina* and found cDNAs similar for coding for proteins such as the growth factor, cytochrome P450, GTP-binding protein, ubiquitin-proteasome and hydrophobin, whereas 17 cDNAs were found to be unique and were predicted to play specific roles in fruiting. Later, Yamada et al. (2008) found that *Fv-pda*, a gene coding for chitin deacetylase (CDA), specifically expressed through the entire stage of fruiting body development, and suggested the role of CDA in the process of the fruiting of *F. velutipes.* The role of the environment, specifically illumination on fruiting was also studied and found that light stimulus helps the pileus to develop and may be due to cell wall-associated protein (PSH) that was differentially induced in the pileus than in the stipe. The amino acid sequence was similar to that of hydrophobin in some aspects. They found that *psh* is specifically expressed during pileus differentiation after light stimulation (Sakamoto et al. 2007). Liu et al. (2018) found that proteins involved in the TCA cycle, carbohydrate metabolism, biosynthesis of fatty acids and branched chain amino acids, carotenoid formation, MAPK signaling pathway are upregulated when fruiting bodies were developed by mycelia in *F. velutipes* (Liu et al. 2018). Kurata et al. (2016) identified 6 genes involved in the fruiting body development (2 hydrophobin genes (*hyd1*, *hyd2*), 1 mitochondrial ATP-synthase gene (*atp*), 1 fruiting body-specific gene (*fds*) and 2 transcription factor genes (*fst, gat*)). Similarly, Park et al. (2014) performed genome analysis and showed various genes associated with mushroom formation which includes mating type genes, hydrophobins and fruiting body-specific genes (*FDS*, *FVFD16*, *FVFD30*).

11.2.3 Taxonomy

Flammulina velutipes has a cap, 2–7 cm wide, broadly convex to flat, moist, sticky, dark orange to yellowish orange in color. Lamellae adnate, whitish to yellowish brown, 0.3 cm broad, crowded. Stipe up to 5–10 cm long, 0.8 cm broad, equal, tough, orange brown to rusty brown, velvety and stuffed. The basidiospores 6–10 × 3.5–5 μm, ellipsoid, inamyloid, smooth. Basidia 24–30 × 10–13 μm, narrowly clavate, thin walled, tertrasporic, sterigmata up to 3 μm long. Pleurocystidia and cheilocystidia both similar in size and shape, scattered, lageniform to ventricose, 28–50 × 10–15 μm, thick walled. Pileipellis a cutis of ixotrichoderm made up of branched, cylindrical to fusoid ventricose elements with clamps up to 15 μm wide. Clamps are present (Kuo 2013).

11.2.4 Genomic Composition

The genome of *Flammulina* is around 34–35 Mb, as reported for strain TR19 and KACC42780, respectively, with around 49% of GC content. The genome possesses 12,218 predicted protein-encoding genes and 287 tRNA genes with a total of 11 chromosomes (Park et al. 2014). The mitochondrial DNA (mt DNA) of *F. velutipes* is circular with 88,508 bp and GC content of about 16.50%. The total codons in mtDNA are 4889 bp (Yoon et al. 2012). The genome of mitochondria in *F. velutipes* contains about 35 genes (Park et al. 2014).

11.2.5 Breeding System and Sexuality

In *Flammulina,* dikaryotic mycelium form after the dedikaryotization of monokaryons. Dikaryotic mycelia grow faster than their parental monokaryons, but in some cases the growth rate of parental mycelium is intermediate compared to the parental monokaryons (Takemaru 1957). Monosporous culture isolates from a single fruit body also show significant variation in growth rates (Simchen 1965). Kniep (1920) observed that unclamped mycelia formed when cultured together give rise to clamped mycelium; and suggest the heterothallic character of *Flammulina*. Bifactorial control of heterothallism is reported by several researchers (Vandendries 1923; Zattler 1924). In *F. velutipes* mating genes responsible for sexual development have been investigated extensively. The tetrapolar mating system is dominated by two factors on different chromosomes: homeodomain (HD) loci including HD-a and HD-b subloci, and pheromone (PR) loci consisting of PR-a and PR-b subloci (Van Peer et al. 2011; Wang et al. 2018a).

11.3 Breeding Objectives

In many countries, *Flammulina velutipes* is a commercial crop. Breeding objectives mainly change for region and time. However, a few current pertinent breeding objectives are discussed below.

11.3.1 Breeding for Yield

Yield is a major constraint in winter mushroom. Various studies have been done to improve the yield through the development of hybrids. The white and yellow strains of *Flammulina* are both used to breed for high yield. Wu et al. (2019) suggested that the transcription factor *pdd1* is responsible for primordial development. They showed that the increased transcriptional level of *pdd1* shortened the cultivation time and increase the yield. The regulation of fruiting genes is also done by *pdd1*.

11.3.2 Breeding for Shape and Quality

Fruiting-body color is one of the most important considerations in breeding programs. Some researchers have hypothesized that the high lysine content in fruiting bodies may be correlated with brittleness. The firmness of the stipe helps in its elongation which is beneficial for bottle cultivation in *Flammulina velutipes* (Liu et al. 2015).

11.3.3 Breeding for High Temperature Fruiting

Flammulina mushroom generally requires less than 15 °C in temperature for fruiting. Moreover, temperatures of 4–6 °C are required for primordial formation. Therefore, for sustainable cultivation in subtropical and tropical regions, high temperature resistance strains are required. Moreover, the difference may be due to the two formae present: *Flammulina velutipes* var. *velutipes* f. *velutipes* and F. *velutipes* var. *velutipes* f. *longispora*. Forma *velutipes* is characterized by its fruiting and can bear all year, while forma *longispora* fruits only in winter (Fultz 1988). Studies were also done to identify the cause of cold induced fruiting in *Flammulina* and it was found that cold signal transduction activates the mitogen-activated protein kinase (MAPK) pathway to initiate pinheads (Wu et al. 2018). Many researchers have tried to develop high temperature resistance strains in *Flammulina* using hybridization, mutagenesis and genetic engineering methodologies. Various studies for high temperature breeding were done on *Flammulina.* In one study, *F. velutipes*

strains that fruit at 22 °C was crossed with conventional strain fruit that fruit at 15 °C. It was found that the hybrids showed incomplete dominance of the low-temperature requirement. Researcher indicated that a minimum of two genes are required for fruiting at lower temperature (Fultz 1988). Kong et al. (2004), developed strains for high temperature conditions using selection, backcrossing and marker-assisted selection. These strains were well suited to elevated temperatures. Mutagenesis was also used in another study to develop high temperature resistance strains. Mutagens such as ultraviolet (UV) radiation, lithium chloride (LiCl), ethyl methanesulfonate (EMS) and combinations thereof were used. Three temperature tolerant strains were developed and were able grow at 33 °C and fruit at 20 °C (Lin-Zhi et al. 2013).

11.3.4 Breeding for Ideotype

Ideotype literally means an ideal individual with all the desirable characters belonging to a specific taxon. Ideotype breeding is a biological model that aims to develop an improved cultivar for a defined environment or purpose. In *Flammulina velutipes* the ideotype plays an important role as bottle cultivation requires an elongated stipe and is a less labor-requiring variety. For example, Geumhyang2ho, a brown cultivar, was developed as a labor-saving variety by crossing cvs. Geumhyang and Garlmoe. During bottle cultivation, Geumhyang2ho cv. does not require the vinyl cone treatment thereby reducing the labor required (Kim et al. 2018) (Fig. 11.3)

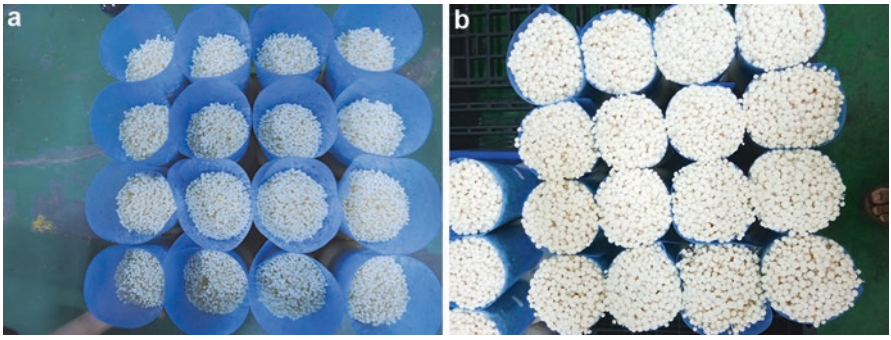

Fig. 11.3 Ideal ideotype of *Flammulina velutipes* for bottle cultivation technology. (**a**) The ideal strain of enoki mushroom with uniform growth, (**b**) Enoki mushroom ready for mechanized harvesting. (Photos by Sudheer Kumar Annepu)

11.4 Genetic Improvement of *Flammulina velutipes*

In the past five decades, several attempts were made by mycologists regarding the domestication and hybridization of *Flammulina velutipes*. Sanming 1 was the first cultivar of *F. velutipes* domesticated from the a wild strain in China in 1974 (Meiying 1997). In 1983, Japanese breeders released a new strain, Xinnong 2, which was developed through hybridization using Sanming 1 as one of the parent strains. This strain might be the first hybrid strain released for cultivation with white color and slender stem characters. Four years later, in 1987, F21, another strain with white and slender stem characters was introduced in China. This pattern indicates that the yellow strains may have been domesticated directly from the wild strains; whereas, the cultivated strains in China and Japan with Jinzhen characters may have developed through hybridization between the wild cultivars (Liu et al. 2016).

11.4.1 Hybridization

Hybridization in *Flammulina* is done to achieve a suitable gene combination to target certain breeding objectives. The majority of *Flammulina* breeding is done using intraspecific hybridization. But some intergeneric studies were also done. For example, somatic hybridization by protoplast fusion between *P. ostreatus* and *F. velutipes* resulted in synkaryons that retained genes from both parents (Yoo et al. 2004). Intraspecific hybridization is predominately used for improving yield and exploiting heterosis.

Hybridization is achieved by isolating spore prints followed by a serial dilution technique on malt extract agar media or potato dextrose agar media incubated at 24 ± 2 °C to create single spore isolates. These single spore isolates are hybridized by placing the different single spore isolate bits in a media petri plate at 1 cm apart. The super-hypal structure at the contact point of both mycelia is considered the putative hybrid (Fig. 11.4). Various research studies which attempted to develop cultivars of winter mushroom are provided in Table 11.1.

11.4.2 Mutation Breeding

Mutation breeding is an important aspect of mushroom breeding. Various mutation studies were done in winter mushroom for various purposes such as development of improved strains, development of superior quality of mushroom, improvement of yield of enzymes and bioactive compounds. Most irradiation studies focused on protoplast and mycelium. Protoplast mutagenesis was widely used in many of the mutation studies. Very few studies indicated changes in chromosome number or shape during the mutation. Various studies done in *Flammulina velutipes* are presented in Table 11.2.

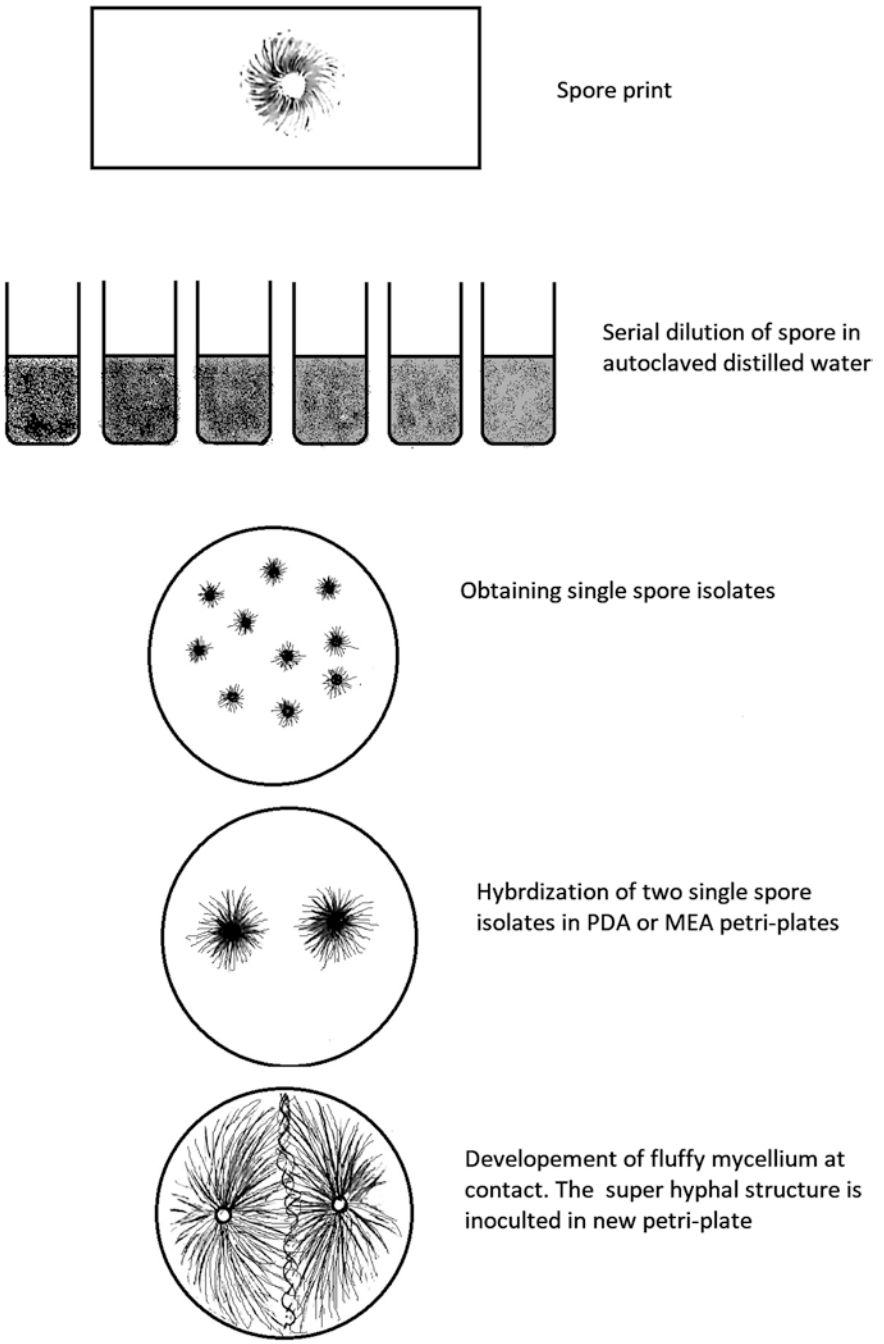

Fig. 11.4 Mechanism of hybridization in *Flammulina velutipes.* (Figure constructed by Babita Kumari and Anupam Barh)

Table 11.1 Intraspecific studies on *Flammulina velutipes*

Intraspecific hybridization	Results	Reference
Mating of two monokaryotic strains	Different white *F. velutipes* strains monokaryons were selected randomly from each parental strain and mating was done. Hybrid FM × F3-W and F10 × F3-W adapted to the standard set of cultivation conditions. Correlation between mycelial growth rate and fruit body yield was not found	Xu et al. (2009)
Mating of two monokaryotic strains	A variety Baek-a having high quality and high productivity was developed	Kong et al. (2013)
Mating of two monokaryotic strains of white and brown strain	Baekjung variety was developed and was adaptable to high temperature. The mycelia growth was at 30 °C and fruiting was achieved at 14 °C	Kim et al. (2015a)
Mating of two monokaryotic monokaryons isolated from Garlmoeand wild strain CBMFV-33	A variety Geumhyang was developed with short cultivation period with around 25% biological efficiency	Kim et al. (2015b)
Mating of two monokaryotic monokaryons isolated from Garlmoe and wild strain CBMFV-10	Dark brown color variety named Heukhyang was developed having short cultivation period and good taste. The BE of variety was 15.8%	Kim et al. (2015c)
Mating of two monokaryotic strains	Baekseung, a new variety of *F. velutipes* was developed	Woo et al. (2017a)

Table 11.2 Mutation studies done on *Flammulina velutipes* for trait improvement

Mutagen	Dose	Exposure duration	Results	Reference
UV irradiation	UV	1 min	Protoplast of *F. velutipes* was irradiated by UV for 1 min. Mutant possess higher the mycelia growth rate by 23.1–52.3% and their fruiting period was 10–14 days earlier than parents	Yali et al. (1995)
^{60}Co γ-rays	^{60}Co γ-rays	–	After irradiation old strains of *F. velutipes* selected for new strain with higher biological efficiency and better quality. They found a unique methodology for *F. velutipes* breeding	Ling et al. (2000)
Irradiation by He-Ne laser	He-Ne laser	–	*F. velutipes* irradiated with He-Ne laser. They found stability of yield in protoplast mutagenesis was higher than mycelium fragmentation and spore mutagenesis	Yaowei et al. (2002)
^{60}Co γ-rays	^{60}Co γ-rays	–	Irradiation on dikaryotic hypha and selection for several generations. Done for resistance against disease, growth and production	Wang et al. (2007)

The irradiation of protoplasts requires protoplast isolation. Protoplast isolation is done by removing the cell wall from the organism. Protoplasts are used in both mutagen treatment and fusion. Protoplast fusion is used to generate novel characters even in distantly related species (Singh and Kamal 2017). The protoplast isolation procedure depends on three major components used in isolation: age of the mycelium, lysis enzyme used for cell wall degradation and osmotic stabilizers. In *Flammulina velutipes,* 5-day old mycelia are effective for protoplast isolation. It is also seen that Novozyme 234 plus cellulase CP at 10 mg/ml concentration for 3 h is suitable for lysis of the cell wall of mycelia of *F. velutipes*. The osmotic stabilizers are important during protoplast isolation as the cell wall pressure needs to be replaced by osmotic pressure in the isolation mixture. The best osmotic stabilizer for the protoplast formation of the mycelium was 0.6 M sucrose with pH 6.2 (Yea et al. 1988). Protoplast regeneration is done in an osmotic stabilizer solution. Mutagenesis is accomplished either by treating the protoplast suspension with UV or a chemical mutagen. If the chemical mutagen is applied, then the protoplasts are washed after the treatment with osmotic stabilizer solution to remove traces of mutagens and plated on regenerating solid medium.

11.5 Biotechnological Advancement

11.5.1 Genetic Transformation

Several researchers have studied transformation using different methods viz. PEG mediated, electroporation, particle bombardment and *Agrobacterium*-mediated transformation. Some of these are developed for a unique purpose like oral vaccine development (Huang et al. 2019). The major concern in *Flammulina* for transformation is its low transformation frequency. Kuo et al. (2004) used the electroporation method as a rapid, simple and convenient method by transforming the hygromycin B phosphotransferase gene from *Escherichia coli* using *gpd* promoter. Shi et al. (2017) used liposome-mediated transformation (LMT) system for genetic transformation in *F. velutipes*. They used the LMT system for laccase gene and found increased transformation frequency with stable integration. Similarly, a unique study was done to develop an oral vaccine for hepatitis B using the stable transgenic hepatitis B virus surface antigen (HBsAg) in *F. velutipes.* The transformation was done using *Agrobacterium*-mediated transformation. They found immunogenicity in pigs by feeding them transgenic HBsAg *Flammulina* (Huang et al. 2019). Various other studies on *Flammulina* transformation are presented in Table 11.3.

Table 11.3 Transformation studies on *Flammulina velutipes improvement*

Transformation method	Objective	Reference
Agrobacterium-mediated transformation	Studied transformation frequency and obtained 16% with stable integration	Cho et al. (2006)
Restriction enzyme-mediated integration	Transformation in *F. velutipes* for alcohol production by pentose using *glyceraldehydes-3-phosphate dehydrogenase* (*gpd*) gene promoter to control the expression of target genes with *hygromycin B phosphotransferase* (*hph*) gene from *Escherichia coli*	Maehara et al. (2010)
Agrobacterium-mediated transformation	To develop mutants for trait improvement and to conduct gene function studies	Park et al. (2010)
Polyethylene glycol-mediated transformation	Two gene *taxadiene synthase* gene (*ts*) and *hygromycin phosphotransferase* gene (*hph*) were co-transformed 44.44% efficiency. The study was conducted to improve the varietal quality of *F. velutipes*	Kang et al. (2013)
Agrobacterium tumefaciens-mediated transformation	Transformed *Flammulina* to express virus like particle for oral vaccine against enterovirus 71 infection	Lin et al. (2015)
Polyethylene glycol-mediated transformation	Transformant was developed having ability to synthesis of intermediate baccatin III required for development of cancer drug paclitaxel, in the transgenic *F. velutipes* using 10-deacetylbaccatin III-10β-O-acetyltransferase (*DBAT*) gene	Shi et al. (2017)

11.5.2 Molecular Marker Breeding

In last three decades, molecular markers have emerged as a powerful tool not only for plants and animals but also for fungi. The multidimensional uses of genetic marker have enabled breeders to use markers in genetic improvement. In *Flammulina velutipes,* molecular markers predominantly were used for genetic diversity studies, identification of traits such as fruit color (Liu et al. 2005), to study breeding history, association mapping, linkage mapping, marker-assisted breeding, varietal identification and species identification. The markers used for various studies in *Flammulina* are provided in Table 11.4.

11.5.2.1 Genetic Diversity of *Flammulina* Species

Diversity studies done before molecular markers became available were based upon morphological diversity; however, after the advent of molecular markers various studies were conducted on the genus *Flammulina* to identify diversity and distribution pattern. Hughes et al. (1999) proposed classification of the genus *Flammulina* based on ITS markers and recognized species such as *F. mexicana, F. populicola, F. rossica, F. ononidis, F. elastica, F. fennae and F velutipes*, distributed in the

Table 11.4 Markers used in *Flammulina velutipes* studies

Marker	Results	References
Restriction fragment length polymorphism (RFLP)	DNA Sequencing and PCR-RFLP analysis of the ribosomal RNA gene (rDNA) regions was performed to differentiate between 7 typical *Flammulina* strains. PCR-RFLP method was found more suitable	Palapala et al. (2002)
Sequence characterized amplified region (SCAR)	A pair of SCAR molecular marker was synthesized, which are linked with fruit body color gene in *F. velutipes*	Liu et al. (2005)
Inter simple sequence repeat (ISSR)	Genetic diversity of 59 strains was analyzed by the use of ISSR markers and morphological characteristics	Yang et al. (2007)
ISSR, SCAR	ISSR primers were used to analyze DNA polymorphism of 7 strains of *F. velutipes*. Among which 1 primer was able to differentiate 1 strain from the other 6 strains. Further, a pair of SCAR primers was developed and validated to recognize strain that strain at molecular level	Su et al. (2008)
Microsatellites or simple sequence repeats (SSR)	Total 10 SSR markers were developed and characterized by FIASCO methods against 14 *F. velutipes* cultivars. 4 SSR loci were found which can be used to differentiate all 14 cultivars	Zhang et al. (2010)
Random amplified polymorphic DNA (RAPD)	RAPD and bulk segregant analysis techniques were used for development of marker associated with fruit body color. 200 RAPD markers were screened and 1 marker found which is stable and associated with color gene	Kong et al. (2014)
SSR	SSR markers developed which are associated with 5 agronomic traits through association analysis for marker-assisted breeding	Lu et al. (2015)
SSR	To understand the genetic background and breeding history of *F. velutipes*, 124 strains (cultivars and wild) were tested and 25 SSR polymorphic markers were developed	Liu et al. (2016)
SSR	A total of 12 polymorphic SSRs markers were developed from SSR-enriched library of *F. velutipes* SSR and these markers were utilized to analyze the genetic diversity of 32 strains of *F. velutipes* collected from Korea, China and Japan	Woo et al. (2017b)
SSR	To efficiently utilize the germplasm core collection (32 strains) was developed from 81 strains by analyzing genetic diversity with the help of 25 SSR markers	Liu et al. (2018)
SSR	The phenotypic and genetic diversity of 37 *F. velutipes* strains from China were investigated by using 7 agronomic traits and 70 SSR markers respectively to find elite breeding strains of *F. velutipes* strains	Wang et al. (2018a)

(continued)

Table 11.4 (continued)

Marker	Results	References
Internal transcribed spacer (ITS) translation elongation factor (tef1-α), RNA polymerase II (rpb2), and homeodomain1 markers	In this study partial sequences of the ITS, *tef1-α*, *rpb2* and homeodomain1 (HD1) of the mating gene were used for phylogenetic analysis and species delimitation	Wang et al. (2018a)
Expressed sequence tag-simple sequence repeats (EST-SSR) and single nucleotide polymorphism (SNP)	Markers were used to understand molecular mechanism of cold-induced fruiting in *F. velutipes*. Unique EST-SSRs and SNP were identified for functional genomics study of cold-induced fruiting in *F. velutipes*	Wu et al. (2018)

Northern Hemisphere. They also ascertained that Europe may be a center of diversity for the above species. Diversity studies on *Flammulina* done in China suggest that species *F. rossica*, *F. velutipes* and *F. yunnanensis* are found in China and strains of *F. velutipes* in China are more closely related to a species found in Canada rather than to those in Europe (Ge et al. 2008). Another genetic diversity study in China of *F. velutipes* using SSR markers found higher genetic diversity in wild strains (Liu et al. 2016). A study by Wang et al. (2018b) on phenotypic diversity showed that white and yellow strains differ in fruiting time, stipe length and yield. In two of the above studies both found yellow strains of *F. velutipes* to be indigenous to China. Other studies revealed that wild strains showed higher genetic diversity and that many economically-important traits are present in these wild strains (Liu et al. 2016, 2018; Tao et al. 2016).

11.5.2.2 Genome Editing in *Flammulina velutipes*

Genome editing and gene editing tools are used for many purposes. In *Flammulina velutipes,* until now, these tools have been little used. However, CRISPER-Cas9 system was used in *F. velutipes*. Two histidine kinase genes HK1 and HK2 were edited using CRISPR/Cas9 system to study the fruiting body development in *F. velutipes* (Ouyang et al. 2018). In another study, RNA interference (RNAi) was used for gene knockdown to study the effect of the *Fvcpc2* gene. The study showed that expression of *Fvcpc2* is linked with high yield and reduced fruiting time (Wu et al. 2020).

11.6 Conclusion and Prospects

Systematic breeding program aims to improve the genetic architecture of cultivated mushrooms through a continuous process. Although enoki mushroom cultivation is gaining huge popularity, but its genetic background remains poorly understood.

Existing studies reveal a higher genetic diversity in wild strains. Breeders should aim to utilize this gene-pool arsenal in future breeding programs. Critical insights are required on genetic analysis and character association in different strains to widen the existing gene pool. The mushroom industry, especially enoki cultivation, is rapidly moving from a labor-intensive activity to one that is mechanized and automated. Hence, the new improved strains should have the potential to meet food industry needs. Newer tools such as gene and genome editing open an avenue for understanding the gene and gene functions. These tools help in understanding various function of genes by knockdown and also can be used in the removal of undesirable agronomic traits. Winter mushroom, due to high medicinal and unique flavor, is important but to reduce costs of production and to increase acceptability in tropical regions, there is a need for high temperature strains. Due to recent increases in global temperature and climate change it is necessary to take into consideration the changing environment while breeding newer mushroom strains. New research and studies are still required to identify responsible genes to enhance adaptability of the enoki mushroom.

Appendix I: Research Institutes Relevant to Enoki Mushroom

Institution name	Research activities	Contact information and website
Indian Council of Agricultural Research (ICAR), Directorate of Mushroom Research	Strategic and applied research on collection, conservation, utilization and production of edible and medicinal mushroom. This include research on genetic improvement of edible mushrooms, mushroom protection technologies, mushroom production technologies, and postharvest and preservation technologies	Chambaghat, Solan, Himachal Pradesh State 173213, India https://nrcmushroom.org/
Shanghai Academy of Agricultural Sciences Edible Fungi Institute	Breeding and production of varieties	35 Nanhua Rd., Minhang District, Shanghai, China http://www.saas.sh.cn/
Gyeongnam National University of Science and Technology	Breeding and production of varieties	33 Dongjin-ro, Jinju, Korea https://www.gntech.ac.kr/web/eng
Mushroom Research Division, National Institute of Horticultural & Herbal Science	Breeding of enoki varieties	100, Nongsaengmyeong-ro, Iseo-myeon, Wanju-gun, Jeollabuk-do, Korea https://www.nihhs.go.kr/eng/about/nihhsOrganization.do

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Chapter 12
Shiitake Mushroom (*Lentinula edodes* (Berk.) Sing.) Breeding in China

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Abstract Shiitake mushroom (*Lentinula edodes* (Berk.) Sing.) is widely cultivated in China, Japan, Korea and many other Asian countries. It is one of the most extensively grown and consumed edible fungi in the world, with an exceptional high agricultural yield. Taxonomically, *L. edodes* belongs to the phylum Basideomycotina and family Agaricaceae. It is broadly distributed in the wild, mainly in the subtropical to temperate regions of the northern hemisphere. Due to its excellent taste qualities, high nutritional value, and medicinal properties, *L. edodes* is of great importance for the food industry and has medicinal applications. Various active medical ingredients such as lentinan, lentin, lectin and eritadenin, have been isolated from *L. edodes* culture media, fruiting bodies or mycelium. Enzymes, such as laccase, produced by *L. edodes*, have potential for industrial applications related to paper production (biopulping), residue treatment and improvement in the digestibility of animal rations. Like for other edible fungi cultivation, the raw materials for shiitake mushroom cultivation mostly constitute agricultural waste such as sawdust, straw and cottonseed husk. Moreover, the waste from the shiitake mushroom cultivation itself can be further used as a bio-organic fertilizer, greatly contributing to the process of crop rotation. Due to the changing natural environment along with the continuous improvement of living standards, mushroom cultivation is facing constant challenges. In order to obtain shiitake mushroom strains adapted to different climatic conditions, different cultivation methods and processing practices, breeders continuously screen for new shiitake varieties implementing diverse methods. In this chapter, we present an overview of the origin, distribution, taxonomic position, genetic characteristics, cultivation patterns and history of shiitake mushroom breeding by traditional and modern breeding methods in China.

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12.1 Introduction

Lentinula edodes (syn. *Lentinus edodes*) also known as xiangxun, was originally described as a large wild wood-decay fungus. Its taxonomic status belongs to the kingdom Eumycota, phylum Basidiomycete, class Agaricomycetes, order Agaricales, family Agaricaceae and genus *Lentinula*. *Lentinula edodes* is now the world's leading cultivated edible mushroom, constituting about 22% of the world's mushroom supply (Royse et al. 2017). China has a long history of mushroom cultivation, and it is said that Wu Sangong, a farmer from the Song Dynasty (960–1279 CE), invented the Mince Flower Method (also known as the Chop Flower Method) of mushroom cultivation. There are many legends about the Mince Flower Method, but the most evidence-based is the invention from Wu Sangong. The specific method is to use a *chopping axe* to cut down the logs, chop axe marks in the bark; the natural mushroom spores from the air germinate on the cuts and hyphae form in the bark. After 1–2 years, the fruiting bodies appear. Later, based on the Mince Flower Method, the Japanese invented a method of the hyphae inoculation, and then introduced it to Taiwan and Guangdong Province in the 1930s and 1960s, respectively. It was not until the 1970s that Shanghai, Fujian and other regions began large-scale *L. edodes* cultivation. In 1997, China's fresh *L. edodes* production reached 1.152 million mt, accounting for 87.2% of the world's total production, making China the world's largest producer, consumer and exporter of *L. edodes* (Zhang and Chen 2003). By 2018, the output of fresh *L. edodes* reached 10.432 million mt, which accounted for 27.2% of the total output of edible fungi in China (China Edible Fungi Association, https://www.cefa.org.cn/syj-door/news.html?id=19).

12.1.1 Origin and Distribution

Lentinula edodes is widely distributed in the subtropical to temperate regions of the Northern Hemisphere. There are no records of *L. edodes* growing in the tropics or cold regions. Cultivation areas are mainly concentrated in Asian countries, such as Japan, China, North Korea the Philippines, Indonesia, Malaysia and others, followed by Sakhalin in the Russian Far East. China has a vast territory, and the wild *L. edodes* germplasm resources are extremely rich, providing natural genetic mushroom variants. With the improvement of living standards and economic development, the requirements for the cultivation and quality of *L. edodes* are increasing as well. This requires cultivation of new varieties to meet different market demands. For example, polysaccharide lentinan has a high medicinal value and is widely used

in clinical practice. However, the content of lentinan in the fruiting body of *L. edodes* is low, which increases its market price but limits its application. Therefore, screening *L. edodes* variants for those with high-polysaccharide production becomes nowadays one of the mainstream areas of mushroom agriculture.

12.1.2 Economic Importance

Lentinula edodes, known as the *Queen of Mushrooms*, has a unique flavor and is favored by consumers. Likewise, *L. edodes* health and pharmaceutical products have high economic and social value. *Lentinula edodes* nutritional value is characterized by high protein, high fiber and low fat (Carneiro et al. 2013). The protein component of *L. edodes* is different from the protein in general food crops, and includes mainly albumin, gluten and prolamin. Eight among the 18 amino acids present in *L. edodes* are essential amino acids. Only about 3% fat comprises each 100 g of dry *L. edodes*, with a high content of unsaturated and essential fatty acids. The *L. edodes* carbohydrate content, which is mainly hemicellulose, is as high as 54%. Besides, *L. edodes* contains numerous minerals and vitamins, and recently a selenium-enriched *L. edodes* cultivar has been developed (Turło et al. 2011; Wu et al. 2012a, b).

The fruiting body of the shiitake mushroom is comprised of caps and stipes, which account for approximately 75% and 25% of the dry fruit body mass, respectively (Li et al. 2018). Recent studies have shown that shiitake mushroom stipes, which are often discarded as waste, contain 82.94 g/kg fiber and 439.56 g/kg carbohydrates, which is significantly higher than that found in the caps (Li et al. 2018). The calcium content in stipes (370.10 g/kg) was shown to be significantly higher compared to the caps as well. Thus, the stipes could be used as dietary supplements for health benefits. However, the nutritional composition of shiitake mushroom will vary with changes in environmental factors and developmental stages. The content of calcium is much higher when pine is used as the substrate than that of Chinese fir (He and Sun 1999). Mycelium contains much more calcium than the fruiting bodies, but the copper ion content is lower than in the fruiting bodies (Po 2003). In addition to the traditional processed products, such as dried or salted *Lentinula edodes*, there are also *L. edodes* snacks (mushroom crisp, biscuit), drinks (mushroom tea, mushroom granules) and seasonings (mushroom chicken, mushroom soy sauce).

Besides its high nutritional value, *Lentinula edodes* is of great medicinal importance (Table 12.1) (Bisen et al. 2010; Chen et al. 2012a, b; Chihara et al. 1969). It is rich in lentinan, double-stranded ribonucleic acid and adenine. Clinical and pharmacological studies have shown that lentinan has antiviral, antitumor, immunemodulating and interferon-stimulating properties. Lentinan was shown to inhibit and prevent postoperative micrometastases in chronic myeloid leukemia, gastric cancer and nasopharyngeal carcinoma. Compared with other antitumor drugs, lentinan has almost no toxic side effects. Adenine and lentinan, obtained from *L. edodes*

Table 12.1 Main active components in *Lentinula edodes* and their treatment effect to various diseases

Components	Target diseases	Treatment effect	References
Lentinan	Glioma	Has a significant inhibitory of human glioma SHG-44 cells	Wan et al. (2019)
	Leukemic	Induces HL-60 cell apoptosis by inhibiting PI3 K/AKT signaling pathway	Ma et al. (2019)
	Lung cancer	Exerts antitumor effect on A549 human lung cancer epithelial cell lines	Wu et al. (2018)
	Inhibits myeloid-derived suppressor cells	Reverses the function of immune suppressor cells	Wu et al. (2012a, b)
	Hepatitis B	Possesses potent anti-HBV activity in vitro.	Zhao et al. (2017)
Peptidomannan	Antiviral and interferon inducing activities	Protected mice against infection and antiviral activities	Suzuki et al. (1979)
By-products	Antioxidative and antimutagenic	Possess strong antioxidant capacity in vitro	Kang et al. (2012)
L. edodes mycelia	Influenza virus	Inhibited influenza virus growth in vitro at early phases of infection, increased the survival rate of infected mice	Kuroki et al. (2018)
Fiber	Cardiovascular disease	Lowered the serum total cholesterol level by enhancement of fecal cholesterol excretion.	Fukushima et al. (2001)
Extracts	Diabetes and obesity	Reduces triglyceride, cholesterol, HDL-cholesterol and LDL-cholesterol levels significantly	Kim et al. (2013)

can promote cholesterol metabolism, lower blood lipids and prevent formation of blood clots (Jeung 2013). *Lentinula edodes* high vitamin D content can prevent rickets and treat anemia (Lee et al. 2009).

Shiitake mushroom is a white wood-decay fungus with a strong ability to secrete enzymes such as laccase and manganese peroxidase, which are valuable for scientific and economic purposes (Fig. 12.1). Considering its great potential for industrial applications, especially for biopulping, residue treatment and improvement in the digestibility of animal rations, extensive studies were carried out on the enzyme production of the shiitake mushroom. Laccase from *Lentinula edodes* has been used in various industrial processes, including dye decolorization (Nagai et al. 2002), bioremediation of olive oil mill effluents (Alessandro et al. 1999) and pollutant removal (Eldridge et al. 2017). The *L. edodes* manganese peroxidase was shown to be the main efficient enzyme for the decolorization of synthetic dyes (Boer et al. 2004).

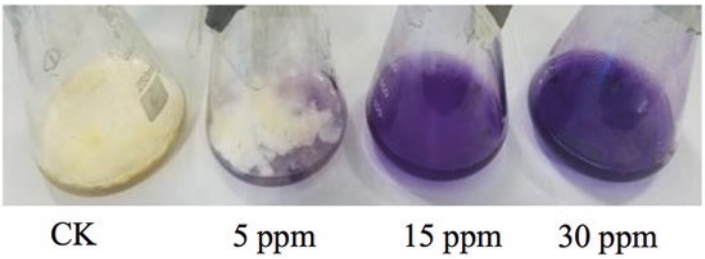

Fig. 12.1 Decolorization of methyl violet by *Ganoderma lucidum.* Different concentrations of methyl violet were added to the liquid medium, and no dye was added to the control (CK). (Figure constructed by Quanju Xiang)

Due to the exceptional nutritional, health and industrial values of *Lentinula edodes*, the demand for its international and domestic production has shown an obvious upward trend in the past 20 years. The market prospects of *L. edodes* in developed countries in Europe, the Americas and Southeast Asia, are broad and highly promising, making *L. edodes* the fastest-growing mushroom crop in the world in terms of the variety of its applications. At the same time, increasing demand for *L. edodes* and its products raises the need for the development of new types of the shiitake mushroom with various specific properties. Similar to animal and plant breeding methods, mushroom breeding includes traditional approaches (natural selection), hybridization and modern techniques of molecular breeding. This chapter provides an overview of the methods for genetic improvement of *L. edodes*, with special emphasis on creating *L. edodes* variants adapted to different temperature conditions.

12.1.3 Impact of Climate Change on Mushroom Breeding and Production

Climate change has a broad effect on ecological functions and the life cycles of all organisms. Likewise, climate change greatly affects various mushroom properties: mycelial interaction, fruiting body development and nutritional value (Gange et al. 2008). Climatic factors, including temperature, moisture, and illumination, play an important role in the diversity of fungal communities and mushroom fruiting stages (Pinna et al. 2010). Some species of edible fungi are adapted to a broad range of temperatures, from very low to very high. It has been reported that shiitake mushroom can survive at −30 °C under wooden logs, whereas its maximum adaptive temperature reaches 35 °C (Antonio 1981; Przybylowicz and Donoghue 1990). Due to the excessive dryness during the summer and autumn seasons in the Mediterranean pine forests, the mushroom yield is low, while in the European forests higher temperatures prolong the fruiting time and considerably increase total fungal biomass (Ágreda et al. 2015).

Production of edible fungi is greatly affected by elevation as well; at high elevations mushroom growth dramatically decreases due to low temperatures and excessive moisture (Boddy et al. 2014). For the cultivation of edible fungi extreme climate is not favorable; production of new strains often requires optimum conditions. Fungal mycelia need energy from the host to accumulate fruiting potential (Krebs et al. 2008). Moreover, the growth of fungal hyphae and the formation of fruiting bodies are affected by illumination. The brown film stage of *Lentinula edodes* is very important for mushroom quality and is highly affected by light exposure (Tang et al. 2016). Excessive light has been reported to influence fungal metabolism and gene expression. Transcriptome analysis of the color-change mechanism of the shiitake mushroom has shown that light is an essential parameter for the color formation and affects the transport and metabolism of carbohydrates and nucleotides (Tang et al. 2013). An optimum amount of moisture is another important parameter for the production and breeding of fungi, which are susceptible to both insufficient and excessive moisture levels. If water availability is low, fungi are unable to obtain the necessary amount required for high production (Büntgen et al. 2012, 2013). On the other hand, an excess of water reduces mushroom pores, leading to poor aeration and ultimately to decreased yield (Allen et al. 2003). Fruiting of fungi is largely dependent on the availability and distribution of nutrients, which is affected by various climatic conditions. Previous studies have demonstrated that the growth of Basidiomycota fruiting bodies is greatly influenced by various environmental factors, both biotic and abiotic (Boddy et al. 2014). The abovementioned facts highlight the dramatic importance of climatic factors on the life cycle, metabolism and breeding of all kinds of fungi. There is a direct link between the mushroom production and the breeding of new strains, since optimum climatic conditions are required for their development.

12.2 Growth, and Development and Genetic Characteristics

12.2.1 Nutrition and Environmental Requirements

Lentinula edodes is a typical wood-decay fungus (white rot fungus). Hardwood or wood chips are the main raw materials for its cultivation, with a preference towards eucalyptus, chestnut, locust, fruit and jujube wood. Cotton husks, corn cobs and other substitutes can also be used as cultivation material (Elif and Aysun 2007). The shiitake mushroom is adapted to a wide range of temperatures. The temperature range for mycelial growth is 5–32 °C, with the optimum at 24–27 °C. At 35 °C shiitake mycelium stop growing, and it dies at 38 °C. For the proper development of *L. edodes* fruiting bodies, different temperatures are required at different stages. For the primordium differentiation, the temperature range is 8–21 °C; 10–12 °C is optimal. The temperature at which the fruiting bodies grow is in the 5–24 °C range; 8–16 °C is optimal. The quality of the fruiting body is evaluated according to the

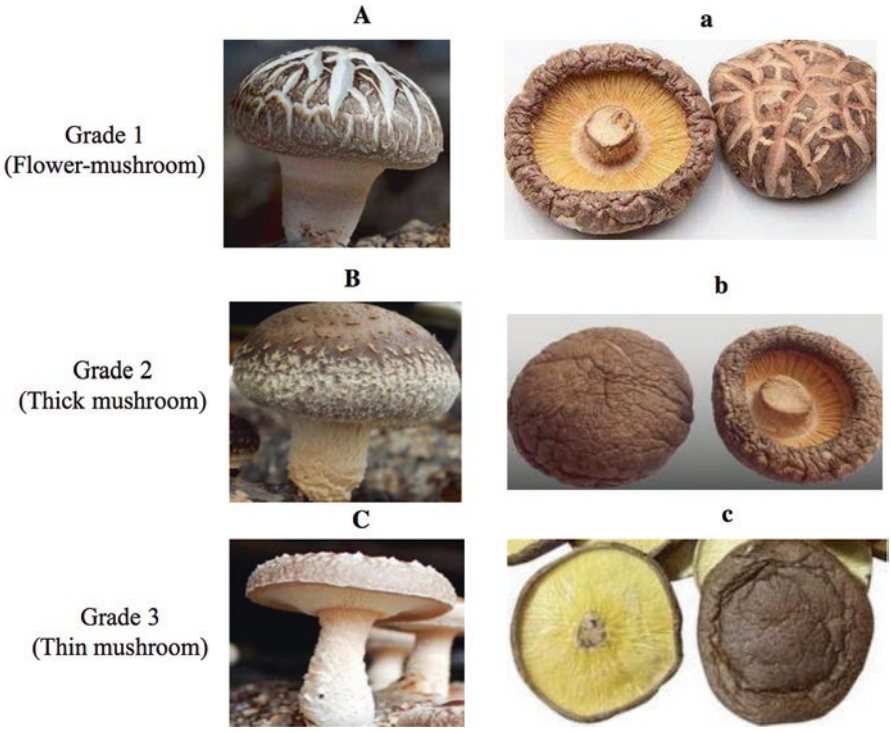

Fig. 12.2 Different grades of fresh (**A, B, C**) and dry (**a, b, c**) *L. Lentinula edodes* fruiting bodies. (Figure constructed by Quanju Xiang)

Table 12.2 Characteristics of *Lentinula edodes* of different grades

Levels	Characteristics
Level 1 (flower mushroom)	There are white yellowish cracks on the mushroom surface, the background color is yellow and white, the cap is thick and not flanging, and the mushroom surface is not less than five cm
Level 2 (thick mushroom)	The same as Level 1 except that there is no white yellowish cracks on the surface
Level 3 (thin mushroom)	No white yellowish cracks on the surface, the background color is yellowish white or dark brown, the cap is thin and cuffed, and the mushroom surface and pieces are not less than 2 cm

three-level grading scale and depends on the temperature at which the mushroom is grown. The first grade (Fig. 12.2, Grade 1) is the flower mushroom, fruiting at 4–8 °C when the air is dry; the second grade (Grade 2) is the thick mushroom, fruiting at 8–16 °C and the third grade (Grade 3) is the thin mushroom, fruiting at higher temperatures, especially above 20 °C (Table 12.2).

During the mushroom bag cultivation process, the water content of the culture material during the mycelia growth stage is 55–60%. During the fruiting stage, the

relative humidity of the air is increased to 85–95%. To produce high-quality commercial *Lentinula edodes*, the formation and growth of fruiting bodies require a certain degree of humidity difference, when dry and wet conditions alternate. *Lentinula edodes* is an aerobic heterotrophic organism, therefore good ventilation should be maintained at all growth stages to ensure timely exchange between carbon dioxide and oxygen. Light is not required during the germinating phase, but some scattered light would hasten the process. On the other hand, for the differentiation and growth of fruiting bodies, scattered light is absolutely required. *Lentinula edodes* prefers a slightly acidic environment with a pH of around 5–6, therefore, there is no need to add lime to the culture material during the cultivation process.

12.2.2 Growth and Development

The growth and development cycle of *Lentinula edodes* is mainly divided into four stages: vegetative mycelial growth and colonization of the substrate (Fig. 12.3a), light-induced brown film formation (Fig. 12.3b), primordium initiation (Fig. 12.3d), and fruiting body development (Fig. 12.3e, f). The *L. edodes* mycelium is a filament which grows from a mushroom spore on a substrate and creates the geometry of the mycelium. The light-induced brown film formation plays a key role in the fungal photoreception, signal transduction, secondary metabolite biosynthesis, and has a great impact on the yield of fruiting bodies (Tang et al. 2013, 2016). The formation of the brown film on the mycelial tissue surface is a critical step which provides a

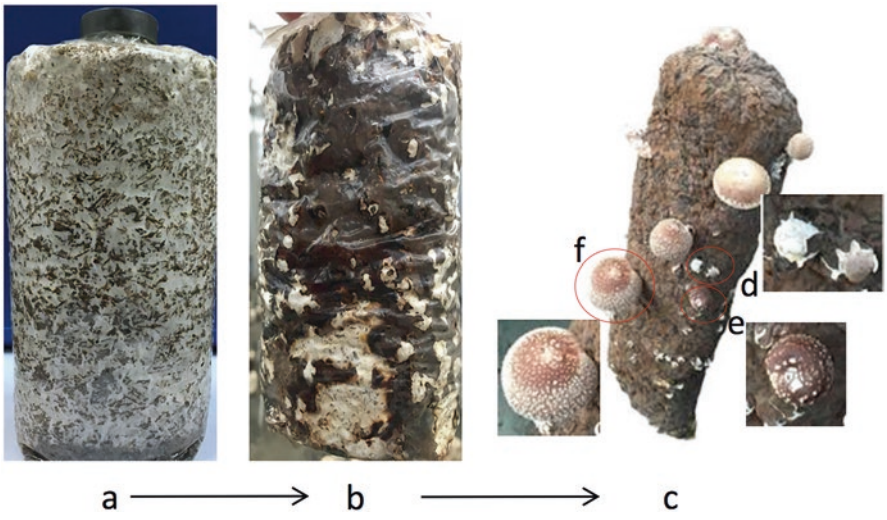

Fig. 12.3 Different development stages of *L. edodes*. (**a**) Mycelium, (**b**) Hyphae color change, (**c**) Fruiting, (**d**) Primordium, (**e**) Young fruiting body, (**f**) Mature fruiting body. (Figure constructed by Quanju Xiang)

protective shield on the medium, similar to the bark of a tree. (Kim et al. 2009). This brown film can maintain water inside the bag to ensure high quantity and quality of the fruiting bodies and protect the mycelium from various pathogenic organisms, such as bacteria, green molds and fungi (Koo et al. 2013). Light has been reported to be essential for brown film formation, blue light being the most beneficial. (Fig. 12.4; Table 12.3) (Tang et al. 2013, 2016). After the mycelium has colonized the substrate and developed the brown film, the filaments begin to form a primordium if the conditions are suitable (scattered light, optimal temperature range, water availability). The primordium further differentiates into a fruiting body, which is a specialized reproductive organ of the mushroom, comprising the cap, pleat and stipe.

The life cycle of *Lentinula edodes* is similar to that of other common basidiomycetes (Fig. 12.5). The main steps are the germination of the basidiospore, the production of four different mating types of monokaryotic hyphae (primary hyphae) and the binding of the two monokaryotic hyphae to form the dikaryotic hyphae (secondary hyphae) with two nuclei in each cell. The cross-section of the two hyphae usually has a clamp connection. Under suitable conditions, the dikaryotic hyphae form a fruiting body, and at the mediostratum of the fruiting body, the apical cells of the dikaryotic mycelium develop into a basidium, which is arranged into a hymenium. In the mature spores, the two haploid nuclei are fused to form a diploid nucleus. The diploid nuclei in the basidium undergo two divisions, including one meiosis, creating four basidiospores. After the spores are ejected, the life cycle is resumed.

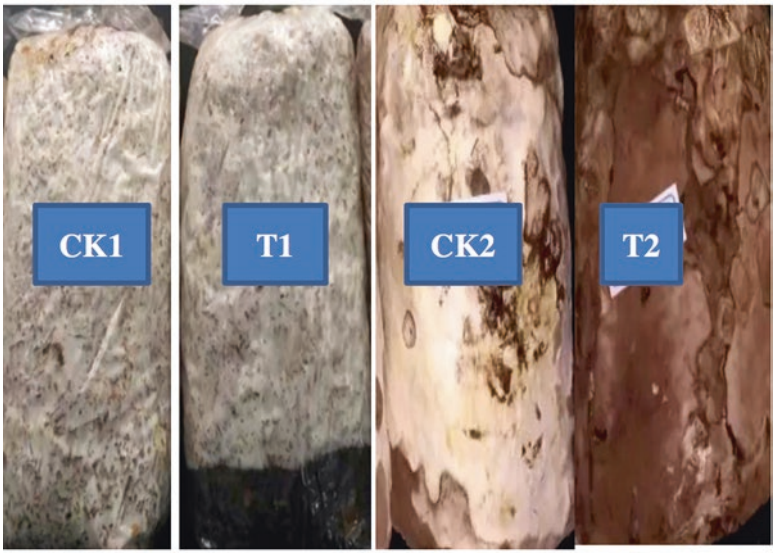

Fig. 12.4 Changes of mycelium surface grown under different regimes. The cultivation substrate was fully colonized by white fungal mycelia after 45 and 54 days incubation in the dark (**CK1**) and light (**T1**), respectively. Subsequently, (**CK1**) were exposed to a 12 h light/dark regime (**T2**) and (**T1**) were still kept in the dark (**CK2**). (Photos by Xinzhu Li)

Table 12.3 Speed of mycelium color changing under different light treatments

Light	Control	Red	Yellow	Green	Blue
Speed(cm^2/d)	19.4	23.69	17.31	15.74	27.45

Source: Data from our research group

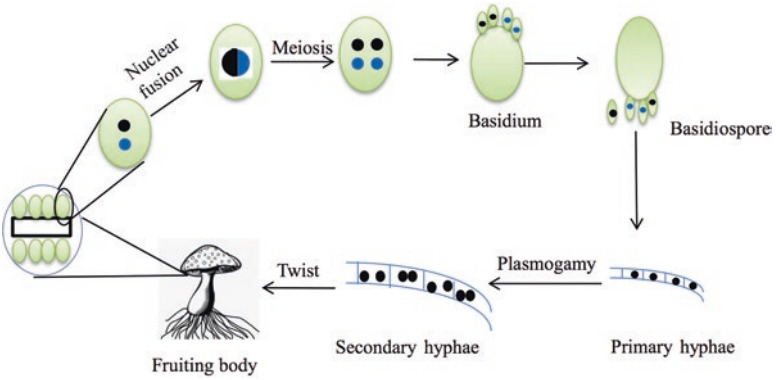

Fig. 12.5 Life history of *Lentinula edodes*. (Figure constructed by Quanju Xiang)

12.2.3 Genetic Characteristics

The complete life cycle of most edible fungi includes two stages: asexual and sexual. A combination of sexual germ cells is not required for the asexual reproduction process, and new reproductive mushroom forms are developed directly from the parent organism, including mycelial break, production of asexual spores (arthrospore of *Lentinula edodes*, secondary spores of *Agaricus bisporus*, chlamydospore of *Volvaria volvacea*, conidiospore of the black fungus) and budding reproduction. Asexual spores are highly resistant to adverse conditions; they can germinate forming the original primary hyphae or bipolar hyphae under suitable conditions and continue the sexual life cycle. The process when new mushrooms are produced through the combination of sexual germ cells is called sexual reproduction. The differences in the spore nucleus genes determine the different sexes of the monocaryotic hyphae that they sprout. Sexual reproduction can be divided into: homozygous (10%) and heterozygous (90%) according to the sex of the mononuclear hyphae.

Most of the edible fungi spores or monocaryotic hyphae have *female* and *male* sex (marked as + and -, respectively). Sex is controlled by genetic factors; only the heterozygous monocaryotic hyphae can be fused into dikaryotic hyphae, while the mycelia of the same sexes are never fused together. This phenomenon is called heterozygous combination, also known as self-infertility. Around 90% of the fungi are heterozygous, and there are two types of heterozygous combinations: bipolar (25%) and tetrapolar (65%). Bipolarity is controlled by one pair of genetic factors (Aa), while tetrapolarity is controlled by two pairs of genetic factors (AaBb). In tetrapolar fungi, there are four spore-bearing sex gene types: AB, Ab, aB and ab, which are

similar to four genders and are called four polarities. For the tetrapolar primary hyphae, only AaBb combination is fertile, therefore the fertility rate is 25% (Table 12.4). *Lentinula edodes* belongs to the tetrapolar heterozygous type. This means that when we use single spore hybridization for breeding, only 25% of the combinations are viable after the combination of the two spores. It is only a success with the combination of cytoplasm without the fusion of the nuclei, both in the homothallic and heterothallic processes. The dikaryotic cells will continue to divide, forming large numbers of new cells, leading to the rapid growth of the mycelium. After reaching a certain growth stage, the mycelium undergoes meiosis to form new monospores with different sexes.

Identification of the mating type genes is important for breeding and cultivation of edible mushrooms. The mating type system of *Lentinula edodes,* which is composed of unlinked A and B loci, plays a vital role in sexual development and formation of the fruiting body. Various genetic factors controlling mating types have been indicated and identified. The matB regions from two compatible *L. edodes* strains (939P26, 939P42) have been cloned and analyzed. The results showed that there are four pheromone receptors and pheromone precursor genes in these two respective strains, and these pheromone receptors and pheromone genes control the B mating type of *L. edodes* (Wu et al. 2013). The A mating type locus was identified from *L. edodes* draft genome sequence, and two polymorphism markers were developed for the rapid molecular determination of the A-type monokaryotic strains (Au et al. 2014). Analysis of the mating type preferences in the 127 *L. edodes* strains collected in East Asia showed that certain A mating types are preferred. It suggests that A mating genes may play an important role in the expression of genes determining mushroom quality. Moreover, it was found that A mating type was highly diverse in the wild strains; this diversity will perhaps further increase as more wild strains are found. The high diversity in such a small area implies a rapid evolution of *L. edodes* A mating genes (Ha et al. 2018).

12.2.4 Cultivation Practices

Cultivation of *Lentinula edodes* mainly includes the following methods: chopping flower cultivation, segmental wood cultivation, mushroom brick cultivation, space package cultivation, and substitute cultivation. The first records of *L. edodes*

Table 12.4 Fertility of a tetrapolar combination of *Lentinus edodes*

Genes sex types	AB	Ab	aB	ab
AB	–	–	–	+
Ab	–	–	+	–
aB	+	+	–	–
Ab	+	–	–	–

+ indicates that they are fertilized; – indicates that the two are infertile, and the fertility rate is 25%

cultivation in China refer to the Western Jin Dynasty (Zhang and Miles 2010). The chopping flower cultivation method is based on growing mushrooms from natural spores, and the yield mainly depends on the weather conditions, which could be unstable. However, it was still the main method of cultivation in China until the 1960s. Later, in 1965, the method of artificial cultivation of pure hyphae has been gradually promoted, which increased the yield and improved the stability of *L. edodes* production. At the same time, due to the acquisition of pure mycelial culture, the cultivation of substitute materials came into existence.

Various materials can be used as substrates for *Lentinula edodes* (Elif and Aysun 2007). Nowadays, there are two main agricultural methods for the *L. edodes* cultivation: log-media-based and sawdust-media-based (Fig. 12.6). Taking into consideration the increasing emphasis on the protection of the ecological environment, the log-based method could be advantageous. However, the materials (wood) for this method are limited. At the same time, sawdust, straw, and other types of agricultural waste are broadly available and need to be recycled. These factors led the sawdust-media-based bag cultivation to become the main technique at present. In addition, it is characterized by higher yield (~ 15%) and shorter cultivation period than the log cultivation method, thereby making the sawdust bag cultivation the number one method for *L. edodes* cultivation worldwide (Koo et al. 2013).

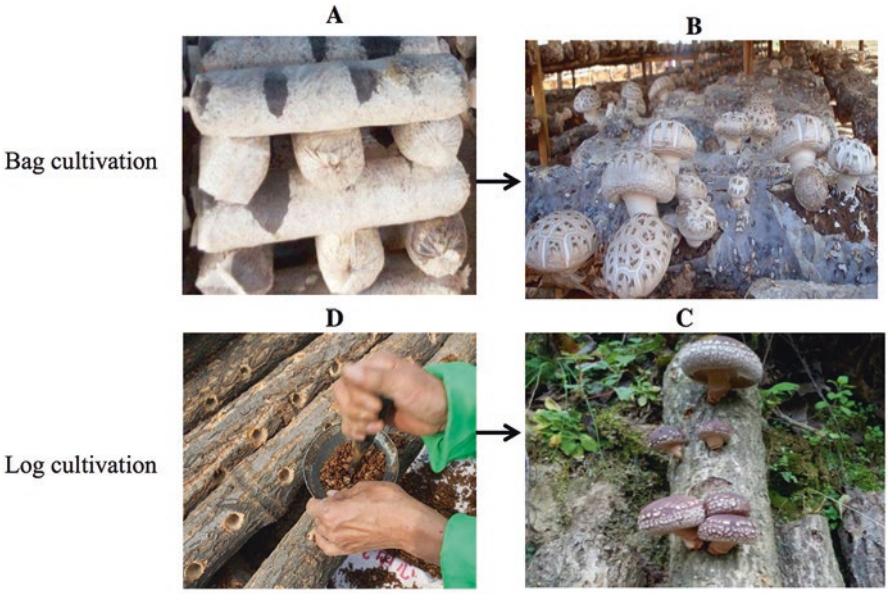

Fig. 12.6 Mycelial (**a, c**) and fruiting body (**b, d**) under bag and log cultivation of *Lentinula edodes*. (Photos by Quanju Xiang)

12.3 Breeding Objectives

Breeding of the shiitake mushroom is focused on the development of new superior variants characterized by specific genetic traits, and the breeding objectives constantly change with the development of social economy. As for other agricultural crops, the basic goal for the edible fungi breeding is to increase the yield, quality, and resistance of the species (Table 12.5). In the new era, the constant pursuit of higher living standards implies improvements in the taste and nutrition values of the edible fungi, creating new goals for the breeding process.

An exemplary *Lentinula edodes* breed could be characterized by a dikaryotic mycelium (each mycelium cell having two nuclei), cells which are divided by septa, and clamp connections (more clamp connection means higher ability to produce mushrooms). The hyphae should grow well, have exceptional stress-resistance and strong adaptability to various conditions. After the mushroom is harvested, the new hyphae should be able to germinate very quickly, ensuring the shortest possible interval between the two sequential mushroom harvests. Besides that, the agronomic traits of the fruiting body should meet the highest standards in terms of shape and taste. Another important trait is the conversion rate of fresh mushrooms, which can reach 30–40% for the best *L. edodes* variants. Nowadays, new breeding goals are emerging, for example, developing strains suitable for intensive industrial farming, with a short growth cycle and high polysaccharide content (Chen et al. 2001).

12.4 Breeding Approaches

Like in other microbial breeding, the breeding history of *Lentinula edodes* includes traditional breeding methods followed by advanced molecular breeding techniques. Traditional breeding is comprised of natural breeding and cross-breeding, while with the new developments of science and technology, molecular breeding is constantly improving and includes various techniques such as the single mutation method and combinations of different mutation methods.

12.4.1 Natural Selection

Natural selection breeding, also known as natural isolation, refers to the breeding method that directly screens microbial cell populations without manual treatment; it is also known as *single colony isolation*. Natural selection breeding is a simple and effective method which is often implemented at early stages of edible fungi breeding as a basis for further breeding techniques. Edible mushrooms are abundant in the wild and need to be collected. The polysaccharide content in mycelium and

Table 12.5 Different breeding target and their representative strain

Targets	Types	Characteristics	Representative *Lentinula edodes* strain
Cultivation substrates	Wood	Drought tolerance and long mushroom-producing period	241, 7401
	Sawdust	Moisture resistance and short mushroom-producing period	L125, L808
	Forage	Strong moisture resistance and short mushroom-producing period. It can be mix-cultivated with sawdust and grass	Cr-04, L66
	Grass	Strong moisture resistance and short mushrooming period. It can be cultivated with grass such as reed and sorghum	LC206, LC236
	Wood and grass	Strong adaptability and can be cultivated with sawdust and grass	8001, 7402
Temperature for primordium differentiation	Low	Fruiting body differentiation temperature range is 5–18 °C, suitable temperature 10–15 °C	7401, 7403
	Medium	Fruiting body differentiation temperature range is 8–22 °C, suitable temperature 15–20 °C	7402, 904
	High	Fruiting body differentiation temperature range is 12–25 °C, suitable temperature 17–22 °C	7405, L18
	Wide	Fruiting body differentiation temperature range is 8–28 °C, fruiting body varies with temperature	Cr-02, Zhongxiang 68
Physiological maturity time of mycelia	Early maturity	Mycelium physiological mature time 60–90 d, effective accumulated temperature 800–1300 °C, most of the mushroom temperature is higher	8001, L18
	Medium maturing	Mycelium physiological mature time 90–120 d, effective accumulated temperature 1400–2000 °C, moderate temperature for fruiting body	L808, Zhongxiang 68
	Late maturity	Mycelium physiological mature time 120–180 d, effective accumulated temperature 2000–2500 °C, low temperature for fruiting body	L135, Xianggu 241-4
Size of fruiting-body	Small	Diameter of 4–6 cm	7402, L18
	Middle	Diameter of 6–9 cm	241-4, L135
	Big	Diameter of 9–20 cm	8001, L808
Thickness of fruiting-body	Thin	< 7 mm	
	Medium	7–12 mm	7402, 7404
	Thick	> 12 mm	L135, L808

(continued)

Table 12.5 (continued)

Targets	Types	Characteristics	Representative *Lentinula edodes* strain
Suitable for sales situation	For dry products selling	Fruiting bodies are tightly organized and have low water content	L135, 241-4
	For common fresh selling	Short growth cycle, uniform individual, lighter mushroom and higher water content	Cr-04, Cr-63
	For insurance fresh selling	The water content is low, the shape is round, and the membrane is not easy to be opened, the stem is short and the flesh is thick, and the commercial value is good	Xianggu 241-4, Cr-04

Source: Data from our research group

fruiting bodies of 99 *Lentinula edodes* strains was significantly different (Table 12.6), so natural selection remains an important step in the breeding process. The main role of natural selection is to purify the strain to obtain a cell population with a uniform genetic background.

Two commonly used methods—single spore separation and tissue isolation—play an important role in the natural selection process. Single spore separation is the separation of individual spores which then germinate to form hyphae, while tissue isolation is the method by which the tissue is separated directly from the fruiting body. The strains isolated by natural selection are tested to evaluate their agronomic traits. Those strains with strong resistance, high yield, improved quality and other promising features are selected at that stage.

Under natural conditions, spores of different *Lentinula edodes* sexes produce a variety of genetic recombinations, providing the raw material for *L. edodes* breeding. The *L. edodes* strains are selected according to the breeding purpose. For example, when temperature adaptation is the target of the selection, the strain should be picked up at the corresponding latitude or elevation. Suitable strains are obtained by tissue isolation or spore collection, and their purity must be verified by cultivation experiments. Several excellent *L. edodes* cvs. such as Guangxiang N0.5, Guangxiang N0.7, Guangxiang N0.9 and 241 were developed by the natural selection breeding (Wu 2005).

Natural selection breeding also includes the method of introducing strains from one area to another for further domestication. For example, Yang and Huang (2000) introduced the *Lentinula edodes* variety cultivated in the tropics of Indonesia which showed good properties after domestication and cultivation experiments. This had an important application value, solving the problem of high-temperature *L. edodes* cultivation during summer periods, thus guaranteeing the stable supply of fresh *L. edodes* all year round.

Due to natural mutations or separation of the heterokaryon and polyploid, the genetic traits of some strains can degenerate over time. This results in impure strains

Table 12.6 Crude polysaccharide content of mycelia and fruiting bodies of different strains

No.	M [a] (%)	FB [b] (%)	No.	M [a] (%)	FB [b] (%)	No.	M [a] (%)	FB [b] (%)
1	4.37	3.96	34	2.47	3.27	67	nd	0.98
2	4.48	3.16	35	2.37	4.73	68	2.03	2.19
3	5.04	2.55	36	2.92	2.31	69	nd	1.12
4	2.06	5.33	37	2.46	2.76	70	nd	0.76
5	3.96	2.41	38	2.85	2.74	71	0.7	1.62
6	1.71	1.19	39	2.08	1.15	72	1.05	1.84
7	4.1	3.65	40	nd	2.9	73	1.94	1.9
8	1.31	2.42	41	nd	1.66	74	nd	1.22
9	1.58	2.42	42	3.18	5.55	75	nd	1.44
10	3.04	3.16	43	2.19	2.42	76	nd	1.32
11	1.69	1.79	44	1.73	2.44	77	nd	1.73
12	2.54	nd [c]	45	1.42	1.11	78	nd	1.35
13	1.8	2.41	46	2.86	1	79	nd	0.8
14	1.75	3.22	47	1.56	1.11	80	1.82	1.88
15	1.16	2.91	48	nd	1.7	81	nd	1.27
16	2.98	2.18	49	1.45	1.51	82	nd	1.71
17	2.69	1.05	50	nd	4.52	83	nd	1.9
18	2.83	2.9	51	2.84	4.2	84	1.76	6
19	3.51	1.31	52	1.09	5.78	85	5.23	2.14
20	1.91	3.81	53	2.04	1.34	86	1.92	1.8
21	2.13	3.08	54	1.29	1.23	87	3.02	4.06
22	3.96	2.21	55	nd	2.14	88	1.49	1.83
23	5.35	4.1	56	1.13	0.75	89	1.87	nd
24	2.4	3.77	57	2.83	2.74	90	1.72	nd
25	1.69	2.69	58	1.89	1.17	91	1.55	1.9
26	2.43	2.35	59	nd	1.21	92	1.28	nd
27	1.15	3.3	60	1.96	1.4	93	1.02	nd
28	5.07	nd	61	nd	4.96	94	1.56	nd
29	4.29	2.04	62	1.05	4.16	95	1.28	2.72
30	2.62	2.18	63	nd	1.32	96	0.8	1.88
31	1.41	1.67	64	nd	1.42	97	1.38	nd
32	3.17	1.12	65	nd	0.88	98	0.91	2.15
33	3.17	1.5	66	nd	0.74	99	1.38	2.43

Notes: [a] Crude polysaccharide content of mycelia; [b] Crude polysaccharide content of fruiting bodies; [c] No fruiting body in artificial cultivation mode, and the polysaccharide content was not determined

Source: Data from our research group

and severe productivity decline. Therefore, strain purification should be carried out frequently in industrial production and fermentation research. Natural selection is one of the main methods for strain purification of edible fungi. The fruiting bodies with excellent qualities can be further used for strain selection. *Lentinula edodes* strains such as 7925 and Hunong No. 1, which are widely cultivated at present, are

excellent mushroom strains selected by the natural breeding method (Zhang and Chen 1996).

12.4.2 Cross-Breeding

Compared with the natural selection, the number of shiitake mushrooms obtained by the cross-breeding method is relatively small. However, cross-breeding contributes a lot to the rapid development of China's *Lentinula edodes* industry (Tan and Ying 2000). Strain selection based on single spores or tissue isolation alone may bring short-term improvements; however, it is unlikely to be as effective as methods involving controlled cross-breeding (Elliott and Langton 1981).

Cross-breeding is the recombination of genetic material at the cellular level. It involves the free separation of genes, gene recombination, gene linkage exchange, cytoplasmic inheritance and inheritance of quantitative traits. For cross-breeding, strains with good productivity, favorable fruiting body traits, temperature adaptations, and specific developmental patterns can be used as parental strains (Noh et al. 2015). These strains should be complimentary by sex and have different genetic traits. Using cross-breeding, it is possible to combine parental traits to produce super-parents or new traits. The new strains would have higher genetic variation and therefore will be able to form new traits, creating new mushroom varieties. However, the hybrid breeding process is cumbersome, costly and requires many operational steps; nevertheless, it remains an effective method for breeding edible fungi. Cross-breeding of edible fungi includes heterozygous and homologous combinations, thereby achieving the purpose of improving heterogeneity and homology within the population. Currently, the most widely used breeding methods are single hybridization and double-single hybridization (asymmetric hybridization).

Single-hybridization refers to collecting monospores from fruiting bodies or obtaining protoplast monokaryons, culturing different mating types of monocaryotic hyphae, and selecting a dikaryotic body with a lock-like combination as a hybrid progeny (Fig. 12.7). Through cultivation tests, it is possible to screen for excellent hybrids that meet the production goal. The content of crude polysaccharides in fruiting bodies of heterozygous strains were significantly higher than that of parent strain (Table 12.7). The Fujian Sanming Fungi Research Institute used foreign strain 7402 to hybridize with the wild strain Lc-01, and produced 16 hybrid strains with a higher yield compared to the control, increasing the yield by 15–30%. Among them, strains Cr-20 and Cr-62 were popularized and are currently cultivated in various parts of the country (Cai and Huang 2000). Tao et al. (1999) used wild *Lentinula edodes* 0426 and the cultivated Le1 as parents and obtained monocaryotic hyphae by regeneration of the single-nuclear protoplast. Single cross-breeding was done between the monocaryotic mycelia of the two parents. After the cultivation test, the new *L. edodes* cv. Shenxiang No. 8 was finally created.

In the double-single hybridization process, the monocaryotic protoplast of the modified strain is used as a recipient, and a dikaryotic strain which carries the

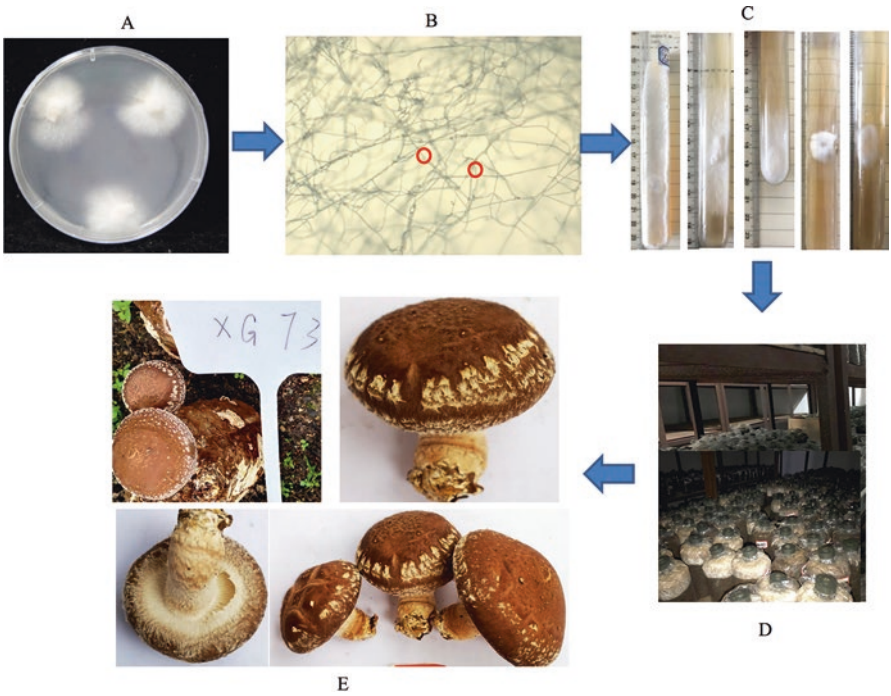

Fig. 12.7 Single-hybridization of *Lentinula edodes*. (**a**) Inoculation of 2 different spores on plate, (**b**) Lock-like combination (highlighted by red circle), (**c**) Heterozygote mycelium growth in test tube, (**d**) Cultivation test of Heterozygote, (**e**) fruiting and agronomic traits test. (Figure constructed by Quanju Xiang)

Table 12.7 Contents of crude polysaccharides in fruiting bodies of parent and heterozygous strains

Strains	Crude polysaccharide (g/100 g)
Parent Strain	1.51
Heterozygote No. 1	1.84
Heterozygote No. 2	1.72
Heterozygote No. 3	2.09
Heterozygote No. 4	1.75
Heterozygote No. 5	1.89
Heterozygote No. 6	2.01
Heterozygote No. 7	1.97
Heterozygote No. 8	1.66

Source: Data from our research group

desired trait is used as a donor. An asymmetric hybridization is done between the monocaryotic and dikaryotic hyphae. This breeding method has the advantages of reducing the screening workload of the hybrid progeny and shortening the breeding time; moreover, the phenotype of the offspring has a higher resemblance to the recipient. Several *Lentinula edodes* strains (26, 939, 135. Shenxiang 16) were developed by this method (Song et al. 2010; Tao et al. 2007). A high-yield cultured Shenxiang 18, with the yield reaching 1 kg of fresh mushrooms per 1 kg of dry material, was obtained by asymmetric hybridization between a late- and a medium-maturing strain (Song et al. 2012).

12.4.3 Protoplast Fusion

Protoplast fusion technology originated in the 1970s (Anne and Peberdy 1976), which provides a new approach for edible fungi genetic breeding and genetic research. Protoplasts are sensitive to mutagens and are not affected by the competent state. Protoplast fusion refers to the process of merging protoplasts of different genetic *Lentinula edodes* strains induced by fusion agents, to obtain partial or complete genome exchange and recombination, creating new *L. edodes* varieties.

This technology includes protoplast repopulation, protoplast mutagenesis, protoplast transformation, protoplast fusion and other protoplasts fusion breeding techniques. Intraspecies, interspecies, intergenus, and even interfamily protoplast fusion techniques for the breeding of edible fungi have been reported previously. The Japanese Fungi Research Center used intergenus breeding to combine the shiitake mushroom and the matsutake mushroom, *Tricholoma matsutake* (intergenus). The Hebei University performed an intergenus fusion between the *Pleurotus ostreatus* (oyster mushroom) and *Lentinula edodes* (intergenus), and Tsinghua University used the same method to combine *L. tigrinus* and the enokitake mushroom, *Flammulina velutipes* (Liu and Tai 1992; Yang et al. 2010).

The Shanghai Edible Fungus Technology Extension Station used the intergenus protoplast fusion technology to carry out cross-breeding of *Lentinula edodes*, creating a new strain named Xiangrong No.1 (Chen et al. 2001). After nearly 4 years of small, regional and production application tests, the strain has shown a 5–10% increase in yield compared to the original strain. Moreover, it is characterized by strong resistance, adaptability, shortened fruiting time, higher fruiting body weight (> 20 g) and rich flavor. The Xiangrong No.1 has become one of the most frequently used cultivated strains in the Shanghai suburbs.

12.4.4 Mutation Breeding

Compared with wild domestication, spontaneous mutation and cross-breeding methods, artificial mutagenesis can increase the frequency of mutations and create traits not found in nature. This method is relatively fast and easy to implement. Therefore, it is widely used by researchers. In China, new varieties of edible fungi such as *Pleueotus ostreatus*, *Auricularia auricula*, *Hericium erinaceus*, *Lentinula edodes*, *Agaricus bisporus* and *Flammulina velutip*es have been selected by using induced mutations. These new varieties have high yield, strong resistance to stress and good quality. A number of auxotrophic and drug-resistant mutants have been also developed. These varieties enriched the germplasm resources of edible fungi, providing genetic markers for hybridization and protoplast fusion breeding.

The mutagenic ^{60}Co γ-ray irradiation was used to fuse *Lentinula edodes* protoplasts to obtain a strain with a high polysaccharide content, 20.6% higher than that of the original strain (Dou et al. 2009). The polysaccharide content of another improved *L. edodes* strain, obtained by ultraviolet (UV) irradiation of the protoplasts, was increased by 18.62% compared to the starting strain (Chen et al. 2001). The mutagenesis method was also used to create a selenium-enriched *L. edodes* strain, with a stable selenium concentration in the mushroom fruiting body, reaching 38.64 μg/g (Wei et al. 2004). A thermo-tolerant *L. edodes* strain, with a fruiting temperature of up to 38 °C, was selected from a wild high-temperature resistant *L. edodes* sample by UV irradiation (Wang 2014).

There are many types of mutagens that could be used for mutagenic breeding. Different mutagens have different genetic effects and different frequencies for inducing a particular trait in an edible fungi strain. For example, the auxotrophic strains could not be obtained with UV, diethyl sulfate, nitrous acid or nitrosoguanidine mutagens, while nine auxotroph strains could be obtained by γ-ray mutagenesis (Han et al. 1994). Different edible fungi or different developmental stages of the same fungus can exhibit various sensitivity levels and dose requirements for the radiation mutagenesis. Ultraviolet lines for protoplast, basidiospore and binuclear hyphae of *Lentinula edodes* are 15 W, 30 ~ 40 cm, 60 ~ 120 s, 5 W, 30 ~ 40 cm, 90 ~ 150 s and 30 W, 30 cm, 60 ~ 120 s, respectively. Recent studies have found that beneficial mutations tend to occur at lower doses, while at higher doses mutations tend to have adverse effects. As the irradiation time is further prolonged, the survival rate rapidly decreases. The protoplast of *L. edodes* is more sensitive to UV rays, and once exposed, the survival rate begins to decline, and with longer irradiation time, the reaction curve decreases slowly. Different stages of *L. edodes* could be arranged by their sensitivity to radiation as following: mononuclear mycelium protoplasts > basal spore protoplasts > binuclear mycelium protoplasts.

Another important process of mutagenesis breeding is the selection and evaluation of the mutant strains. At present, the selection of high-yield mutant strains remains largely traditional, through experimental cultivation and verification of the traits. This method takes a long time and is inefficient. In order to find a simple and effective method, shorten the breeding cycle and reduce the workload, a lot of

useful explorations were carried out. For the rapid mutagenesis evaluation, Ando Masao, according to Yang et al. (1998), used the following method of cultivation. First, the mushroom hyphae were cultivated on PDA (potato sucrose agar) for 43 days at 25 °C under red light. Then, the temperature was lowered to 15 °C for 10 days. After that, the primordia appeared, and the temperature was raised to 20 °C for the next 7 days for the formation of the fruiting bodies. According to this method, the mushroom waste material and 0.2% of ammonium tartrate should be added to the PDA medium. After inoculation, strains are cultured at 25 °C under red light for 40 days, then placed in a refrigerator at 4 °C for 3–5 days, and then moved to 15 °C under scattered light for 7–10 days, after which time the fruiting bodies are formed on the plate. By studying the nutrients and environmental factors required for the formation of *Lentinula edodes* fruiting bodies, leatham has developed a synthetic medium which promotes the rapid development of *L. edodes*. The fruiting body usually appears 27–36 days after inoculation. This synthetic medium can also induce *Pleueotus ostreatus* and *Fistulina hepatica* to form fruiting bodies, and thus can be used for the identification of the fruiting of these mushrooms.

The physical mutagenic sources for the fungi mutagenesis breeding are no longer limited to UV rays and gamma rays. Nowadays, new mutagenic sources such as neutrons, beta rays, ion beams and lasers are used more frequently. Recently, the atmospheric and room temperature plasma (ARTP) was found to be a new powerful mutagen (Xue et al. 2014). The new mutant strain of the *Hericium erinaceus*, created through the ARTP mutagenesis, has a 22% increased yield and the polysaccharide content improved by 16% compared with the original strain (Zhu et al. 2019).

At the same time, new chemical mutagens are continuously screened for. Using a combination of different mutagenic factors (especially physical and chemical factors) could be beneficial, creating a synergistic effect, thereby increasing the efficiency of mutagenesis. Mutagenesis breeding can help not only with cultivating new mushroom varieties with high yield, high quality and strong resistance, but with applying them in production as well. It is possible to create new mutants with unique characteristics. The comprehensive traits of these mutants may be not prominent, but, nevertheless, highly valuable in some aspects, such as disease resistance, fruit body morphology and sporulation performance. Even if they might not be suitable for direct application in production, they can be used as a parent material to expand the genetic basis for further mutagenesis studies. Induced mutations can be combined with breeding methods such as cross-breeding and protoplast fusion.

Induced mutations can create new genes and improve bad parental traits, while hybridization can combine excellent traits from different parents. Therefore, using the mutants as hybrid parents can fully exert induced mutations to create new genes and improve parental strains.

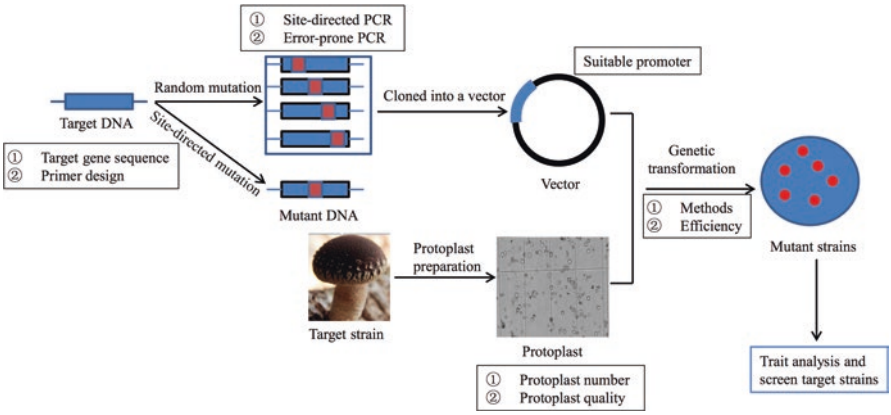

Fig. 12.8 Brief operation procedure of molecular breeding. Note: Some key factors in the relevant steps are noted with boxes. (Figure constructed by Quanju Xiang)

12.4.5 Molecular Breeding

Molecular breeding is the application of biological techniques to bring about genetic changes at the molecular level (Fig. 12.8). Due to the relatively low market value of mushrooms and a lack of large investments, the breeding of edible fungi is behind the times, especially in terms of molecular breeding. However, in recent years it has developed very quickly, due to the relatively small genome size and the short life cycle of edible fungi.

The acquisition of target genes is the primary step of the genetic engineering breeding (Fig. 12.8). Plenty of functional genes of *Lentinula edodes* have been cloned and studied (Akiyama et al. 2002; Miyazaki et al. 2007; Ng et al. 2000). Moreover, *L. edodes* whole genome sequencing is gradually improving (Chen et al. 2016; Chum et al. 2011; Shim et al. 2016), making it easier to identify the functional genes. Effective transformation technology is another key step for molecular breeding. The commonly used genetic transformation methods for *L. edodes* are PEG (polyethylene glycol)-mediated transformation, *Agrobacterium*-mediated transformation, electric shock and the restriction enzyme mediated method (Sato et al. 1998; Sun et al. 2001). The glyceraldehyde-3-phosphate dehydrogenase and chitin synthesis gene promoters from *L. edodes* have been proved to be efficient promoters for high-level expression of heterologous *L. edodes* genes (Hirano et al. 2000; Kuo and Huang 2008; Sato et al. 2011). Recently, researches used *L. edodes* spores and mycelia as experimental materials to successfully transform the expression vector containing *gpd* promoter and *gus* into *L. edodes* by electroporation, and the transformation efficiency reached 30–150 transformants per microgram of DNA. The manganese (II) peroxidase (MnP) cDNA derived from *Pleurotus ostreatus* was transformed into *Coprinus cinereus* protoplasts, and the transformants clearly showed higher lignin-decolorization (Ogawa et al. 1998).

Table 12.8 Some molecular markers recently used for genetic characterization in *Lentinula edodes*

Marker type	Purpose	Country	References
SSR	Genetic diversity in shiitake mushroom varieties	Korea	Moon et al. (2017)
SNP	Identifying the functional	China	Jie et al. (2015)
ISSR and SRAP	Genetic diversity among 89 Chinese *L. edodes* cultivars	China	Liu et al. (2015)
SCAR	Genetic diversity among large scale cultivated *L. edodes* strains	China	Wei (2011)
RAPD, ISSR and SRAP	Evaluate the genetic diversity among 23 *L. edodes* strains in China.	China	Fu et al. (2010)
RAPD, ISSR, SRAP and SSR	Construction of a molecular maker genetic linkage map	China	Cheng et al. (2013)
SCAR	Distinguishing commercial strains of *L. edodes*	China	Wu et al. (2010)
SCAR	Select polymorphisms of 24 main commercial strains of *L. edodes* cultivated widely in China.	China	Liu et al. (2012)
SSR	Molecular characterization and identification of SSR markers	Korea	Lee et al. (2017)
SSR	Genetic diversity and population structure	China	Xiang et al. (2016)

SSR Simple sequence repeat, *SNP* Single nucleotide polymorphism, *ISSR* Inter-simple sequence repeat, *SRAP* Sequence-related amplified polymorphism, *SCAR* Sequence characterized amplified region, *RAPD* Random amplified polymorphic DNA

Molecular marker-assisted breeding is widely used for the identification, genetic diversity analysis, gene mapping and molecular-assisted selection. Molecular-marker technology reflects differences between organisms by detecting changes in the individual genes or genotypes (Dudley 1993; Zhao et al. 2010). There are many labeling methods used for the genetic breeding of edible fungi. The most commonly used are RAPD (random amplified polymorphic DNA), RFLP (restriction fragment length polymorphism), ISSR (inter simple sequence repeat), SRAP (sequence-related amplified polymorphism), AFLP (amplified fragment length polymorphism) and SCAR (sequence-characterized amplified regions) (Table 12.8). The genetic background of 11 *Volvariella volvacea* strains was analyzed by RAPD, and 2 strains with distant relationship were selected to carry out single-spore hybridization, which produced low-temperature-resistant and high-yield *V. volvacea* strains (Zhan et al. 2017). The SCAR markers were used to evaluate the genetic diversity of 24 commercially cultivated *Lentinula edodes* strains. It was found that the genetic diversity of these strains was small, suggesting that the wild *L. edodes* strains should be introduced during the breeding program.

With the development of molecular biology, new molecular-marker technologies, such as SSR (simple sequence repeat), SNP (single nucleotide polymorphism), TRAP (target region amplified polymorphism), IRAP (inter-retrotransposon amplified polymorphism) and EMAP (retrotransposon-microsatellite amplified polymorphism), are gradually being developed and applied. Based on the development of the SSR, TRAP, IRAP and REMAP-labeled primers, the applicability of these

molecular markers was evaluated in a study of genetic diversity of *Lentinula edodes* germplasm resources and laid the foundation for further breeding of the elite varieties (Lee et al. 2017; Xiang et al. 2016; Xiao et al. 2010a,b). Because *L. edodes* is a typical tetrapolarity heterothallic edible fungus, a lot of work has been done on the two pairs of mating genes: mating type A and mating type B, which play a decisive role in the genetic regulation of key developmental processes such as mating and fruiting. Recently, the genes and their structures of *L. edodes* mating type A and B were investigated, which laid a theoretical foundation for further development of molecular markers to assist *L. edodes* breeding (Au et al. 2014; Wu et al. 2013). Moreover, the diversity of A mating type in *L. edodes* and mating type preference in 127 cultivated strains were studied, and the result showed that there is a preferred selection of certain A mating types in the process of strain development, suggesting potential role of A mating genes in the expression of genes governing mushroom quality (Ha et al. 2018). The genetic maps of different *L. edodes* strains were constructed, and the QTL (quantitative trait loci) mapping of the important genes or traits has been rapidly developing in recent years, providing a strong basis for further molecular breeding (Li 2016; Liu et al. 2012; Lu 2012).

12.5 Conclusion and Prospects

Among all breeding methods, the cross-breeding cycle is relatively long, but the breeding objectives are direct, and the outcome can be better predicted. Therefore, the cross-breeding technique may still remain the main method of *Lentinula edodes* breeding for a long period of time. However, with the completion of *L. edodes* genome sequencing and the development of biotechnology, the combination of various molecular markers and traditional breeding techniques will better serve the shiitake mushroom breeding process.

Since most *Lentinula edodes* are medium-low temperature strains, fresh shiitake mushrooms for the summer market are currently in short supply. Consequently, breeding high-temperature strains will have good economic benefits. At the same time, due to the fact that the traditional method of mushroom cultivation is time-consuming and inefficient, the factory-based mode of *L. edodes* production, which relies on science and technology and is not restricted by natural environmental conditions, is rising day by day. In order to create shiitake mushroom varieties of greater commercial value and various beneficial characteristics, a higher intensity of selection is required. For now, new gene editing technologies, such as CRIPSER (clustered regularly interspaced short palindromic repeats), have not yet been used in the breeding of edible fungi. In the production process of *L. edodes*, new problems are constantly arising, and *L. edodes* breeding is not only an urgent matter, but also a long-term, arduous and important task.

Appendices

Appendix I: Major Institutes Engaged in Research on Shiitake Mushroom

Institution name	Specialization and research activities	Contact information address	Website
College of Biology science, China Agricultural University	Physiological active substances of edible fungi and circular agriculture	No.2 Yuanmingyuan West Road, Haidian District, Beijing, 100094, China. Phone: 0086-10-62733874 Email:wlzx@cau.edu.cn	http://en.cau.edu.cn/
College of Resource, Sichuan Agricultural University	Breeding of *Lentinus edodes* and *Hypsizygus marmoreus*	No. 211 Huimin Road, Wenjiang District, Chengdu, Sichuan Province, 611130, China. Phone: 0086-028-86290982	https://www.sicau.edu.cn/
Department of Microbiology, Shanxi Yuanping Agricultural School	Cultivation of edible fungi	No. 13 Qianjin Road, Yuanping City, Shanxi Province, 034100, China. Phone: 0086-0350-8223857	http://www.ypnxy.com/
Edible Fungi Research Institute of Shanghai, Shanghai Academy of Agricultural Sciences	Breeding, cultivation and deep processing of *Lentinus edodes*	No. 35 Nanhua Road, Minhang District, Shanghai,201106, China. Phone: 0086-021-62200538	http://zhsyj022.emushroom.net/
Edible Fungi Technology Engineering Center of Henan, Henan Academy of Agricultural Sciences	Breeding and cultivation of edible fungi	No. 28 Huayuan Road, Zhengzhou City, Henan Province, 450008, China. Phone: 0086-0371-65722860	http://www.hnagri.org.cn/
Edible Fungus Center, Guangdong Academy of Agricultural Sciences	Breeding and cultivation of edible fungi	No. 7 Qianrong Road, Wuxi City, 214000, China. Phone: 0086-0510-5515957	http://www.gdaas.cn/
Edible Fungus Research Center of Horticulture, Institute of Zhejiang Academy of Agricultural Sciences	Breeding of *Lentinus edodes*	No. 139 Xinshiqiao Road, Hangzhou City, Zhejiang Province, 310021, China. Phone: 0086-0571-86404017	http://www.zaas.ac.cn

(continued)

Institution name	Specialization and research activities	Contact information address	Website
Fujian Edible Fungi Association, Fujian Agricultural and Forestry University	Breeding and cultivation of *Hypsizygus marmoreus*	No. 53 Baimazhong Road, Fuzhou City, Fujian Province, 350003, China. Phone: 0086-0591-3368144	https://skxy.fafu.edu.cn/
Fujian Sanming Fungi Research Institute	Breeding of *Agaricus bisporus*, and make seed production	Building 156, Luyan Contry, Liedongxinshi Norse Road, Sanming, Fujian Province, 365000, China. Phone: 0086-10-05988243994	http://gjxy.fjsmu.cn
Hebei Zunhua Liqiang editable fungi research institute	Strain breeding, training, consultation and promotion of raw and auxiliary materials	No. 26, North Second Ring Road, Zunhua City, 064200, Hebei Province, China. Phone: 0086-0315-6636248	http://zhsyj022.emushroom.net/
Horticultural Institute Center of Editable Fungi, Inner Mongolia Academy of Agricultural Sciences	Domestication of wild edible fungi in grassland	No. 246 Wulanchabu East Road, Hohhot city, 010010, China. Phone: 0086-0475-4929474	http://www.imaaahs.ac.cn/
Horticulture Research Institute, Chengdu Academy of Agriculture and Forestry Science	Breeding of *Lentinus edodes* and *Stropharia rugosoannulata*, and cultivation of *Dictyophora indusiata*	No. 200 Nongke Road, Wenjiang District, Chengdu, Sichuan Province, 611130, China. Phone: 0086-028-82747352	http://www.cdnky.com/
Institute of Edible Fungi, Chaoyang City, Liaoning Province	Cultivation of *Auricularia auricula*	No. 37-4, Section 2, Xinhua Road, Chaoyang City, Liaoning Province, 122000, China. Phone: 0086-0421-2812022	https://www.laas.cn/index.aspx
Institute of Edible Fungi, Institute of Edible Fungi, Hunan Agricultural University	Breeding and cultivation of edible fungi	Hunan Agricultural University, Furong District, Changsha City, Hunan Province, 410128, China. Phone: 0086-0731-4618175	https://www.hunau.edu.cn/
Institute of Edible Fungi, Linkou County, Heilongjiang Province	Breeding and cultivation of *Black fungus*	No. 68 Daoliquzhaolin Street, Haerbing, Heilongjiang Province, 157600, China. Phone: 0086-04533580031	http://imb.has.ac.cn

(continued)

Institution name	Specialization and research activities	Contact information address	Website
Institute of Microbiology, Chinese Academy of Sciences	Preservation and identification of edible fungi strains, formulation of product standards and technical procedures, deep processing of edible fungi	No.1 Beichen West Road, Chaoyang District, Beijing 100101 China. Phone: 0086-10-64807462 Email: office@im.ac.cn	http://www.im.cas.cn/
Jiangsu Microbiology Institute Center of Bacteria	Laboratory and testing center of bioactive substances	No. 7 Qianrong Road, Wuxi City, 214000, China. Phone: 0086-0510-5515957	http://www.jsim.cn/
Jilin Agricultural University	Conservation of resources, breeding and standardized cultivation of edible fungi, analysis of active components and deep processing of edible fungi	No. 2888, Xincheng Dajie, Changchun, Jilin Province,130118, China. Phone: 0086-0431-84533101	https://www.jlau.edu.cn/jyjx.htm
Liaoning Edible Fungus Technology Development Center	Breeding of *Lentinus edodes*, *Cordyceps militaris*,	No. 84 Dongling Road, Shenhe Distriact, Shenyang City,110034, China. Phone: 0086-024-86126921	https://www.laas.cn/index.aspx
Research Center for Edible Fungi Development, Sichuan Academy of Agricultural Sciences	Breeding and cultivation of *Morel* and *Auricularia auricula*	No. 20 Jingjusi Road, Chengdu, 610066, Sichuan Province, China. Phone: 0086-28-89576964	http://www.chinawestagr.com/
Shandong Jinxiang Institute of Fungi, Institute of Fungi Shandong Jinxiang	Breeding and cultivation *Coprinus comatus*, *Pleurotus ostreatus*	Jitian District, Jinxiang County, Jining, Shandong Province, 272208, China. Phone: 0086-0537-8851472	https://wenming193.etlong.com/
Shanxi Institute of Microbiology, Chinese Academy of Sciences	Artificial cultivation technology of characteristic medicinal fungi in Qinling Mountains	No. 8 Xiying Road East, Xi ′an City, Shaanxi Province, 710013, China. Phone: 0086-029-85525097	http://sxim.xab.cas.cn/
Strain Experimental Center of Huazhong, Huazhong Agricultural University	Germplasm resources evaluation, genetic breeding, cultivation techniques	Shizishan Street, Hongshan District, Wuhan City, Hubei Province,430070, China. Phone: 0086-027-87386167	http://www.hzau.edu.cn/

Appendix II: Genetic Resources of Shiitake Mushroom

Strain	Temperature form	Suitable fruiting temperature (°C)	Culture time (d)	Application (Number refers to the elevation of the origin place)
L-856	Moderate and low	8~22	60~65	300~500 m, can get flower-mushroom in autumn cultivated in the open field
Nong 7		8~22	65~70	
L-087		8~22	65~70	
Cr-02		8~22	55~60	
9018		12~20	60~65	
L-135		6~18	180~200	> 600 m, shelf culture in spring, and get flower-mushroom in autumn and winter
9015		8~22	90~120	
Nanhua 103		8~22	100~180	
Le-13	Low	8~18	60~65	Inoculate in autumn in open field, fruiting in spring, winter and autumn in Northwest China
9101		7~18	60~65	Inoculate in autumn on soil, fruiting in spring, winter and autumn in Northeast China
N-06		8~20	60~70	Inoculate in autumn on shelf, fruiting in spring, winter and autumn in North China
241-4		7~20	160~200	> 600 m, inoculate in spring in open field, fruiting in spring, winter and autumn
939		8~20	160~180	> 600 m, inoculate in spring on shelf, fruiting in winter and autumn
Cr-66	Medium	9~23	60~75	300~500 m, inoculate in autumn in open field, fruiting in spring, winter and autumn
Cr-62		9~23	60~70	
L-26		10~24	65~70	
Shenxiang No.9		12~18	60~70	

(continued)

Strain	Temperature form	Suitable fruiting temperature (°C)	Culture time (d)	Application (Number refers to the elevation of the origin place)
Suxiang No.1	Medium and high	10~25	60~75	< 300 m, inoculate in autumn in open field, fruiting in spring, winter and autumn; 300~600 m, Inoculate in spring and buried, fruiting in spring and autumn
Yuanya No.1		10~25	70~75	Inoculate in autumn in open field, fruiting in spring and winter in low altitude areas; 300~600 m, inoculate in spring and buried, fruiting in winter and autumn
Cr-04		10~23	70~80	Inoculate in autumn in open field, fruiting in spring and winter in low altitude areas; 300~600 m, inoculate in spring and buried, >700 m, keep in open field, fruiting in summer and autumn
Cr-20		12~26	70~80	< 300 m, inoculate in autumn in open field, fruiting in winter and spring; >300,<600 m, buried in spring; >700 m, keep in open field, fruiting in summer and autumn
Wuxiang No.1	High	16~25	70~80	>500m, off-season inoculation in spring, and buried, > 700m, keep in open field, fruiting in summer and autumn
8001		14~26	70~75	
Guangxiang 47		14~28	70~80	>300 m, inoculate in autumn, and fruiting in winter and spring; >500 m, inoculation in spring and buried,>700m, keep in open field, fruiting in summer and autumn
8500		13~26	70~80	
XinglongNo.1		14~28	70~80	Off-season cultivation in cold regions of northern, fruiting in summer and autumn in open field

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Part IV
Truffles

Chapter 13
Desert Truffles (*Terfezia* spp.) Breeding

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Abstract Desert truffles are hypogeous edible fungi that have been exclusively harvested in wild areas for hundreds of years. Land-use changes coupled with shifts in precipitation pattern and volume, as a result of climate change, have led to a decline in wild production of these fungi. Due to their high nutritional value, as well as rising market prices, efforts were stepped toward domestication more than 20 years ago. The present chapter describes the achievements made to understand the biology and diversity of these desert truffles which have helped to make this resource more sustainable. Most efforts to domesticate this natural resource have begun primarily with *Terfezia claveryi* Chatin. Biotechnological processes for mycorrhizal plant production as well as plantation management practices are analyzed with the experience accumulated to date. *Terfezia* cultivation is a totally organic crop, with minimum water irrigation, without the consumption of fertilizers or phytosanitary products and using native fungal and plant species. Thus, the long-standing tradition of desert truffle harvesting looks to the future, by adapting its domestication to modern agriculture.

Keywords Desert truffle · *Terfezia* · Breeding · Organic crop · Arid areas

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13.1 Introduction

Global agriculture is facing the challenge of climate change that will shift crops from one region to another. Selection of crop alternatives adapted to drought is a must, in light of an uncertain climatic scenario. The hypogeous edible ascocarps of Ascomycetes fungi belonging mostly to the Pezizaceae family are known as *desert truffles* due to their habitat, which is typically arid and semiarid areas, mostly in the Mediterranean Region (Morte et al. 2009; Zambonelli et al. 2014). Some species of *Terfezia* and *Tirmania* have a long history of use in cooking and traditional medicine because they are rich in nutrients and bioactive compounds (Shavit 2014) and are an important economic resource for local populations. However, declining rainfall and changes in land use have led to a decline in wild populations of these fungi and scientists are therefore considering their domestication to create a new drought-adapted crop. These desert truffles are fungi that form mycorrhizal symbiosis with different plant species and this symbiosis is really what adapts to the drought in the areas where they live. This drought adaptation has been well characterized and studied and is based mainly on strategies to improve plant nutrition and photosynthesis (Kagan-Zur et al. 2014a; Morte et al. 2000; Turgeman et al. 2011), the water use efficiency (Morte et al. 2010), mycorrhizal root colonization (Navarro-Ródenas et al. 2012a, 2013), expression of fungal and plant aquaporins (Navarro-Ródenas et al. 2012b, 2013) and catalase genes (Marqués-Gálvez et al. 2019).

Terfezia claveryi Chatin was the first desert truffle to be cultivated in mycorrhizal symbiosis with plants of *Helianthemum almeriense* Pau in 1999 in southeastern Spain (Honrubia et al. 2001; Morte et al. 2008). Since then, most of the data related to the biotechnological aspects and plantation management practices to domesticate it have been compiled in several publications (Honrubia et al. 2014; Morte and Andrino 2014; Morte et al. 2008, 2009, 2012, 2017, 2020). Moreover, many other aspects related to the biology and uses of desert truffles are included in the first book dedicated to desert truffles (Kagan-Zur et al. 2014b).

The crop's cumulative demand, in Spain and also in other countries, has resulted in the search for new ways to increase the production of these fungi in the field, improve the quality of productive mycorrhizal plants and deepen the knowledge of biodiversity and the biological cycle of these edible fungi. This chapter outlines the main achievements toward these objectives and the latest results obtained on desert truffle yield.

13.2 Biodiversity of the Edible Species of Desert Truffles

The most appreciated desert truffles species belong to the genera *Terfezia* and *Tirmania*; *Terfezia claveryi*, *Terfezia boudieri* Chatin, *Tirmania nivea* (Desf.) Trappe and *Tirmania pinoyi* (Maire) Malençon (Fig. 13.1) are the most prized species on the market.

Fig. 13.1 (**a**) *Terfezia claveryi*, (**b**) *Terfezia boudieri*, (**c**) *Tirmania nivea*, (**d**) *Tirmania pinoyi*, (**e**) *Helianthemum almeriense*, (**f**) *Helianthemum lippii* (L.) Dum. (Photos a, b, d are by A. Rodríguez; c, e, f by A. Morte)

Recently, several new species of *Terfezia* (Bordallo et al. 2012, 2013, 2015; Crous et al. 2019; Kovăcs et al. 2011; Zitouni-Haouar et al. 2018) and *Tirmania* (Crous et al. 2018) have been identified. The ITS-rDNA sequence, host plant and soil pH seem to be key to discovering new desert truffle species (Morte et al. 2017). These species (or their host) seem to be able to adapt to a wide range of soil pH, edaphic conditions and texture (Bonifacio and Morte 2014). In desert truffles, as

mycorrhizal fungi, the preference-specificity factor of the host is manifested as an important feature to understanding their life cycle and ecology.

All hypogeous species belonging to the desert truffle genera are edible fungi but not all of them are profitable to market. What makes a species interesting to market is that it reaches a medium to large size and that its peridium is easily cleaned.

At least 14 edible desert truffles are well identified for being consumed by people. These fungal species have characteristic host plants and soil pHs, which define their mycorrhizal symbiosis and ecology (Morte et al. 2017). These fungal species are: *Choiromyces magnusii* (Mattir.) Paol.; *Mattirolomyces terfezioides* (Mattir.) E. Fisch.; *Picoa juniperi* Vittad.; *Picoa lefebvrei* (Pat.) Maire; *Terfezia arenaria* (Moris) Trappe; *Terfezia boudieri*; *Terfezia canariensis* Bordallo & Ant. Rodr.; *Terfezia claveryi*; *Terfezia crassiverrucosa* Zitouni-Haouar, G. Moreno, Manjón, Fortas & Carlavilla; *Terfezia fanfani* Mattir.; *Terfezia leptoderma* Tul. & C. Tul.; *Tirmania honrubiae* Morte, Bordallo & Ant. Rodr.; *Tirmania nivea*; *Tirmania pinoyi*; *Tuber lacunosum* Mattir. and *Tuber oligospermum* (Tul. & C. Tul.) Trappe (Morte et al. 2017). In fact, some of these species (*Terfezia arenaria* and *Terfezia claveryi*) are included in official documents to regulate their collection in public and private forests in some regions of Spain, such as Extremadura, Castilla-La Mancha, Andalusia and Murcia (Oliach et al. 2020). However, only two species have been successfully cultivated until now, *Terfezia claveryi* in Spain (Honrubia et al. 2001; Morte et al. 2008, 2009, 2010, 2012) and *Terfezia boudieri* in Tunisia (Slama et al. 2010) and Israel (Khagan-Zur and Sitrit pers. comm). More recently, mycorrhizal plants with *Picoa lefebvrei* and *Terfezia crassiverrucosa* have been planted in the spring 2018 in Murcia, producing the first truffles in the spring 2020 (Morte pers comm).

Carpophores of *T. claveryi* represent more than 90% of the desert truffle collections in semiarid environments of Spain in basic soils. To the scientific and commercial interest of this desert truffle, we must add the fact that it is the only species of fungus that was suggested as the object of management measures for its use in the Murcia Region, according to the Red Book of Protected Wild Flora of the Region of Murcia (Sánchez-Gómez et al. 2002). It is also considered among the natural resources of this Region (Honrubia et al. 2003). It is undoubtedly a species of great interest, given the sequencing of its genome (https://mycocosm.jgi.doe.gov/Tercla1/Tercla1.home.html) at the Joint Genome Institute. It is not known whether conservation measures exist for other desert truffle species in the different countries where they are wild-collected.

13.3 Production of Desert Truffle Mycorrhizal Plants

The first step toward desert truffle cultivation is the selection and production of suitable, quality mycorrhizal seedlings adapted to different cultivation locations. Both micropropagated plants and seedlings of *Helianthemum* species, in conjunction with *Terfezia* mycelium and spores, have been used to carry out mycorrhizal

synthesis (Morte and Andrino 2014; Morte et al. 2008, 2009). The use of seeds and spores is the most widely used system for the production of mycorrhizal plants because it costs less than using micropropagated plants and mycelium. However, each of these combinations has its own advantages and disadvantages, and we use them depending mostly on the availability of fungal inoculum.

13.3.1 Fungal Inoculum

The spore solution is commonly used for two main reasons; first, the erratic growth of mycelium under in vitro conditions; second, and most important, it increases the possibility of including spores with a complementary mating (Marqués-Gálvez 2019). The spore solution, obtained by blending mature spores, could be inoculated directly to the roots or using different carriers, where the spores are attached to their surface (Andrino et al. 2012; Morte et al. 2012). The use of this carrier technique allows a 40% reduction in the amount of spores (Morte and Andrino 2014). However, the problem with the spore inoculation techniques is that pathogens, pests and other fungal spores can still contaminate the plants (Iotti et al. 2016). Much effort has been made to improve cultural conditions, nutrients, and to understand the factors or inhibitors that can limit *Terfezia* growth. Cano et al. (1991) reported that the best growing medium for *T. claveryi* was modified Melin-Norkrans (MMN) medium (Marx 1969) with a pH adjusted to 7. Navarro-Ródenas et al. (2011) showed that *T. claveryi* and *Picoa lefebvrei* mycelia need some adjustment of the water potential of the in vitro culture medium, as they grow better under moderate water stress of −0.45 and − 0.72 kPa for *T. claveryi* and *P. lefebvrei*, respectively. At the base of that tolerance to water stress was found an increased expression of the *T. claveryi* aquaporin TcAQP1 gene, a membrane protein that acts as a water channel and other substances facilitating the transport of water between cells (Navarro-Ródenas et al. 2012b). Moreover, the use of cyclodextrins (CD), especially β-CD, could stimulate the growth of *T. claveryi* mycelium to a final diameter and growth rate five times greater than that of the control without CD (López-Nicolás et al. 2013). More recently, Arenas et al. (2018) found that the limiting factor of in vitro growth of *T. claveryi* is neither nutrients nor growth conditions, but rather the deficiency in certain growth factors, such as vitamins involved in glucose catabolic pathways, which the fungus may not be able to synthesize. The best results were obtained using glucose as carbon source, buffering the pH at 5.0, adding a pool of vitamins and using 15 and 0.6 g L^{-1} as carbon and nitrogen sources of the MMN medium, respectively. Biomass production of *T. claveryi* in the bioreactor increased from 0.3–3 g L^{-1} dry weight and productivity increased from 10.7–95.8 mg L^{-1} day^{-1} dry weight, thus providing a suitable amount of mycelium for large-scale mycorrhizal inoculation (Arenas et al. 2018).

13.3.2 Host Plants

The other important factor in mycorrhizal plant production is the choice of the appropriate host plant. This choice must take into account the edaphic and bioclimatic conditions of the plantation site. It is logical and better to use a perennial species than an annual one. *Terfezia claveryi* forms mycorrhizal symbiosis with perennial and annual species of the *Helianthemum* genus, belonging to the Cistaceae (Morte and Andrino 2014). Perennial species chosen as host plants in Spanish plantations are *H. almeriense* Pau (Fig. 13.1), *H. violaceum* (Cav.) Pau and *H. hirtum* (L.) Miller. Different elevations better suit each of these species, making this symbiosis widely adapted from almost 0 to 800–1000 m elevation. For the cultivation of *Terfezia boudieri* and *Tirmania nivea*, the plant species of *H. lippii* (L.) Dum. (= *H. sessiliflorum*) (Fig. 13.1) has been selected to produce mycorrhizal plants for being well adapted to arid zones where these desert truffles occur naturally.

In general, seed scarification is necessary to increase germination rates of *Helianthemum* species due to their erratic seed germination. In addition, high mortality of the germinated seedlings is very frequent during the first 2 months after germination in nursery conditions (Morte et al. 2012). Micropropagation techniques have been used for plant production, as they improve in vitro seed germination and plant survival (Morte and Andrino 2014; Morte et al. 2008, 2012).

In order to improve plant production from *Helianthemum* seeds in the nursery, several PGPR (plant growth promoting rhizobacteria) have been isolated and characterized from the mycorrhizal roots, mycorrhizosphere soil and peridium of desert truffles collected at desert truffle productive plantations (Navarro-Ródenas et al. 2016). Some of these bacteria had a positive effect on seed germination and survival of *H. almeriense* plants, with an increase of 40–122% in comparison with the treatment without bacteria. The auxin-producing bacteria were highly relevant during the mycorrhization stage increasing the root-stem ratio and colonization percentages by 47–154% in comparison with plants without bacterial inoculation (Navarro-Ródenas et al. 2016). Moreover, one strain of *Pseudomonas mandelii* was able to considerably increase mycorrhizal colonization but not the plant growth, being considered as mycorrhiza-helper-bacteria (MHB) (Navarro-Ródenas et al. 2016). Further studies have demonstrated that the mycorrhization percentage in plants inoculated with *P. mandelii* (40%) or in plants inoculated with bacteria in combination with a drought treatment (60%) were higher than control plants (20%) without bacteria inoculation or water stress. Moreover, this strain is able to mitigate the negative effect of water stress, maintaining both shoot water potentials of drought stressed and control plants at similar levels (Espinosa-Nicolás 2017; Martínez-Ballesteros 2018).

Therefore, the mycorrhizal roots, mycorrhizosphere soil and peridium of desert truffles are bacteria-enriched environments that can increase plant quality in the desert truffle plant production system at a semi-industrial scale (Navarro-Ródenas et al. 2016). Recently, we began to apply these MHB bacteria in the irrigation water

in young plantations to try to stimulate truffle fructification, although we still do not have enough results to demonstrate this effect in field conditions.

13.3.3 Desert Truffle Mycorrhizal Plants

For the final production of desert truffle mycorrhizal plants, the time required is 4–9 months, depending on the type of plant propagation system and the source of inoculum used (Morte et al. 2017). After that time, it is very important to check the percentage of mycorrhizal colonization before using the mycorrhizal plants for cultivation. For this purpose, we suggest that 12 plants should be examined for each batch of 1000 plants and we consider a mycorrhization percentage exceeding 33% as a statistically good indicator of plant quality (Morte et al. 2012).

The evaluation process consists of morphological examining the entire root system by stereomicroscope, detecting the mycorrhizal root morphotypes characteristic of this symbiosis (Gutiérrez et al. 2003).

The type of fungal colonization that *T. claveryi* forms inside *H. almeriense* roots is mostly intracellular under natural field conditions, inter- and intracellular without a sheath in pot cultures, and intercellular with a characteristic sheath and Hartig net in vitro (Gutiérrez et al. 2003; Morte et al. 2008).

We demonstrated that this colonization varies from ecto- to endomycorrhiza according to plant water availability. The scarcer the water, the more intracellular the colonization is found (Navarro-Ródenas et al. 2012a, 2013). Therefore, this symbiosis is considered as an *ectendomycorrhizal continuum* (Navarro-Ródenas et al. 2012a) and water availability modifies the relative amount between intra- and intercellular hyphae in this *continuum*. For the mycorrhizal certification of plants, the roots have to be examined by staining them with 5% blue ink in acetic acid or 0.01% acid fuchsine solution and observe them under the microscope. The *Terfezia* mycelium shows an easily identifiable moniliform shape inside the root cortical cells or in the intercellular spaces of the root cortex (Fig. 13.2). To calculate the mycorrhization status, 100 root segments per plant are observed microscopically and quantified for colonization according to McGonigle et al. (1990).

13.4 Desert Truffle Plantations and Yield

The novel cultivation of desert truffles accumulates only 20 years of experience. Since then, basic and applied researches have focused on understanding the phenology of the mycorrhizal plants in natural areas. In southeastern Spain, the cultivation of desert truffles is called *turmiculture*, after the local name *turma* to designate the desert truffle (Honrubia et al. 2014).

Numerous plantations have been established in Spain using *Helianthemum almeriense*, *H. violaceum* or *H. hirtum* as host plants and *T. claveryi* as desert truffle

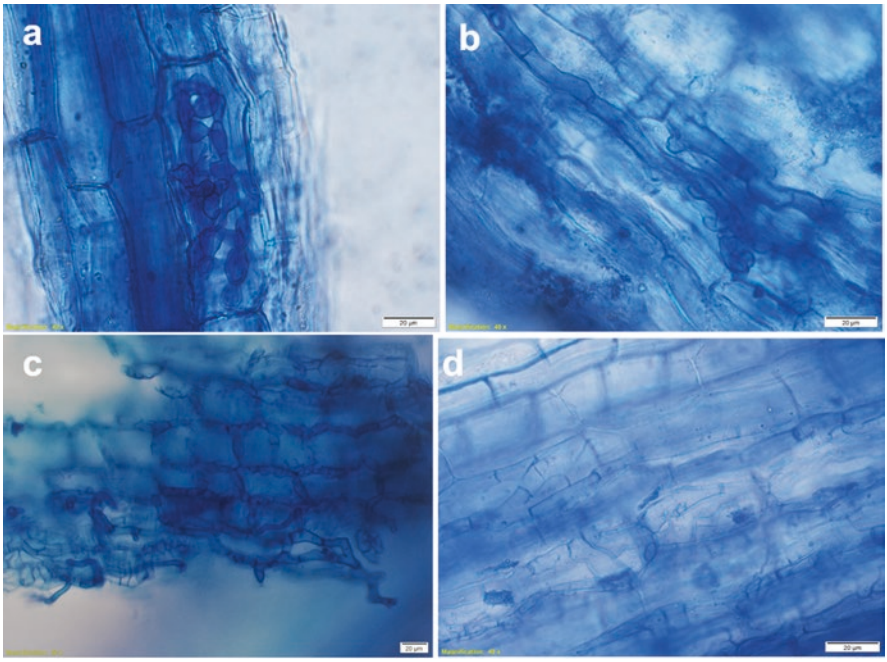

Fig. 13.2 (**a**) Mycorrhizal colonization of desert truffles with moniliform mycelium inside the root cortical cells, (**b**, **c**, **d**) the intercellular spaces of the root cortex. (Photos by J.E. Marqués-Gálvez)

(Fig. 13.3) with around 8 ha spread over different provinces but mostly in Murcia, Alicante, Castellón, Albacete, Granada and Jaén. Moreover, experimental results of the cultivation of *T. boudieri* in Tunisia (Slama et al. 2010) and Israel (V. Kagan-Zur and Y. Sitrit pers. comm) have been obtained. These two species of *Terfezia* cultivated until now are typical of alkaline or basic soils.

Desert truffle fructification should occur 1–3 years after planting, depending on the quality of the mycorrhized seedlings, site suitability, frame and season of the plantation as well as field management practices, mainly irrigation and weed control (Morte et al. 2017). But ultimately, all these factors are subject to environmental conditions whose parameters have been little studied so far. In this section, all these variables are analyzed.

13.4.1 Desert Truffle Yield

In order to determine trends in desert truffle crops and how they are influenced by climate, a study of the oldest *Terfezia* plantation in Spain was recently published (Andrino et al. 2019). This plantation was established in May 1999 with 60 *H. almeriense* plants well-mycorrhized with *T. claveryi* (above 90% of mycorrhization)

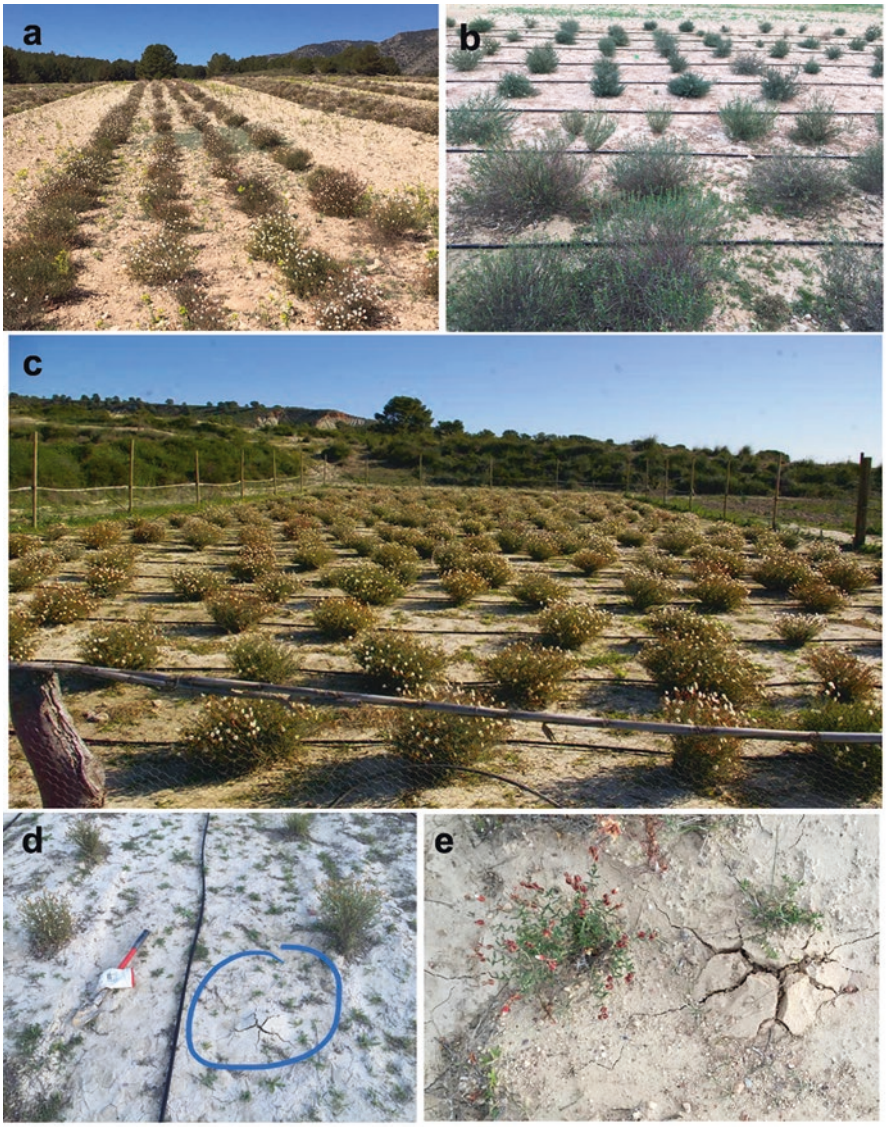

Fig. 13.3 *Terfezia claveryi* plantations with *Helianthemum almeriense* in different zones of the Region of Murcia: in (**a**) Caravaca de la Cruz, (**b**) Torre Pacheco, (**c**) Corvera, (**d,e**) cracks and bumps on the soil indicating the presence of desert truffles. (Photos a,b are by A. Morte; c,d,e are courtesy of Paco de Lara)

(Gutiérrez 2001). The plantation was located in Zarzadilla de Totana, Lorca, Murcia (37°52′15.5”N 1°42′10.5”W) at an elevation of 870 m, with annual precipitation of 289 ± 106 mm/year. The planting pattern was 0.5 x 0.5 m in a total area of 20 m^2. In order to promote a proper establishment of the seedlings, they were watered with

15 l/m^2 every 15 days for the first 3 months until August 1999. During August and January 1999–2000, 50 l/m^2 were supplied at each watering time. In spring of 2001, the first desert truffles were picked. Following the first fructification, no further watering was provided and the orchard has subsequently grown solely on natural rainfall.

From 2001 to 2015, the orchard increased its average annual crop yield almost linearly until 2009 (Fig. 13.4), reaching a cumulative average harvest of 379 kg/ha and remained almost constant, with a standard deviation of ±14 kg/ha, throughout the remaining years. By the end of the study, the average desert truffle crop was 355 kg/ha/year. However, the yearly yield showed large interannual fluctuations with a standard deviation of ±318 kg/ha (Fig. 13.4). After the first fructification, the crop was zero (2014) or less than 2 kg/ha (2005, 2006) in only three very dry years. The largest harvest was 2009 with 1069 kg/ha (Fig. 13.4).

The cumulative mean value was taken to determine two groups, depending on whether they showed low productivity years (L) or high productivity years (H), in comparison with the cumulative mean values over the 2000–2015 period. In the following section, the values of ten different meteorological parameters, for each group L and H, were compared to identify which periods of the year showed significant differences in the desert truffle production for each meteorological parameter (Andrino et al. 2019).

Another case study is the plantation established during October 2015, in Corvera (Murcia, Fig. 13.3c). The area receives an average rainfall of 260 mm/year. Irrigation was applied in dry autumn and/or spring and the first truffles were obtained in the

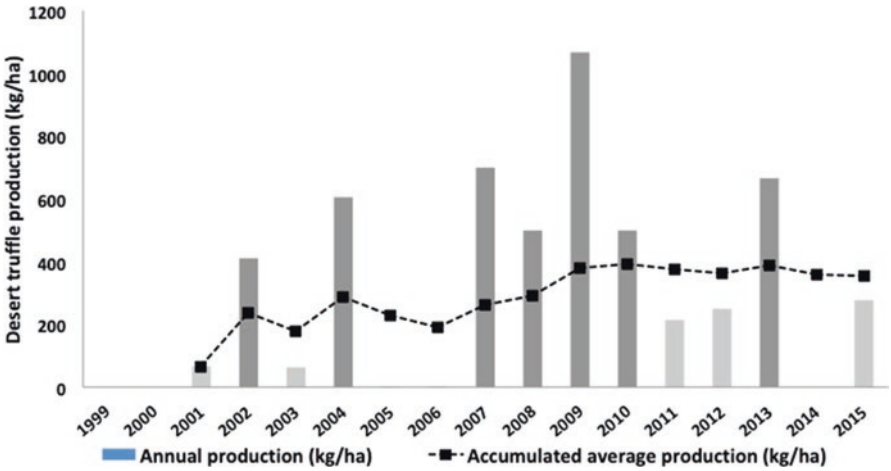

Fig. 13.4 Annual desert truffle yields (kg/ha) 2001–2015. The dashed line shows the average annual harvest (kg/ha/y) since planting. The bars represent the total annual truffle production per year. The light gray bars represent years when yields fell below the annual average (kg/ha/year) and are categorized as low-harvest years (L). The dark gray bars are years when yields were higher than the annual average (kg/ha/year) and are categorized as high-harvest years (H). There are no bars when the yield was zero (2014) or less than 2 kg/ha (2005, 2006). (Source: Andrino et al. 2019)

spring of 2017, with a production of 74 kg/ha, 54 kg/ha in 2018 and 105 kg/ha in 2019. The trend of increasing production each year can be amended by irrigation during dry years. However, excess irrigation decreases the mycorrhization rate and water use efficiency of mycorrhizal plants (Morte et al. 2010) and truffle production is negatively affected. Our ongoing research aims to answer questions on when and how much water has to be applied, in order to correct the lack of rainfall and stabilizing the production.

13.4.2 Phenology and Seasonal Influence of Agroclimatic Parameters

Phenology is defined as the study of periodic events in the life cycles of organisms, as influenced by the environment. The annual phenology of *H. almeriense* is typical of other Mediterranean summer-deciduous or semi-deciduous shrubs (Gulías et al. 2009; Haase et al. 2000; Nilsen and Muller 1981) and consists of a vegetative period that lasts from autumn (bud break) to spring, blooming events that start at the end of winter and finish in spring and leaf senescence at the end of spring. Maximum photosynthesis is found in winter and truffle fruiting coincides with the plant blooming (Fig. 13.5). Mycorrhizal colonization in the field is mainly intracellular at above 40% of mycorrhization average, except for summer, when few signs of mycorrhiza are found (Gutiérrez et al. 2003; Marqués-Gálvez et al. 2020; Morte et al. 2010). Therefore, the start of the desert truffle year can be taken as 1 June. This gap between plant and fungal phenologies means that there are several times during the year when environmental conditions could be decisive in their interaction.

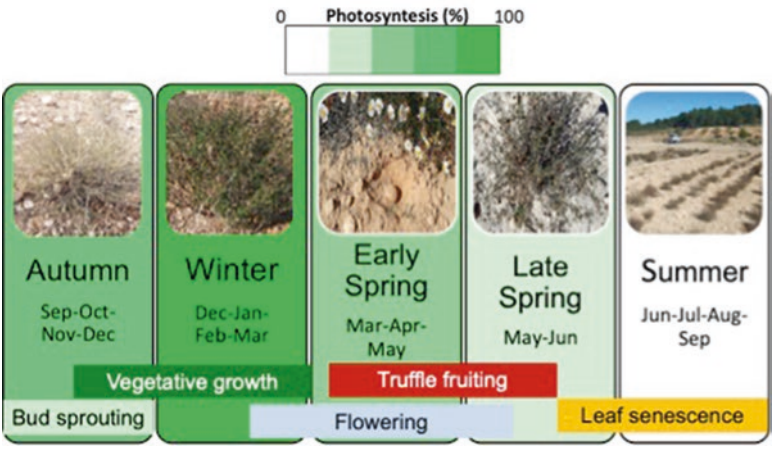

Fig. 13.5 The annual phenology of *Helianthemum* desert truffle plants and the intensity of the photosynthesis in Mediterranean semiarid zones. (Figure constructed by A. Navarro-Ródenas and J.E. Marqués-Gálvez)

Although most studies related to desert truffle production point to the sole importance of precipitation (Bradai et al. 2014, 2015; Mandeel and Al-Laith 2007; Morte et al. 2012), a seasonal influence of eight out of ten parameters (aridity index, evapotranspiration of reference-ET0, mean temperature, mean relative humidity, precipitation, soil water potential, soil water potential anomaly, air vapor pressure deficit-VPD) was observed for the *T. claveryi* crop (Andrino et al. 2019). Results show that *T. claveryi* production in a semiarid Mediterranean climate has two key periods during its annual cycle: autumn (Sept–Oct) and spring (end of March). The aridity index (calculated as precipitation divided by ET0) and soil water potential seem like the most controllable parameters in the field by applications of irrigation for the periods stated above (Andrino et al. 2019).

According to Andrino et al. (2019), during summer (Jun–Aug), in general, climatic parameters, particularly drought conditions, are not critical for desert truffle, since *H. almeriense* is a summer deciduous plant (Andrino et al. 2019).

However, in autumn (Sept–Nov) all agroclimatic parameters, with the exception of temperature, showed significantly correlations with the truffle crop (Fig. 13.6). Precipitation and aridity index are the parameters most strongly related to yield

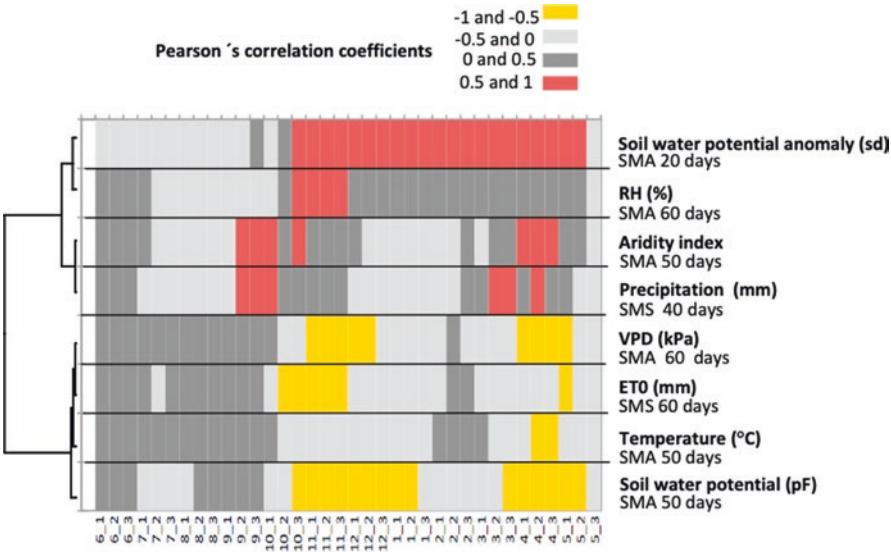

Fig. 13.6 Heatmap that groups the significant ($P < 0.05$) positive (red) and negative (yellow) correlations of Pearson between agroclimatic parameters and truffle harvesting in 10-day periods. Dark gray and light gray indicate that there are no statistically significant Pearson correlations. The average period (days) (simple moving average, SMA) or cumulative period (simple moving sum, SMS) used to calculate the Pearson correlations is indicated under the name of each weather parameter. The SMA or SMS period shows the highest number of significant correlations. On the x-axis: month numbers represents the periods of the year and each month is divided into 10-day sub-periods. Abbreviations are *RH* relative humidity, *VPD* vapor-pressure deficit, *ET0* evapotranspiration of reference. (Source: Andrino et al. 2019)

(Fig. 13.6), and show statistically significant different annual profiles during autumn (Figs. 13.7a, c).

It is particularly at this season, when there is a period of approximately 50 days (10 Sept–20 Oct) when accumulated rainfall of around 80 l/m^2 would result in a year of high productivity (H) (Andrino et al. 2019). Nevertheless, when the accumulated rainfall in this window is less than 26 l/m^2, the harvest of the current year would be strongly impaired and values below 89 kg/ha are expected (Andrino et al. 2019). In any case, the overall effect of rainfall during autumn may be influenced by other parameters such as ET0 (Fig. 13.6), which makes water available to the plants within a more or less defined period of time. The aridity index, precipitation divided by ET0, was found to be the climatic parameter with the most significant differences during the autumn between the H and L years (Fig. 13.7c).

A high correlation with agroclimatic parameters out of time supports the hypothesis of the early formation of truffle primordia during autumn (Bordallo 2007; Pacioni et al. 2014). Furthermore, the correlation with soil water potential, the highest correlation between the crop and any other agroclimatic parameter (Fig. 13.6), is also apparent in late autumn (Fig. 13.7d).

The host plant *H. almeriense* shows the highest gas exchange activity and quantity of mycorrhizal roots during the winter (Dec–Feb), although few, and only slightly significant, correlations were found with the investigated parameters (Fig. 13.6). Soil water potential was the parameter that exhibited the highest

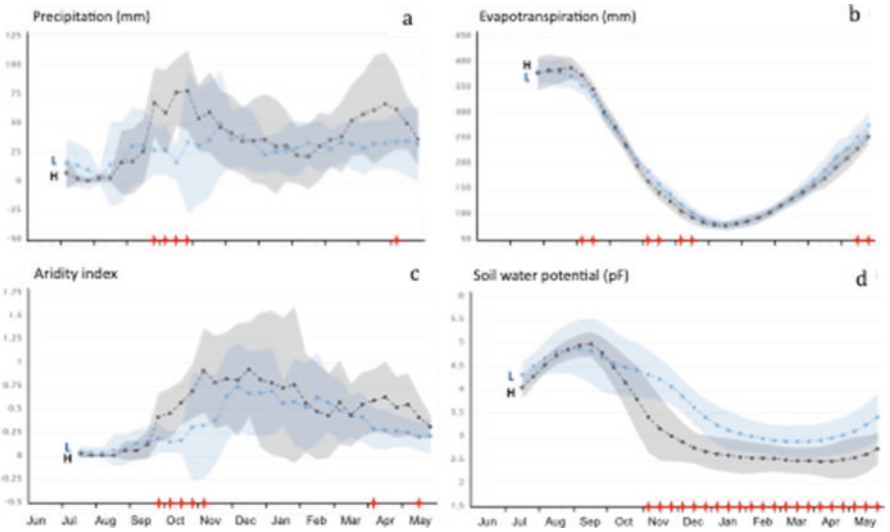

Fig. 13.7 Annual profile of agroclimatic parameters displaying average value (dashed line with circles) and standard deviation (colored shadow) of the various agroclimatic parameters depicted for high productive years (H, black color) and low productive years (L, blue color). The abscissa axis shows the months of the year arranged in 10-day periods and the significantly different values between the productions L and H are labeled with a red star where it corresponds, as a result of the Mann-Whitney U test ($P < 0.1$). Source: Andrino et al. (2019)

correlations with truffle cultivation along this season (Fig. 13.6). Morte et al. (2010) and Navarro-Ródenas et al. (2013) observed a decline in gas exchange parameters under drought conditions, so high soil water potential might favor the production of photoassimilates that might later lead to truffle formation.

During the spring (Mar–May), when *T. claveryi* tends to fruit, rainfall could supplement autumn rainfall when it is sufficient, and partially compensate the yield when rainfall is not sufficient (Andrino et al. 2019). During spring until summer, photosynthetic activity progressively decreases (Marqués-Gálvez et al. 2020). The decline in host plant photosynthesis might be the factor triggering *T. claveryi* fructification. In addition, mild temperatures and consequently mild VPD may increase desert truffle yield. Previous reports indicated a decrease in photosynthesis when atmospheric demand (VPD) achieves certain values and beyond senescence and leaf drop, thus high VPD values during the fruiting stage may cause a precocious end of the fruiting period leading to a drop in yield (León-Sánchez et al. 2018; Marqués-Gálvez et al. 2020; Morte et al. 2010). In addition, the soil water potential exhibited a strong correlation from late autumn, during winter and until late spring (Fig. 13.7d). Mycelial growth of *T. claveryi* is enhanced by moderate drought stress (Navarro-Ródenas et al. 2011); however, as with other hypogeous fungi, fruiting bodies also evolve over a period of months and growing truffles are sensitive to desiccation. Therefore, it is necessary to maintain an adequate soil water potential along the cropping season (Bruhn and Hall 2011); the implementation of soil water potential sensors in prospective desert truffle plantations may improve their maintenance.

Summarizing, soil water potential and aridity index are the most important agroclimatic parameters determining annual desert truffle production. Agroclimatic parameters role long before the desert truffle fruiting season, contrary to other edible mycorrhizal fungi. Major agroclimatic parameters can be controlled by using field irrigation at identified periods of autumn and spring, thereby making it possible to optimize desert truffle yields (Andrino et al. 2019).

13.4.3 Irrigation

Suitable irrigation involves delivering the right amount of water needed, at the right time of application. Morte et al. (2012) reported a statistical correlation, according to Pearson's test, with the amount of rainfall during the fall (Sept–Nov) of a given year and the subsequent truffle harvest of *T. claveryi* in the spring of the next year. From their own experience, Honrubia et al. (2014) recommended that irrigation should be provided in late summer/early autumn and, if dry conditions continue, additional irrigation of 50–80 l/m^2 at the beginning of the fruiting season would significantly increase yield. We, however, propose three different approaches to handle *T. claveryi* plantations in in a semiarid Mediterranean climate, according to the results obtained recently by Andrino et al. (2019) and based on the resources and facilities available in the plantations:

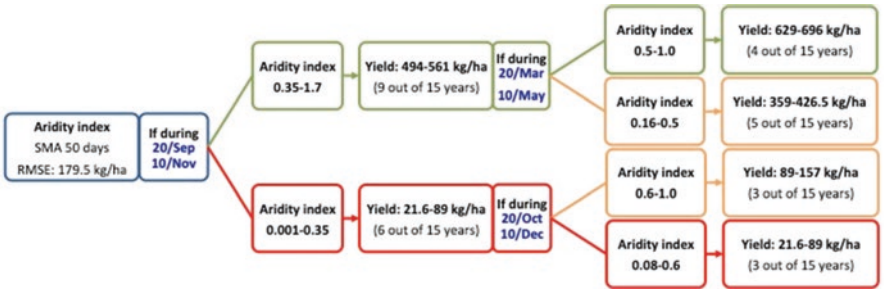

Fig. 13.8 Classification and regression tree (C&RT) on aridity index values. On the left, the first box (blue) shows the optimal SMA or SMS values calculated from the heatmap and used to compute the C&RT. This chart also shows the calculated RMSE value between the observed and predicted truffle yield. On the right, the box below displays the C&RT forecasted dates with the greatest impact on truffle harvesting. On the right, the two nodes indicate the expected range of values of the different agroclimatic parameters, the ranges of truffle cultivation in the desert, and the number of years included in each son node (i.e. the node derived from the previous one). The green nodes display the best scenario, the orange nodes display the suboptimal scenarios, and the red nodes display the unwanted scenario. Source: Andrino et al. (2019)

(a) Using the aridity index (Fig. 13.8): ET0 must be surveyed during the 50 days prior to 10 October and watering applied to maintain the aridity index at least above the threshold of 0.35 (Table 13.1) and during the 50 days prior to 10 May at least above the threshold of 0.50 (Table 13.1). The values of ET0 and precipitation can be acquired from a nearby weather station at the plantation or from the closest official weather station.

The final irrigation, according to these parameters, can be calculated as:

$$RW_p = AI_t ET0_{p,} I_p = RW_p ? P_p$$

where RW is the required water, AI_t is the aridity index threshold, $ET0_p$ is the evapotranspiration during the period, I_p is the irrigation during the periods, P_p is the precipitation during the periods, according to Andrino et al. (2019).

(b) According to the soil water potential (Fig. 13.7d): Watering must be carefully monitored from 10 November to keep the soil water potential (pF) always below the mean value of L years and as near as possible to the mean value of H years according to the values in Fig. 13.7d. The pF values must be monitored with field probes such as the MPS-2 or MPS-6 Dielectric Water Potential Sensors (Decagon Devices, Inc. Pullman WA) or equivalent sensors, capable of recording the spectrum of data observed in our survey.

(c) Considering a combination of aridity index and soil water potential: Irrigation should be controlled and implemented during the fall (50 days before 10 October) to keep the aridity index above the threshold and, from November onwards, taking into account the soil water potential, which should not be allowed to exceed L years. In spring (50 days before 10 May), irrigation has to

Table 13.1 Proposed management based on the aridity index (AI) threshold with the ET0 and the average rainfall of the 3 years with the lowest production and the proposed irrigation

A Periods	ET0	Precipitation	AI	AI threshold	Required water*	Irrigation**
Sep 21–30	36.7	1.2	0.033	0.35	12.85	11.65
Oct 01–10	31.1	2.2	0.0708	0.35	10.89	8.60
Oct 11–20	28.8	1.5	0.0503	0.35	10.08	8.58
Oct 21–30	29.5	2.0	0.0662	0.35	10.33	8.33
Nov 01–10	22.3	1.2	0.0538	0.35	7.81	6.61
Mar 21–30	31.2	7.0	0.2238	0.50	15.60	8.6
Apr 01–10	35.4	4.3	0.1223	0.50	17.70	13.40
Apr 11–20	33.8	6.7	0.1969	0.50	16.90	10.2
Apr 21–30	48.1	9.5	0.1963	0.50	24.05	15.05
May 01–10	34.1	11.9	0.3496	0.50	17.05	5.15
Total		47.5			143.26	**96.17**

*$RW_p = AI_t \cdot ET0_p$; **$I_p = RW_p - P_p$; where RW is required water, AI_t is the aridity index threshold, $ET0_p$ is the evapotranspiration during the period, I_p is the irrigation during the periods, P_p is the precipitation during the periods. Source: Andrino et al. (2019)

be defined according to the aridity index or the soil water potential and irrigation has to be implemented only when one of these two parameters achieve their critical threshold values (Andrino et al. 2019).

The above recommendations must be carefully adjusted at each growing site, considering other environmental factors that could also influence the truffle production, such as soil type, slope, elevation and orientation.

As far as the irrigation system is concerned, drip, sprinkler or pivot irrigations can be used, although the latter two are recommended, whenever possible, because they are more similar to rainfall.

13.4.4 Soil Management and Frame of Plantation

Both *Terfezia* species or their host are capable of adapting to a wide range of soil pH, texture, fertility and edaphic conditions (Bonifacio and Morte 2014). The development of these truffles under a wide range of conditions may be related to the possibility of the presence of several *Terfezia* species of commercial interest or having several hosts (Bonifacio and Morte 2014).

Before planting the mycorrhizal seedlings in the field plots, a soil tilling around 30–40 cm deep with rotovator or plough should be applied to aerate the soil and remove any weeds present. Small holes of about 10–15 cm in depth and diameter are sufficient for planting the seedlings. A fence around the plantation is necessary to prevent truffles from being eaten by wild animals and to keep out undesirable desert truffle hunters.

Soil fertilization has never been applied to old or new plantations made in Spain and mycorrhizal seedlings continue to produce *T. claveryi* truffles annually. As with other types of mycorrhiza, nutrient-poor soils favor the mycorrhization of plants, which could encourage future truffle formation.

So far, no pests have been detected affecting this crop. However, it is important to be aware that the intensification and densification of plantations, as well as the tendency towards monoculture, can lead to the appearance of pests and diseases, as has happened with the cultivation of black truffles, which in a balanced system do not exist as such.

Different frames of plantation have been tested, distributed alternately in rows, ridges and groups (9–12 plants) (Honrubia et al. 2014). According to our experience, a narrow frame (0.5 × 0.5 m, in groups) allowed to obtain desert truffles the first year after plantation (Morte et al. 2012). This could favor the hyphal anastomosis and that more mycelium will be produced, giving an unknown amount of mycelial biomass that should be sufficient to start the production of truffle primordia in the soil (Honrubia et al. 2014). However, a wide frame facilitates mechanical soil tilling and prevents the plants from overlapping and covering the ground as they grow. We have to keep the plantation open so that the solar radiation reaches the soil properly, so if the plants grow a lot they should be pruned in summer. A successful plantation frame was 1.5 x 1.5 m spacing in 4–5 rows forming a block, with 2–3 m separation between blocks (Morte et al. 2020). This design produced the first ascocarps after 2 years. The small size of these shrubs allows them to be placed closer together and thus optimize the cultivated area. Therefore, a plantation of 5000–6000 plants/ha could be amortized after 5 years if the production is appropriated (200–450 kg/ha) (Morte et al. 2017).

Weed control is essential during the initial 2–3 years of planting to avoid competition for water between weeds and the truffle mycelium that begins to develop in the soil from the mycorrhized roots. The elimination of weeds should be carried out mechanically because the utilization of herbicides is unadvisable at any time (Honrubia et al. 2014). It has also been the practice to allow flocks of sheep into the plantations to eat the weeds but not the *Helianthemum* plants. In addition, sheep leave their droppings on the ground, thus fertilizing the soil naturally. Weed elimination should be done mainly in autumn, early winter and summer and it is not advisable during or close to the fruiting season.

Other management practices have been carried out recently in the plantations, such as irrigation with MHB bacteria *Pseudomonas madelii* and plant thinning, but more data are needed to statistically demonstrate their effect in increasing desert truffle crop. Moreover, the recent discovery of the heterothallic presence of MAT genes in the genome of *Terfezia* species, involved in the sexual reproduction of desert truffles (Marqués-Gálvez et al. 2021; Marqués-Gálvez 2019), opens a new perspective and the possibility of introducing the *truffle trapping* technique to stimulate desert truffle fruiting. This technique, which consists of making holes inside the potential productive area and filling them with a substrate containing ascospores, has been applied in black truffle plantations with good results, increasing the fruiting of this truffle mostly inside the holes (Murat et al. 2016).

13.4.5 Desert Truffle Silviculture

Alternatively to desert truffle cultivation, ecosystems with wild desert truffle production can be managed in order to preserve and increase their productivity, it is what we call *desert truffle silviculture* (Honrubia et al. 2014). The sustainability of desert truffle ecosystems implies a compromise between their exploitation and the respect of all interests involved. Only by producing (through the exploitation of resources), conserving (following sustainability criteria) and improving (biodiversity and the multifunctionality of the area) the management of these ecosystems offer guarantees for the future development of rural areas (Honrubia et al. 2014).

The most used practices consist of pruning plants to open up the ecosystem, subsoiling to avoid excessive compaction, the introduction of mature spores and plant seeds and irrigation at the beginning of the autumn and early spring. Thus, the production of wild *T. boudieri*, *T. pinoyi* and *T. nivea*, in natural production areas in Abu Dhabi (UAE), were stimulated by fungal spore inoculation and irrigation of areas with the presence of host plants, and fenced to avoid consumption of truffles by animals (Gouws et al. 2014). Other experiences with pivot irrigation of flat areas with the presence of host plants from the Arabian Peninsula have been successful in stimulating production (https://youtu.be/m51kTgGCMyk), although we do not have exact production data per hectare.

13.4.6 Desert Truffles Harvesting

These fungi produce fruit bodies, ascocarps or truffles, that ripen just beneath the surface of the soil, creating a pattern of cracks and bumps on the surface (Figs. 13.3d,e). People know to collect desert truffles by recognizing these cracks and bumps on the sand (Mandeel and Al-Laith 2007; Trappe 1990). After removing the truffle, it is very important to cover the hole where the truffle was found with the surrounding soil. This helps to avoid disturbing the mycelium of the soil so that it continues to grow and expand throughout the plantation. It is good practice to leave a minimum of truffles (no less than 10%) in the soil to ensure spore dispersion that allow the fungus to remain. This can be done by limiting the permitted harvesting season, with special attention to the end date of the harvesting season, as made for other wild mushrooms.

In southeastern Spain, harvesting is done from the beginning of the year to mid/late March and prolonged until mid-May in abundantly rainy years. However, with the effects of temperature change due to global climate change, there is a trend to bring forward the start of production to the beginning of December.

Harvesting of desert truffles is a manual job for well-trained people who are able to recognize the crack formed by the truffle in the soil around the host plant. As the desert truffle hunter knows how to recognize the cracks in the soil, dogs are not necessary to find them (Morte et al. 2012). However, this task is labor-intensive and

therefore considerably expensive. Moreover, as production is not simultaneous throughout the plantation, but in waves that extend over several months; it requires great attention and dedication on the part of the collector. For this reason, as the number of hectares under cultivation increases, it will be advisable to train dogs that can detect the truffles more effectively and quickly.

13.5 Desert Truffle Market

Desert truffles have been known as a foodstuff since the dawn of Mediterranean cultures. It is known that they were commercialized by Greeks and Romans, who imported them from Libya and sold them in different markets of the empires (Trappe 1971). Since then, their consumption and commercialization has remained linked to the three Mediterranean cultures associated with the Christian, Jewish and Muslim faiths.

In the Region of Murcia, references to desert truffles have been made since the time of Pliny, who mentioned the origin of truffles ... *it is a callus of the earth, it is born spontaneously and cannot be sown* (García y Bellido 1997).

Today, the vast majority of the world's traded desert truffles are of wild origin and come from North African countries. There are three routes that these collected desert truffles can take (Fig. 13.9): (1) most are sold to local intermediaries in Saudi Arabia (Awameh and Alsheikh 1979), Algeria (Gast 2000), Morocco (Khabar et al. 2001) and Bahrain (Mandeel and Al-Laith 2007); (2) the collectors sell to small local shops, restaurants or directly to other local consumers and (3) a small part is consumed by the collectors themselves, their family members and in the surrounding area. Depending on the country of origin and the destination of these truffles, the price to the collector is close to 10–20 EUR/kg (Volpato et al. 2013). In Spain, although they have been consumed for a long time and it is estimated that wild production must be similar to countries in North Africa, their commercial value is very

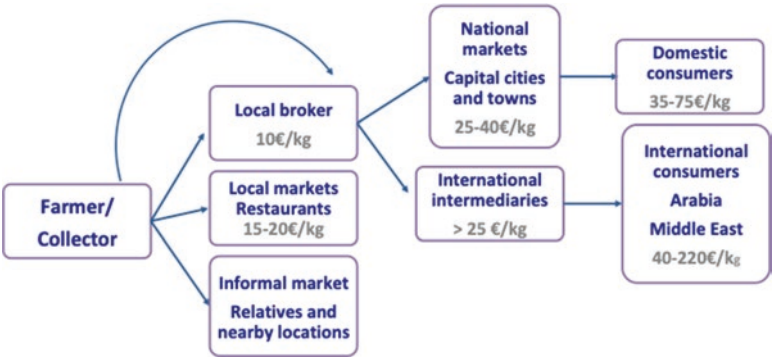

Fig. 13.9 Diagram of the different links in the desert truffle market. (Figure by A. Navarro-Ródenas and A. Morte)

recent and most of what is collected is still consumed by residents or served in local restaurants. A desert truffle plantation has an average yield of around 350 kg/ha from the sixth year (Morte et al. 2017), so the annual income for a desert truffle farmer would be around 7000 EUR/ha.

Local intermediaries usually sell to international intermediaries in Arabia, the Middle East or Europe. In this transaction, the price of the desert truffles is increased to 25 EUR/kg or more (Fig. 13.9). In countries like Saudi Arabia, Kuwait and the Maghreb states (Mauritania, Algeria, Morocco, Tunisia) these truffles are highly appreciated and their consumption and trade are an important resource for collectors and intermediaries (Mandeel and Al-Laith 2007; Trappe et al. 2008a, b).

The United Arab Emirates, Kuwait, Saudi Arabia or Qatar are currently the largest importers of desert truffles, reaching prices of 40–220 EUR/kg (Volpato et al. 2013). However, in Spain there is not yet an established commercial network, as it is a product with a local market.

It is very difficult to estimate the volume that the trade of desert truffles represents worldwide. Some data provided by officials of the cargo division of Air Algérie report that more than 594,000 EUR worth of desert truffles passed through the Houari Boumédiène Airport in Algiers during the month of February 2009, destined for the Persian Gulf States and Syria (Fethi 2009 pers. comm, Algerian truffles gain international fame).

The counterpart to this consistently high demand and high prices is that conditions are being created for desert truffle harvesting to have a long-term negative impact on natural populations, particularly if harvesters do not fill the holes in the ground after finding the truffles. Competition among harvesters and high demand for the product trigger an early harvest of immature truffles, which are consequently smaller and less valuable. In addition, some of the traditional desert truffle areas are being lost due to land use changes (Morte et al. 2009) and decreased rainfall because of climate change (Morte et al. 2017).

The main end-use of truffles is to be eaten fresh, although it is possible to find canned food in brine or frozen at the market. In the Saharan culture it is common, in times of an excess of desert truffles, to prepare them in sheets and dry them in the shade to be able to consume them in other times of the year (Volpato et al. 2013). Nevertheless, to maintain its quality, texture, aroma, shelf life, nutritional composition and economic value, different industrial preservation techniques have been tested with different results depending on the truffle species (Murcia et al. 2014).

The Spanish Turmiculture Association (https://trufadeldesierto.com/) is an association of desert truffle growers that has been created in 2017 to carry out the Turmiculture Project. The aim is to establish and consolidate the innovative cultivation of desert truffles or *turmas* as an alternative crop in the Region of Murcia and to highlight the value of the consumption of these edible truffles in order to promote the rural development of the less productive areas of the Region (Morte et al. 2019). The objectives of the project are: (1) establishment of plantations in three different climatic and soil zones, (2) training people in techniques of plantation management, marketing, enterprise and mycoturism and (3) valorization of the consumption of desert truffle in Spain by studying their conservation after harvest and their use in

the gastronomy by prestigious restaurateurs. To achieve these objectives, researchers, farmers and gastronomy experts are working closely together, each contributing their knowledge to enhance the value of this crop. This project has financial support from the European Agricultural Fund for Rural Development and the Regional Government of Murcia (FEADER, CARM). This association has been a great impetus in the revaluation of desert truffles in Spain and has helped to place these truffles in the kitchens of Michelin-starred restaurants, with elaborated recipes, and to be appreciated by great chefs of Spanish gastronomy.

13.6 Conclusions and Prospects

The cultivation of *Terfezia* is very new if we compare it with other cultivated mushrooms or plants. However, in the 20 year history of this crop, much progress has been made on the knowledge of its genome, its mycorrhizal symbiosis and biotechnological techniques to improve it, as well as agronomic tools for field cultivation and increase the production of desert truffles. It is a totally ecological crop, which does not require fertilizers or phytosanitary products, with low water requirements and which avoids the desertification of the arid regions where it is grown. The prospects and future of this crop are focused on extending it to other species of desert truffles, sequencing more genomes of desert truffle species, as well as preserving and enhancing the areas where they occur naturally through mycosilviculture. Results obtained so far will help us to select fungal strains and host plants well adapted to drought in order to adapt this novel crop to climate change.

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Appendix I: Research Institutes Relevant to Desert Truffles

Institution name	Address/Country	Contact information and website
University of Murcia	Department of Plant Biology (Botany), Faculty of Biology, Campus of Espinardo, Murcia 30,100, Spain	E-mail: amorte@um.es Website: https://www.um.es/web/biologia-vegetal/contenido/pdi https://www.researchgate.net/profile/Asuncion_Morte https://orcid.org/0000-0002-6426-0202 https://scholar.google.es/citations?user=8Q7fe9cAAAAJ&hl=es&oi=ao
The Jacob Blaustein Institute for Desert Research	Ben-Gurion University of the Negev, Beer-Sheva 84,105, Israel	E-mail: sitrit@bgu.ac.il Website: https://in.bgu.ac.il/en/bidr/FAAB/Pages/sitrit.aspx https://www.researchgate.net/profile/Yaron_Sitrit
University of Oran	Department of Biotechnology, Oran, Algeria	E-mail: torfez2000@yahoo.fr Website: https://www.univ-oran1.dz/ https://www.researchgate.net/profile/Zohra_Fortas
Mohammed V-Agdal university	Department of Biology, Faculty of Sciences, Rabat, Morocco	E-mail: l.khabar@yahoo.fr Website: http://www.um5.ac.ma/um5/ https://scholar.google.es/citations?user=globZs0AAAAJ&hl=es&oi=ao

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Chapter 14
Enhancing White Truffle (*Tuber magnatum* Picco and *T. borchii* Vittad.) Cultivation Through Biotechnology Innovation

Alessandra Zambonelli, Mirco Iotti, Federico Puliga, and Ian R. Hall

Abstract *Tuber magnatum* Picco, the Italian white truffle, and *T. borchii* Vittad., the bianchetto truffle, are two European white truffles that command high prices because of their excellent gastronomic properties and unique aromas. Indeed, *T. magnatum* is one of the most expensive culinary delicacies. Although cultivation of *T. borchii* began nearly 200 years after the Périgord black truffle (*Tuber melanosporum* Vittad.), it has rapidly met with market approval and is now cultivated not only in Europe but in countries outside of Europe where this truffle does not grow naturally. In contrast, reliable methods for cultivating *T. magnatum* have only just been developed which hopefully will quickly lead to its widespread cultivation. In this chapter, we present an overview of the distribution, economic importance, a taxonomic appraisal, genetic resource characterization and conservation, and review current cultivation practices. We also review recent biotechnology developments and their potential application for cultivation and domestication of *T. magnatum*. The future prospects of genetic engineering for improving truffle strain characteristics are also addressed.

Keywords Bianchetto truffle · Biology · Cultivation · Italian white truffle · Mycelial cultures · Strain genetic selection

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14.1 Introduction

A hypogenous life cycle is common in nearly every major group of fleshy fungi within the phyla Ascomycota, Basidiomycota and Mucoromycotina (Tedersoo et al. 2010). Underground fruiting structures are formed in most of the Ascomycetes families (Trappe et al. 2009) but *true truffles* are considered to be only those species in the genus *Tuber*, family Tuberaceae, order Pezizales (Zambonelli et al. 2016). The origin of the genus *Tuber* is estimated to be in the early Cretaceous, around 142 Mya (Bonito et al. 2010; Jeandroz et al. 2008) evolving from an epigeous ancestor (Bonito et al. 2010). Spores are sequestered inside the fruiting bodies and generally are dispersed by mycophagous animals that are attracted by a wide range of powerful aromas (Zambonelli et al. 2017). Most truffles live in ectomycorrhizal (ECM) relationships with shrubs and trees in temperate forests mostly in the Northern Hemisphere although the desert truffles inhabit semi deserts of the northern Mediterranean and Africa (Bonito et al. 2013).

The genus *Tuber* includes around 200 species (Bonito et al. 2010) but only a handful have economic value, with the European species the most valuable, such as the white truffles *T. magnatum* Picco (Italian white truffle) and *T. borchii* Vittad. (bianchetto truffle) and the black truffles *T. melanosporum* Vittad. (Perigord black truffle) and *T. aestivum* Vittad. (summer truffle). Other edible black truffles that are locally commercialized in Europe are *T. brumale* Vittad. (winter truffle), *T. mesentericum* Vittad. (truffe mèsentèrique) and *T. macrosporum* Vittad. (smooth black truffle) (Hall et al. 2007). Several other *Tuber* species from China, such us the Chinese black truffles, *T. indicum* Cooke and Massee, *T. sinoaestivum* J.P. Zhang and P.G. Liu, the Chinese white truffle *T. panzhihuanense* X.J. Deng and Y. Wang (Deng et al. 2013; Wang 2012; Zhang et al. 2012) and white North American truffles such as *T. oregonense* Trappe, Bonito and P. Rawl., *T. gibbosum* Harkn. and *T. lyonii* Butters (Lefevre 2012) are locally consumed or exported to Europe where they are often passed-off as their more expensive European cousins.

In this chapter, the new biotechnological approaches for improving cultivation techniques and for germplasm conservation of the European white truffles *Tuber magnatum* and *T. borchii* are detailed and their very different lifestyles contrasted.

14.2 *Tuber magnatum* and *T. borchii* Characteristics

14.2.1 Morphology

Truffles present different morphological features during the three different phases of their life cycles: vegetative as free-living mycelium, symbiotic as ectomycorrhizas, and reproductive ascomata (the fruiting bodies).

The hyphae of all *Tuber* spp. are 3–8 μm in diameter, septate, hyaline and simply branched, showing frequent anastomoses, rare coils and vesicles (Fig. 14.1a) (Iotti

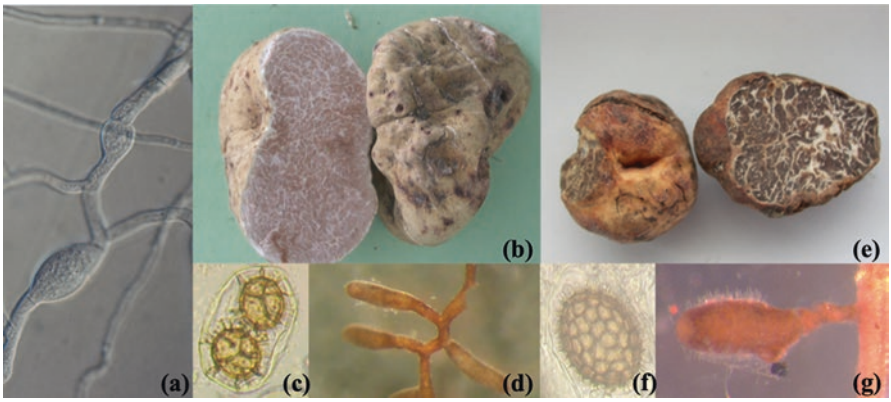

Fig. 14.1 Morphological features of the *Tuber magnatum* and *T. borchii*: (**a**) *T. borchii* mycelium, (**b**) *T. magnatum* ascoma, (**c**) *T. magnatum* spores, (**d**) *T. magnatum* mycorrhizas, (**e**) *T. borchii* ascomata, (**f**) *T. borchii* spore, (**g**) *T. borchii* mycorrhiza. (Figure constructed by A. Zambonelli)

et al. 2002). In *T. borchii* vesicles are more numerous, assuming the characteristics of chlamydospores in unfavorable conditions such as in the presence of adverse bacteria (Barbieri et al. 2005), when cultures age (Iotti et al. 2002) or when iron is deficient (Picceri et al. 2018). The mycelial colonies are regular, whitish in color with most of the biomass growing into the agar or completely submerged when in liquid culture. In contrast, the fruiting bodies and the mycorrhizas differ greatly and are characteristic for each species.

Despite the often very dark to black patches on the surfaces of *Tuber borchii* and *T. magnatum* both are regarded as white truffles because both have ascomata with a smooth peridium and are generally straw-colored. In contrast, the black truffles have a brown to black peridium ornamented by warts, for example, *T. melanosporum, T. aestivum, T. mesentericum* and *T. brumale*. The peridium of *T. magnatum* is smooth, olive-yellow in color; the gleba at maturity is ocher to pale brown marbled with numerous white thin sterile veins (Fig. 14.1b). Occasionally, *T. magnatum* ascomata show pink to red patches on the surface and within the gleba, probably due to bacterial infection by *Microbacterium* and/or *Chryseobacterium* (Amicucci et al. 2018). However, these do not appear to affect the aromatic characteristics of the ascomata. The spores are subglobose to broadly ellipsoid, light-yellow, yellow-ocher at maturity, ornamented with an irregular reticulum (2–) 3–5 (−7) μm high, with 2–4 meshes across the width of the spores. (Fig. 14.1c). In contrast, the peridium of *T. borchii* is minutely pubescent caused by a layer of yellow reddish-brown colored hyphae with setose hairs and the gleba at maturity is dark brown with wide, white veins (Fig. 14.1e). The spores are ellipsoid to broadly ellipsoid, ocher-brown at maturity, ornamented with a regular reticulum with meshes 3–7 (−10) μm high, 3–10 across the width of spores (Fig. 14.1f) (Zambonelli et al. 2000a).

The mycorrhizas of both species are macroscopically similar, simple or ramified with whitish-ocher, fulvous colors, in *Tuber borchii,* and ocher to grayish-white in *T. magnatum* (Mello et al. 2001; Zambonelli et al. 1993). Microscopically the

mantles are similar, composed of epidermoid cells ornamented by awl-shaped hyaline cystidia. These are never ramified in *Tuber borchii* ectomycorrhizas and 70–140 µm long (Fig. 14.1g), whereas they are often branched in *T. magnatum* and up 480 µm long (exceptionally 580 µm) (Giomaro et al. 2000; Mello et al. 2001; Riccioni et al. 2016) (Fig. 14.1d).

14.2.2 Distribution

Until the turn of the millennium *Tuber magnatum* was considered an almost exclusive Italian truffle restricted to the north and center of the country with smaller patches, in Istria, Croatia southeastern France and the Ticino Canton of Switzerland (Hall et al. 1998). However, recent studies have found it in Sicily (Vasquez et al. 2014), the Geneva Canton of Switzerland (Büntgen et al. 2019), Hungary and several Balkan regions (Bratek et al. 2007; Riccioni et al. 2016). Intriguingly, is has also been found in Thailand (Suwannarach et al. 2017). In contrast, *T. borchii* has one of the widest distributions of any truffle being found throughout Europe, from southern Finland to Sicily and from Ireland to Hungary and Poland (Hall et al. 2007; Shamekh et al. 2009) (Fig. 14.2).

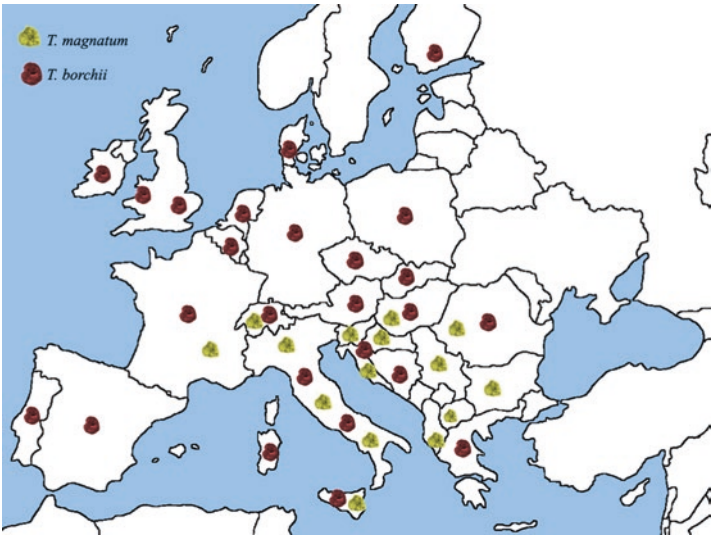

Fig. 14.2 Geographical distribution of *Tuber magnatum* (green symbols) and *T. borchii* (brown symbols). (Sources: Gogan-Csorbai et al. 2018; Hall et al. 2007)

14.2.3 Economic Importance

The high economic value of truffles is due to their unique aromas and flavors, which make them one of the most expensive food delicacies. *Tuber magnatum* is the truffle that commands the highest prices although this varies depending on the size of the ascoma and from season to season. In poor harvest years such as 2017, the retail prices in Italy were very high ranging between 3500 EUR/kg and 5000 EUR/kg. The production in 2018 was greater and prices fell to 1000–2100 EUR/kg (https://www.termometropolitico.it/1332538_2019-05-02-prezzo-tartufo-bianco.html). Outside Italy even higher prices can be paid, for example, *T. magnatum* was sold in Harrods, London, for GBP 6500/kg on 25 September 2010 (Badalyan and Zambonelli 2019; Hall, personal communication). *Tuber borchii* commands lower prices than *T. magnatum* and *T. melanosporum*, although recently it has become more appreciated and prices have risen somewhat. In 2019 retail prices in Italy were 105–305 EUR/kg depending on the size (https://www.andareatartufi.com/wordpress/quotazioni-tartufo-bianchetto/rom). In contrast, the off-season price of *T. borchii* produced in New Zealand that are not mixed with inferior species of white truffle can fetch much higher prices that hover around NZD 3000/kg (about 1700 EUR/kg (http://www.trufflesandmushrooms.co.nz/Tuber%20borchii%20web.pdf). Unlike black truffles, white truffles are used either uncooked or added to dishes just after cooking in order to retain the delicate, volatile aromas such as when flavoring pasta or salads.

14.2.4 Ecology

Tuber magnatum has specific ecological requirements, which limit its distribution in Europe. It is generally found below 600 m elevation, but in Southern and Central Italy it can be found up to 900 m elevation. It develops in woods with more or less closed canopies, as well as areas with relatively sparse vegetation. It often fruits in floodplains or around stream beds probably because its mycelium displays a high demand for water for growth in soil (Iotti et al. 2018; Marjanović et al. 2015). The host plants are: *Alnus cordata* Desf. (Italian alder), *Corylus avellana* L. (hazelnut), *Ostrya carpinifolia* Scop., *Populus alba* L. (white poplar), *P. tremula* L. (European trembling aspen), *P. nigra* L. (Lombardy poplar), *Quercus cerris* L. (Turkish oak), *Q. ilex* L. (holm oak), *Q. pubescens* Willd. (downy oak), *Q. robur* L. (English or common oak), *Salix alba* L. (white willow), *S. caprea* L. (pussy willow), *Tilia cordata* Mill. (small-leaved lime), *T. platyphyllos* Scop. (large-leaved lime) (Hall et al. 2007) and *Fagus sylvatica* L. (Büntgen et al. 2019). It is also, though rarely, found under *Pinus* spp. (Zambonelli, personal communication) but the ectomycorrhizal association with conifers has never been verified with molecular tools. *Tuber magnatum* requires well-drained soils without gravels, having a preference for neutral to extremely alkaline soils (pH 7–8.5), with abundant active $CaCO_3$ (Hall et al. 1998).

The peculiar characteristics of *T. magnatum* soils are good aeration and very soft consistency due to its elevated volume (around 15%) occupied by interconnected macropores (Bragato and Marjanović 2016). The climatic requirements include a winter temperature range of 2–8 °C (in January) and summer temperatures of 18–26 °C (in June). Ideal precipitation ranges between 500–2000 mm and spread more or less evenly throughout the year, although in summer this tends to be in the form of thunderstorms (Hall et al. 2007). The ascomata are sensitive to winter frost and summer drought which helps explain their preponderance in riparian habitats (Iotti et al. 2018; Le Tacon 2016).

Tuber borchii has broad ecological requirements and is found from sea level to 1100 m elevation (Tanfulli et al. 1999). It forms ectomycorrhizal associations with a wide range of host plants including several species of conifers including pines and cedars (Table 14.1). It is most commonly found on *Pinus* spp. particularly *P. pinea* and *P. pinaster* in the coastal areas of the Mediterranean Sea. Recently it was found to form mycorrhizas with commercially interesting fruit plants such as the strawberry tree (*Arbutus unedo*, Lancellotti et al. 2014) and pecan (*Carya illinoinensis,* Benucci et al. 2012). Surprisingly, it can also form mycorrhizas with orchids (Tešitelová et al. 2012) confirming its capacity to form symbiotic associations with herbaceous plants, as predicted by Mannozzi-Torini (1988).

Tuber borchii grows in moderate to strongly calcareous, very sandy to silty soils (Fig. 14.3) such as those in coastal areas of Italy, as well as in the same alkaline soils where the Italian white truffles are harvested in the Apennines. In Italy it is also found in neutral and slightly acidic soils where the pH ranges from 6–7. Occasionally the pH can be as low as 5.2 (Gardin 2005) but such soils are more likely to be the home of inferior white truffles such as *Tuber maculatum* Vittad. (Hall, personal communication). The climate of areas where bianchetto is found in Italy and New Zealand ranges from cool temperate to Mediterranean, with annual rainfall of 600–1600 mm.

14.2.5 Aroma Characteristics

The aroma of truffles is due to the volatile organic compounds (VOCs) which are produced during ascoma formation and maturation. Aroma production is a biological strategy used by truffles to attract mycophagous animals which disseminate their spores (Zambonelli et al. 2017). Although around 300 VOCs have been reported in truffles (Splivallo et al. 2011) only 10–20 odorants per species are responsible for the typical truffle odors perceived by the human nose (Schmidberger and Schieberle 2017). Some of these are common to several truffle species particularly 2- and 3-methylbutanal, 2- and 3-methylbutan-1-ol and oct-1-en-3-ol. Others are species-specific such as 2,4-dithiapentane in *Tuber magnatum* and thiophene derivatives in *T. borchii* (Fiecchi et al. 1967; Gioacchini et al. 2005; Splivallo and Ebeler 2015; Splivallo et al. 2011).

Table 14.1 Putative host plants of the white truffles

Family	Species	*Tuber borchii*	*T. magnatum*
Betulaceae	*Alnus cordata*	*	*
	Corylus avellana	*	*
	Ostrya carpinifolia	*	*
Fagaceae	*Fagus sylvatica*	*	*
	Quercus cerris	*	*
	Q. ilex	*	*
	Q. petraea	*	*
	Q. pubescens	*	*
	Q. robur	*	*
Tiliaceae	*Tilia americana*	*	
	T. cordata	*	*
	T. × *europaea*	*	*
	T. platyphyllos	*	*
Juglandaceae	*Carya illinoinensis*	*	
Pinaceae	*Abies alba*		*
	Cedrus atlantica	*	*
	C. deodara		
	Larix spp.	*	
	Picea excelsa	*	
	Pinus brutia	*	
	P. nigra ssp. *nigra*, *P. nigra* ssp. *nigricans*	*	
	P. pinaster var. *atlantica*	*	
	P. pinea	*	*
	P. strobus	*	
	P. sylvestris	*	
Salicaceae	*Populus alba*	*	*
	P. nigra	*	*
	P. tremula		*
	Salix alba	*	*
	S. caprea	*	*
Ericaceae	*Arbutus unedo*	*	
Cistaceae	*Cistus albidus*	*	
	C. incanus		
	C. monspeliensis		
	C. salviaefolius and other *Cistus* spp.		
Orchideaceae	*Epipactis* spp.	*	

Sources: Benucci et al. (2012), Büntgen et al. (2019), Hall et al. (2007), Lancellotti et al. (2014) and Tešitelová et al. (2012)

Truffle products aromatized using synthetic scents are quite different and generally less appealing than fresh ascomata, primarily because they contain a markedly higher quantity of 2,4-dithiapentane and two VOCs, dimethyl sulfoxide and dimethyl sulfone, which are absent from ascomata (Wernig et al. 2018).

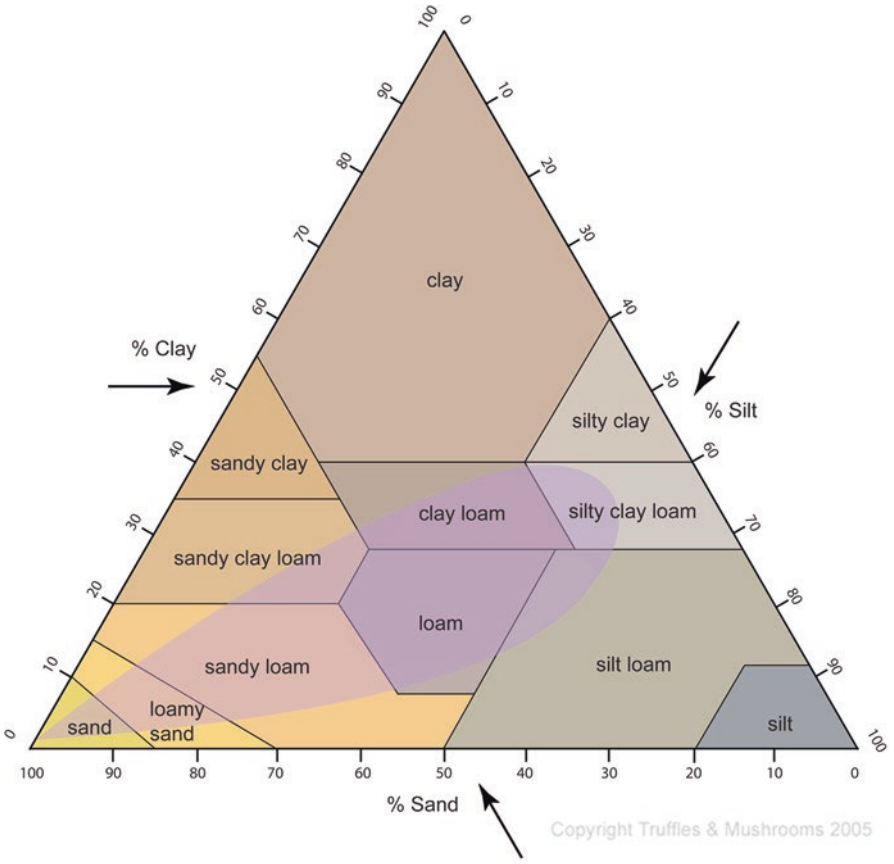

Fig. 14.3 Soil textural triangle for the bianchetto truffle. The area marked in violet is suitable for this truffle. The arrows indicate the direction the reader needs to read the graph. (Source: Hall et al. 2007)

Not only does genotype affect aroma (Splivallo et al. 2012) but also associated bacteria, yeasts and other fungi that also produce VOCs and contribute to truffle aromas (Buzzini et al. 2005; Pacioni and Leonardi 2016; Splivallo and Ebeler 2015; Splivallo et al. 2014; Vahdatzadeh et al. 2015). Truffle aroma is also influenced by other environmental factors such as soil composition, climate and the host plant (Splivallo and Culleré 2016). It is therefore not surprising that as ascoma maturity, methods for storing, changes in microbiome composition, season and geographical location, all affect VOCs profiles and perceived quality (Gioacchini et al. 2008; Pennazza et al. 2013; Vahdatzadeh et al. 2019; Vita et al. 2015; Zeppa et al. 2004). Mycelial fermentation techniques have been used to produce natural truffle aromas but these were found to be less complex (Splivallo et al. 2007; Tirillini et al. 2000). For example, 3-methyl-4,5-(2 H)thiophene is produced only during the sexual stage of *Tuber borchii* fruiting body formation. However, the VOCs composition of *Tuber*

mycelium can be improved by supplying axenic cultures of truffle mycelium with leucine, isoleucine, phenylalanine and methionine inducing the synthesis of the VOCs derived from amino acid catabolism (Ehrlich pathway) (Li et al. 2012; Liu et al. 2013; Splivallo and Maier 2016; Vahdatzadeh and Splivallo 2018; Xiao et al. 2015). It is hoped that strain selection may be used to improve truffle mycelium VOC production (Vahdatzadeh and Splivallo 2018) as well as repeated freeze-thaw cycles (Xiao et al. 2015).

This capacity of *Tuber* mycelium to produce VOCs may be commercially exploitable and lead to the production of natural flavor with a higher consumer acceptance of truffle-flavored food products (i.e. truffle-flavored olive oil) that currently predominantly contain synthetic flavors (Splivallo and Maier 2016).

14.3 Cultivation

14.3.1 Traditional Cultivation Practices

The first techniques for cultivating truffles were developed for *Tuber melanosporum*, possibly independently by Josef Talon and Pierre Mauléon in the nineteenth century (Hall and Zambonelli 2012). They simply sowed acorns collected under oaks that were producing truffles and then transplanted these into adjacent soils. Only in the 1970s was modern truffle cultivation introduced into France and Italy after the discovery of the mycorrhizal nature of truffles. This consists of producing mycorrhizal plants in the greenhouse and then out planting them into areas with suitable soils and climate. Using these techniques, *T. aestivum*, *T. borchii*, and *T. brumale* are now cultivated as well as *T. melanosporum,* although the latter is still the most extensively cultivated, not only in Europe but in countries outside of Europe (Hall et al. 2017; Reyna and Garcia-Barreda 2014). In recent years *T. borchii* cultivation has become more widespread in Italy (Zambonelli et al. 2015), Spain, Portugal (https://micofora.com/en/growing-tuber-borchii/) and in France. *Tuber borchii* is also successfully cultivated in New Zealand, Australia the USA (Zambonelli et al. 2015) and China (Wang Yun, personal communication).

The production of *Tuber* mycorrhizal plants represents the first important step in modern truffle cultivation. The plants are preferably colonized with a single *Tuber* species of interest and are preferably free of other contaminating ectomycorrhizal fungi. Generally, the quantification of mycorrhizal formation is assessed morphologically. Under a stereomicroscope the percentage of root tips colonized by *Tuber* spp. is counted or estimated (Andrés-Alpuente et al. 2014) although the use of this method requires trained personnel (Sisti et al. 2010).

Although, the link between the level of mycorrhizal formation on inoculated seedlings prior to planting has not been conclusively demonstrated, planting seedlings with high degrees of truffle mycorrhizal colonization is recommended to improve the chance of truffle production in plantations (Murat 2015). For this

reason in Italy and France *Tuber* mycorrhizal plants can be commercialized only if the degree of mycorrhization (number of root tips colonized with *Tuber*/total number of tips ×100) is above 30% (Donnini et al. 2014; Govi et al. 1997; Regione Emilia-Romagna 2018). *Tuber borchii* commercialized plants usually exceed this level of mycorrhizal infection. In contrast, the mycorrhizas of *T. magnatum* can be difficult to obtain and only a company in France is actually selling *T. magnatum* mycorrhizal plants at prices 5–10 times higher than *T. borchii* or black truffle mycorrhizal plants. Morphological and molecular methods have now been perfected in order to identify and quantify mycorrhizal formation on root systems (Alvarado and Manjon 2013; Andrés-Alpuente et al. 2014; Fischer and Colinas 1996; Mello et al. 2006; Rocchi et al. 1999; Zambonelli et al. 1993, 2012a). In contrast, to ectomycorrhizas of black truffles (*T. aestivum*, *T. brumale* and *T. melanosporum*) (Zambonelli et al. 1993), the morphological characters of all white truffles are similar, and in particular the mycorrhizas of *T. maculatum*, *T. oligospermum*, *T. dryophilum* and *T. borchii* (Boutahir et al. 2013; Zambonelli et al. 1999) which require the use of molecular techniques to be sure. Species specific primers for *T. borchii* and *T. magnatum* were designed to be used in simple, multiplex or quantitative PCR (Amicucci et al. 1998, 2000; Iotti et al. 2012a; Mello et al. 1999; Rubini et al. 2001) (Table 14.2). Spore inoculation is used by the vast majority of companies that produce *Tuber* mycorrhizal plants because it is simple and effective. First, fresh, dry or frozen truffles are ground in water to create a spore suspension which is then used to inoculate sterile seedling or cuttings a few months old. However, with spore inoculation techniques, plants can become contaminated with undesirable truffle species if the truffles are not carefully selected and identified. Moreover, pests and pathogens can be introduced with the inoculum. Furthermore, because ascospores are produced sexually, each plant is potentially colonized by a different suite of fungal genotypes from every other with unknown characteristics.

In order to achieve ascoma production the infected plants have to be planted in the field in environmental conditions (soil, climate) that suit both the host plant and the truffle. *Tuber borchii* tends to fruit more quickly than *T. melanosporum.* In experimental *Pinus pinea* plantations the first ascomata were found only 3–4 years after planting (Hall, personal communication; Zambonelli et al. 2000b). Although, *T. borchii* cultivation is becoming more common, no specific research on how to manage *T. borchii* cultivation has been carried out and no handbook on *T. borchii* cultivation is available in the market, although a booklet is available from Ian Hall (2017) that reviews what are thought to be the ideal soil and climatic conditions. However, farmers tend to copy guidelines for *T. melanosporum* cultivation which in low pH soils can result in excessive applications of lime and depressed soil trace element concentrations.

Some success has recently been reported by Bach et al. (2021) in the production of *T. magnatum* in France and it is to be hoped that this will lead to its routine cultivation rather than the erratic positive results obtained in Italian experiments of the 1990s and 2000s (Bencivenga et al. 2009).

Table 14.2 Molecular markers used for genetic characterization in *Tuber borchii* and *T. magnatum*

Tuber species	Marker type	Sequence (5′–3′)/repeat motif [1]	Primer name/ locus name[1]	Reference
Tuber borchii	ITS-rDNA	TGTATGGGATGCCCTATCGGACT	TboI (fwd)	Amicucci et al. (1998)
		CTATTACCACGGTCAACTTC	TboII (rev)	
	ITS-rDNA	TGCCCTATCGGACTCCCAAG	TBA (fwd)	Mello et al. (1999)
		GCTCAGAACATGACTTGGAG	TBB (rev)	
	ITS-rDNA	GAAGTTGACCGTGGTAATAG	rTboII (fwd)	Amicucci et al. (2000)
		TCCTCCGCTTATTGATATGC	ITS4 (rev)	
	SSR	$(TATTTT)_{10}$	Tb1	Leonardi et al. (2019)
		$(AGGC)_8$	Tb11	
		$(AAC)_8$	Tb151	
		$(GGA)_{12}$	Tb155	
		$(GAG)_8$	Tb156	
		$(TTTAGA)_5$	Tb17	
		$(CCTT)_8$	Tb206	
		$(GAGGGA)_6$	Tb244	
		$(AGAAGG)_5$	Tb293	
		$(CTTTT)_5$	Tb43	
		$(TACC)_8$	Tb43bis	
		$(AGA)_9$	Tb46	
		$(AAAG)_8$	Tb704	
		$(GACT)_8$	Tb83	
Tuber magnatum	ITS-rDNA	GGATGCGTCTCCGAATCCTGAAT	Tmag1[a] (fwd)	Amicucci et al. (1998)
		CGGGCCCTTTCTCAGACTGCTG	Tmag2 (rev)	
	ITS-rDNA	TCCTACCAGCAGTCTGAGAAAGGGC	P7 (fwd)	Mello et al. (1999)
		TGAGGTCTACCCAGTTGGGCAGTGG	M3[b] (rev)	
	ITS-rDNA	TCCTCCGCTTATTGATATGC	ITS4 (rev)	Amicucci et al. (2000)
	ITS-rDNA	GTCACTGAAAACCCACTCACG	TSMAGN (fwd)	Rubini et al. (2001)
		TGAGGTCAACCCAGTTGGACAGT	ITSBACK3 (rev)	
	β-tubulin	CCTCCCAATTTGCAATACAC	tubmagnf (fwd)	Zampieri et al. (2009)
		AAAGACGAAGTTATCTGGCCTGA	elytubr (rev)	
	ITS-rDNA	GCGTCTCCGAATCCTGAATA	TmgITS1for (fwd)	Iotti et al. (2012a)
		ACAGTAGTTTTTGGGACTGTGC	TmgITS1rev (rev)	
		TGTACCATGCCATGTTGCTT	TmgITS1prob	
	SSR	$(AC)_{20}(TC)_{18}$	MA2	Rubini et al. (2004)
		$(GA)_{17}$	MA4	
		$(AC)_{18}(TC)_{10}$	MA5	
		$(GT)_{16}$	MA7	
		$(CT)_{16}(T)_{14}$	MA12	
		$(TC)_{16}/(TC)_7$	MA13	
		$(GA)_{30}$	MA14	
		$(TG)_{15}$	MA19	

(continued)

Table 14.2 (continued)

Sources: Parladé et al. (2016) with modifications; Zambonelli et al. (2012a)
[1]Sequence and primer name are referred to internal transcribed spacer (ITS) of nuclear ribosomal DNA (rDNA), i.e. ITS-rDNA. β-tubulin markers, repeat motif and locus name for short sequence repeat (SSR) markers
[a]In multiplex PCR with ITS4 as reverse primer
[b]In multiplex PCR with ITS1 as forward primer

14.3.2 New Biotechnologies for Cultivating Tuber borchii

Tuber borchii is considered the model species for genetic and biological studies on truffles because its mycelium can be cultured in vitro more easily than other species of truffle. In contrast, difficulties in making pure cultures of *T. magnatum* have always hindered insights into its biology.

The first mycorrhizal plants with *Tuber borchii* mycelium were produced in greenhouses on *Pinus strobus* and poplar by the Italian researchers Fontana and Palenzona (1969). Later a technique to produce *Tuber* mycorrhizal plants under in vitro conditions on *Populus alba* micropropagated plantlets was perfected by Zambonelli et al. (1989), and later on with *Tilia platyphyllos* (Sisti et al. 1998). The method involves raising micropropagated plantlets in a medium with a low concentration of auxin (0.2 mg/l of NAA) to allow the differentiation of roots with multiple secondary roots with limited growth. The mycelium of *T. borchii* is then isolated onto PDA agar in Petri dishes and then bulked up in MMN liquid medium. The mycorrhizal synthesis is performed in vermiculite moistened with MS/2 liquid medium at pH 6.3 (Giomaro et al. 2005). Subsequently, *Tilia platyphyllos-Tuber borchii* mycorrhizal plants became a model to study plant/fungus interactions (Menotta et al. 2004; Polidori et al. 2002, 2007; Zeppa et al. 2002).

Tuber borchii mycorrhizal plants inoculated with mycelia have never been produced commercially for a variety of reasons. Spore inoculation is easier and gives good results with *T. borchii*. Moreover, nurseries producing *Tuber* mycorrhizal plants are usually not equipped with laboratories for isolating and cultivating *Tuber* mycelium in vitro. Although *T. borchii* mycelium generally grows more quickly than other *Tuber* spp. (Iotti et al. 2002) it is still more difficult to manipulate compared with other mycorrhizal mushrooms. It was not until a few years ago that there was evidence that plants inoculated with mycelial-pure cultures could produce truffles. In fact, Paolocci et al. (2006) hypothesized, and later confirmed by Martin et al. (2010), that when the *T. melanosporum* genome was sequenced and the mating type idiomorphs characterized, that truffles were heterothallic and that two strains carrying different mating types would be needed to mate in order to produce the fruiting bodies (de la Varga et al. 2017; Rubini et al. 2014).

Iotti et al. (2016) reported that the first experimental *Tuber borchii* truffle orchard inoculated with 5 different *T. borchii* strains individually or mixed had started to produce and 99 ascomata were eventually collected between February and April with a total fresh weight of 722 g. This demonstrated for the first time that it was possible to produce truffles with mycorrhizal plants inoculated with pure cultures.

Subsequently, a study conducted on this truffière by Leonardi et al. (2019) showed that the paternal genotypes originated by recombination of the inoculated strains and only in a few cases from alien genotypes. Furthermore, 4 of the 5 inoculated strains persisted 9 years after establishing the plantation and remained confined to their inoculated plants without migrating into the neighboring strain areas.

The isolation and genetic characterization of different strains from geographical regions with varying soil and climatic conditions could lead to strains that produce ascomata with enhanced organoleptic characteristics and suited to particular environmental conditions. Recent work conducted by Vahdatzadeh and Splivallo (2018) on the mycelium of nine *Tuber borchii* strains has highlighted how genetically-different strains produce different aromas. Other work conducted by Leonardi et al. (2017) has shown how *T. borchii* strains differ in their tolerance to heat stress, demonstrating that it should be possible, in this regard at least, to select the most suitable strains for a specific cultivation area.

14.4 Genetic Diversity and Germplasm Conservation

14.4.1 Genetic Diversity

A proper understanding of genetic variation within and among *Tuber* species and populations, as well as their phylogeography, is indispensable for efficient utilization of a valuable truffle.

The first studies on the genetic diversity of *Tuber* species involved the analysis of random amplified polymorphic DNA (RAPD) and sequence characterized amplified region (SCAR) markers (Amicucci et al. 1997; Bertini et al. 1998; Gandeboeuf et al. 1997a, b; Lanfranco et al. 1993; Potenza et al. 1994; Rossi et al. 2000). However, ITS regions of nuclear rDNA, alone or in combination with other loci, quickly become the elective markers to explore the genetic diversity within *Tuber* species and to infer their phylogenetic relationships (Amicucci et al. 1996; Bonito et al. 2010; Bonuso et al. 2010; Guillemaud et al. 1996; Halász et al. 2005; Henrion et al. 1994; Huang et al. 2009; Iotti et al. 2007; Mello et al. 1996; Merényi et al. 2014; Murat et al. 2004, 2005; Paolocci et al. 1995, 2004; Qiao et al. 2018; Riccioni et al. 2019; Sica et al. 2007; Wang et al. 2006; Wedén et al. 2005). Microsatellite markers were first investigated for truffle typing (Amicucci et al. 2002; Longato and Bonfante 1997) but they were found more useful for studying population genetics (Bertault et al. 2001; Figliuolo et al. 2013; Leonardi et al. 2019; Linde and Selmes 2012; Molinier et al. 2015, 2016a, b; Murat et al. 2013; Riccioni et al. 2008; Rubini et al. 2005; Taschen et al. 2016).

The first studies of *Tuber magnatum* and *T. borchii* have surprisingly deduced that the two species fall into two distinct clades of the genus *Tuber* (Percudani et al. 1999). *Tuber magnatum* is co-located inside the Aestivum clade which includes species that are morphologically quite different: *T. aestivum, T. mesentericum* and

T. sinoaestivum, three species of black truffle characterized by a warty peridium and reticulate ascospores, and *T. panniferum* which is a unique truffle with a wooly peridium and very spiny ascospores (Bonito and Smith 2016; Bonito et al. 2013; Jeandroz et al. 2008). In contrast, *T. borchii* is co-located inside the Puberulum clade, which includes at least 63 morphologically-similar species distributed across Europe, Asia, North America, South America and North Africa (Bonito et al. 2013; Lancellotti et al. 2016).

Several studies on *Tuber magnatum* genetic intraspecific variability have been carried out to find molecular markers that may define its geographic origin. The first studies, carried out by RAPD (Amicucci et al. 1997; Gandeboeuf et al. 1997a; Lanfranco et al. 1993; Pomarico et al. 2007; Potenza et al. 1994) showed a very low intraspecific polymorphism in *T. magnatum* with respect to other *Tuber* spp. This low genetic diversity in *T. magnatum* was later confirmed by several researchers (Iotti et al. 2012a; Mello et al. 2005; Pomarico et al. 2007) who analyzed ITS regions and other genetic markers. Only simple sequence repeat loci (SSR) proved to be suitable as markers for population genetic studies of *T. magnatum*. Rubini et al. (2004) first selected 8 polymorphic SSRs and used them to analyze 370 ascomata from 5 main areas. They defined 28 populations, one of which was from the Istria region of Croatia and 4, 12, 10 and 1 from the north, center, center-south and south of Italy, respectively.

Recently a new transcriptome assembly of the Italian white truffle has allowed the identification of 2581 gene-based SSR markers (Vita et al. 2018). In addition, the *T. magnatum* genome has now been sequenced (Murat et al. 2018a) and a bioinformatics workflow was applied in order to mine for microsatellite sequences. As a result, 11,189 microsatellite markers specific to *T. magnatum* were found with 3377 marker loci matching with *T. magnatum* proteins (Uncu and Uncu 2019). This will provide some important new tools to explore *T. magnatum* genetic diversity and to find genetic markers that might define the genetic origin of the ascoma.

After the first studies on the genetic diversity of *Tuber borchii* using RAPD (Bertini et al. 1998; Gandeboeuf et al. 1997a) and IGS (Ciarmela et al. 2002), a multigene phylogeny was carried out on 61 representative specimens with a broad distribution throughout Italy (Bonuso et al. 2010). In this study two cryptic species of *T. borchii* were identified.

Recently the *Tuber borchii* genome has also been sequenced (Murat et al. 2018b) and showed 1111 SSRs. Among them 14 SSRs were highly polymorphic and were able to differentiate single genotypes after testing on 50 ascomata harvested in Italy and Hungary (Leonardi et al. 2019).

14.4.2 Genetic Resources Conservation Approaches

The conservation of truffle genetic resources is becoming more important than ever because of the increasing threat from deforestation, overharvesting, climate warming and changing agricultural practices.

Truffle cultivation could be an efficient method to ex situ conserve *Tuber* genetic resources (Varese et al. 2011). To achieve this goal, it would be important to use only spore inoculation methods from locally-harvested truffles in order to secure their genetic diversity. Although truffles growing in natural areas are assumed to be genetically adapted to the site's climatic and edaphic conditions, it is also possible that they represent the vestiges of a distribution when conditions may have been quite different. Regardless, the creation of a germplasm bank of *Tuber* spp. is an imperative in the future once mycelial inoculation becomes the method of choice for nurseries producing *Tuber* mycorrhizal plants for cultivation.

14.4.2.1 In Vitro Conservation of *Tuber* spp. Genetic Resources

In the conservation of mycelial pure cultures in vitro it is very important to ensure that their purity, vitality and genetic integrity is maintained over time (Smith 2004; Voyron et al. 2007). Currently, many conservation methods are available (Nakasone et al. 2004) and the choice of the most appropriate method should depend on the species under consideration and on the purpose of conservation.

The short-term conservation of *Tuber* mycelial subcultures, obtained by taking small plugs of mycelium from the edge of a colony, is easiest and inexpensive, but the risk of nutrient medium contamination is high. Furthermore, *Tuber* mycelium is known to grow very slowly in pure culture (Iotti et al. 2002, 2012b) and repeated transfers may lead to the loss of infectivity, viability and induce mutation or changes in gene expression (Coughlan and Piché 2005; Smith 2012).

Another preservation technique is cryopreservation in liquid nitrogen, which consists of preserving the biological material at ultra-low temperature after the addition of several cryoprotectants. In this process, the material is gradually cooled down to a temperature close to −196 °C, and then stored at that temperature. In this condition all biological activity, including biochemical reactions that would lead to cell death, are blocked and prevents any cellular and genetic damage (Smith et al. 2001). Moreover, cryopreservation reduces work load and saves space needed for preservation on agar, ensures the continued infectivity of strains, and streamlines the accessibility of the collection. The greatest disadvantages of this technique are the large initial investment and ongoing operating costs primarily for the continuous supply of liquid nitrogen which needs to be constantly controlled and maintained. Cryopreservation of *Tuber borchii* mycelium was tested for the first time by Stielow et al. (2012) although the survival ratio on charcoal filter paper strips after thawing was low. In 2017, Piattoni et al. (2017) used a different cryopreservation protocol, employing sorbitol, sucrose and dimethyl sulfoxide as cryoprotectans which allowed cryopreservation of *T. borchii* mycelium without reducing its infectivity.

A similar technique to that of cryopreservation, with the high survival rate of biological material, is ultra-freezing (Kitamoto et al. 2002). This differs from cryopreservation at a higher storage temperature, ranging from −80 to −130 °C and the use of an ultra-freezer instead of liquid nitrogen. This technique, applied to various ectomycorrhizal mushrooms, has shown differing results depending on the

cryoprotectans used and the protocol applied (Crahay et al. 2013; Kitamoto et al. 2002; Obase et al. 2011). A recent protocol, applied to the medicinal mushroom *Ganoderma lucidum* (Curtis) P. Karst., developed by Leonardi et al. (2018) also showed very promising results with *Tuber* spp. and currently tests are underway to verify the survival of *Tuber* spp. mycelia at ultra-freezing temperatures.

14.4.2.2 In Situ Conservation of *Tuber magnatum*

Tuber magnatum has yet to be successfully cultivated so the only way to commercially exploit it without damaging natural production is to conserve it in situ. Intensive harvesting of truffles has been shown to cause decreases in truffle production in Europe and China as a consequence of physical damage to *Tuber* ectomycorrhizas, the soil mycelium and habitat, and perhaps suppressing fertilization. In order to protect this important resource, it is essential to enforce strict rules during harvest to protect their unique environment, to ban the harvesting of unripe ascomata by, for example, raking and to stop overharvesting in some areas allowing spore diffusion.

Regretably, fungi seldom receive legal protection and examples of in situ conservation are rare (Venturella et al. 2011) despite the Italian national law (L. 752/1985, https://www.gazzettaufficiale.it/eli/id/1985/12/21/085U0752/sg) that covers the most important rules for harvesting, cultivation, conservation and marketing of truffles. Some positive aspects of this law are, for example, that immature truffles are protected, dogs (rather than pigs) must be used to locate truffles, a special small trowel must be used to excavate the truffles and excavations are to be covered immediately after a truffle is harvested to avoid damage to the mycorrhizas. However, the biggest concern for *T. magnatum* in situ conservation is the ever-increasing number of truffle harvesters, which currently stands at around 45,000 in Italy (Ministry of Agriculture, Food and Forestry Policies and Tourisms https://www.politicheagricole.it/flex/cm/pages/ServeBLOB.php/L/IT/IDPagina/11100). Sadly, these collectors have open access to extensive oak forests and woodlands and other lands not under cultivation and occupied by *T. magnatum.* Furthermore, there is a lack of political motivation to reduce the number of collectors or to create truffle reserves or ecological corridors, where truffle harvesting is forbidden.

The preservation and plantation of *Tuber magnatum* host plants and the protection of the specific truffle environments are the only effective conservation measures proposed in some parts of Italy. In some municipalities of Tuscany, for example, the owners of *T. magnatum* productive trees receive a reward if they do not cut the trees. In some regions, the local government can give landowners the exclusive right to collect truffles but only after it has been verified that their productive forest has been significantly improved through an adequate thinning of the undergrowth.

Until recently, traditional cultural practices have often been applied in an attempt to improve *Tuber magnatum* areas without knowledge of their subsequent effects. This is because *T. magnatum* ectomycorrhizas are rarely detected in the field (Leonardi et al. 2013; Murat et al. 2005) and so cannot be used as an indicator of its

diffusion in soil, and the production of ascomata is too scattered and variable to follow in short-term experiments.

However, a molecular monitoring method based on quantitative PCR has been perfected (Iotti et al. 2012a) in a recent Italian regional project (MAGNATUM) that allows the quantification of the mycelium of *Tuber magnatum* in soil. For the first time we can now develop a simulation model to predict the mycelial dynamics of *T. magnatum* at varying soil temperatures and moisture conditions (Iotti et al. 2018) and measure the effects of managing summer irrigation. This also provides the first management guidelines aimed at maintaining and improving the environmental conditions we believe are suited to *T. magnatum*, such as soil tillage to relieve soil compaction (Salerni et al. 2014) and the selective removal or thinning of invading shrubs like brambles (*Rubus* spp.) (Zambonelli et al. 2012b).

14.5 Conclusion and Prospects

Tuber magnatum and *T. borchii* are two morphologically-similar white truffles which are traditionally important natural economic resources in Italy and a few other European countries. However, the less valuable *T. borchii* has become increasingly popular over the past 20 years following its successful cultivation first in Italy, followed by New Zealand. It has a very wide ecological range and can be easy cultivated. In contrast, *T. magnatum* has defied routine cultivation but there are few signs that it will be commercially cultivated anytime soon.

In addition to traditional cultivation methods, new techniques are also available for *Tuber borchii*. In particular, a technique for inoculation of plants with pure cultures has been perfected that opens up the possibility of selecting genotypes with particular characteristics, for example, specific climatic or edaphic zones.

The genome sequencing of *Tuber borchii* should help reveal the set of genes that control the adaptability of *T. borchii* to different environmental conditions and its productivity and quality. Genetic transformation of *Tuber* mycelia has been scarcely investigated (Grimaldi et al. 2005; Poma et al. 2005) and the proposed techniques not further developed. However, the discovery of the CRISPR/Cas9 immune system of bacteria and archaea and their repurposing for genome editing has elicited a new era in genetic engineering for filamentous fungi (Kwon et al. 2019) that may be applied to improve the characteristics of isolated *Tuber* strains increasing their ability to produce aromas and/or their adaptability to extreme climatic conditions. This might increase the cultivation area and help to overcome the risks of declining truffle production in the southern parts of Europe caused by predicted global warming (Thomas and Büntgen 2017). Obviously, to better exploit its genetic variability, the creation of a germplasm bank to preserve *T. borchii* genetic resources is mandatory. Cryopreservation techniques of mycelia have been shown to be an efficient tool for long-term preservation of truffle strains without affecting their viability and infectivity.

The cultivation of *Tuber magnatum* may receive a boost in the new genomic era. For example, comparative genomics could help to identify those genes which control *T. magnatum* mycorrhiza formation and nutrition, and to find conditions that favor its mycorrhiza development and in vitro cultivation of mycelia.

Appendix: White Truffle Research Institutions

Institution name	Specialization and research activities	Address	Contact person and website
University of Bologna	Truffle cultivation and biology	Via Fanin 44,40127 Bologna, Italy	Alessandra Zambonelli alessandr.zambonelli@unibo.it https://www.unibo.it/sitoweb/alessandr.zambonelli
University of L'Aquila	Truffle phylogeny	Via Vetoio, 67100 Coppito – L'Aquila, Italy	Mirco Iotti https://mesva.univaq.it/?q=docenti/scheda/Iotti%20Mirco
Truffles and Mushrooms (Consulting) Ltd.	Edible ecomycorrhizal fungi cultivation	P.O. Box 268, Dunedin 9054, New Zealand	Ian R. Hall truffle@trufflesandmushrooms.co.nz https://trufflesandmushrooms.co.nz/
INRA- Nancy (France)	Truffle genomics and cultivation	54280 Champenoux France	Claude Murat claude.murat@inra.fr http://www.nancy.inra.fr/
Ispr. CNR	Truffle ecology and metagenomics	Strada delle Cacce 73, 10135 Torino, Italy	Antonietta Mello antonietta.mello@ipsp.cnr.it http://www.ipsp.cnr.it/
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