Shubhpriya Gupta
Preeti Chaturvedi *Editors*

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Preface

Plantation and horticultural crops provide rural employment opportunities, supplement the dietary needs of the people, and contribute significantly to farmers' income. Unlike cereal crops, these are primarily grown in economically and environmentally susceptible regions, yet contributing considerably to farm income and agri-business growth. Being perennial in growth pattern, plantation crops are exposed to climatic stresses and experience climate change in their life cycle. Hence, the production of quality planting material is the foremost requirement of the plantation sector. Plant tissue culture technology offers this opportunity for the production of large-scale quality planting material. Another major contrivance made possible by tissue culture is the development of disease-free crops. The conventional practice of vegetative propagation is a prolonged process having seasonal dependence. Besides, it prevents species genetic diversity, which can lead to a reduction in crop yield and hence is economically not very feasible. In vitro propagation can multiply plants much faster than conventional propagation. Selection of explants, modification of media, use of novel plant growth regulators and novel techniques such as nanoparticles as growth supplements for explant establishment, plant-fungus co-culture system, and other biotic and abiotic elicitors can be used for regeneration of high-quality plantation and horticultural crops. The public and private sectors involved in the commercial tissue culture of plantation and horticulture crops aim to produce thousands of planting material in short period of time. For this, innovative techniques are desired that may help in the mass production of healthy and quality plants along with efficient marketing, packaging, and transportation of plantlets. The in vitro approach also helps in value addition of the products for achieving better returns to the growers.

This book will address the recent developments and future prospects of micropropagation procedures for the generation of quality planting materials for substantial plantations crops (such as coffee, tea, cocoa, areca nut, sugarcane, opium, oil seeds, oil palms, rubber trees) and horticultural (such as banana, grapes, potato, papaya, cassava, orchids, cardamom, ginger, turmeric, potato, and tomato).

This book, *Commercial Scale Tissue Culture for Horticulture and Plantation Crops*, addresses the recent advances that need to be incorporated into commercial

plant culture systems to improve the overall efficacy of production. The book primarily focuses on novel plant growth regulators, cost-effective media, selection of explants, disease management of ex vitro plants, use of endophytes, biostimulants in tissue culture, somatic embryogenesis, and genetic stability of in vitro plants. Furthermore, this book also elaborates on using nanotechnology in tissue culture, upliftment of rural economy through in vitro micropropagation approaches, improvement of plant survival, and expediting the acclimatization process in commercial plant tissue culture laboratories for fast and improved production of healthy micropropagated plants. This will eventually help in the modernization of the plantation sector.

This book aims at bringing out a comprehensive collection of information on the commercial tissue culture of plantation and horticulture crops. The main focus of this book is to address the rapid production of high-quality, disease-free, and uniform planting material for plantation and horticulture crops on a commercial scale. The book, overall, provides a suitable package of practice for capacity building of commercial tissue culture units involved in the production of plantation and horticulture crops.

We are grateful to the production team at Springer, who have been highly supportive and professional in preparing this manuscript. We greatly appreciate the contributions and time of anonymous referees for reviewing the chapters of this book, which not only assisted us in reaching decisions but also enabled the contributing authors to disseminate work of the highest possible quality. We acknowledge the University of KwaZulu-Natal and the National Research Foundation (Grant Number-145740), South Africa and G.B Pant University of Agriculture and Technology, Pantnagar, India for supporting our academic and research goals. Finally, we are most grateful to all our contributing authors for their valuable contributions. We trust this collection of chapters will be highly informative and valuable for a wide range of readers, including academicians, degree students, research scientists, horticulturists, agriculturists, industrial entrepreneurs, and agro-industry people.

Pietermaritzburg, South Africa
Pantnagar, Uttarakhand, India
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About the Editors

Shubhpriya Gupta completed her Ph.D. in Botany from G.B. Pant University of Agriculture and Technology, Pantnagar, India. Currently, she is working as a Post-Doctoral Researcher at Palacký University Olomouc, Czech Republic, and as a visiting Scientist at the University of KwaZulu-Natal, Pietermaritzburg, South Africa. She is the Founder and Director of Gustaden Manuscript Services, India. Her research field includes plant hormones, biostimulants, plant tissue culture, seed physiology, analytical chemistry, and biotechnology. She is the author/co-author of 20 peer-reviewed articles, which have been published in ISI-rated journals. She is the review board editor of the South African Journal of Botany. She has received an Y1 (Young researcher) rating from the National Research Foundation (NRF), South Africa in January 2022.

Preeti Chaturvedi is Professor and Head, Department of Biological Sciences, G.B. Pant University of Agriculture and Technology, Pantnagar. She did her M.Sc. and Ph.D. in Botany from Kurukshetra University, Kurukshetra, India. Her research interests include in vitro morphogenesis, conservation biology, bryophyte diversity, and bioprospecting. She has successfully developed micropropagation protocols for several high value medicinal plants *viz*., *Aconitum balfourii*, *Picrorhiza kurroa*, *Centella asiatica*, *Polygonatum verticillatum*, and *Rheum emodii*. She has 65 research publications to her credit in national and international journals of repute. She has completed four research projects on micropropagation of medicinal herbs including one on bioprospecting of bryophytes.

Chapter 1
Improved Sterilization Techniques for Successful In Vitro Micropropagation

Govindaraju Atul Babu, Kithiyon Mosa Christas, Elumalai Kowsalya, Manikandan Ramesh, Soo-In Sohn, and Subramani Pandian

Abstract Biotechnological solutions based on in vitro plant tissue culture paved the way for assessing desirable features by increasing the efficiency of in vitro regeneration methods, such as the production of a large number of high-quality plants in a short period of time. Contamination during in vitro regeneration operations, on the other hand, is one of the most serious issues that could stymie progress in this approach. Due to their rapid growth features in the media, numerous bacteria have posed a significant risk to in vitro cultures. The efficiency of the in vitro sterilization procedure has a direct impact on the establishment and maintenance of plants in in vitro cultures. The effective sterilization of biological material (e.g., initial explant) is required for successful in vitro culture initiation. A simple and effective approach to sterilizing explants employing different sterilants such as Nistatin, Flugal, Bavistin, Ridomil gold, and Mercuric chloride has been discussed in this chapter. Standardization of these methods can improve the survival and regeneration ability of large numbers of candidate explants, which is critical for enhancing the efficiency of plant tissue culture transformation systems. This chapter outlines enhanced sterilization procedures, including adequate sterilant concentrations, duration of explant exposure to various sterilants, and sequences of applying these sterilants, for effective in vitro tissue culture programs that could facilitate the large-scale micropropagation process.

Keywords In vitro culture · Contamination · Sterilization · Explant · Sterilants · Micropropagation

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1 Introduction

Tissue culture is the in vitro aseptic culture of cells, tissues, organs, or whole plants under regulated nutritional and environmental factors to produce plant clones (Thorpe 2007). For large-scale plant multiplication, plant tissue culture technology is frequently employed. Plant tissue culture techniques have recently gained industrial importance in the areas of plant propagation, disease removal, plant enhancement, and secondary metabolite production, in addition to their use as a research tool (Sharma et al. 2002). Clones may be produced in quick succession and with uniformity utilizing the tissue culture procedure, making it easier for producers to deliver consistent quality products on demand. Plant tissue culture cultivators and researchers frequently use somaclonal and gametoclonal variations to propagate specific varieties of plant species for crop improvement (Sarasan et al. 2011; Bhoite and Palshikar 2014; Tazeb 2017). The plant tissue culture procedure has aided in the development of plantlets with transgenic properties and weedicide resistance, with the aim of serving growers in the development of new plants that are free of airborne microbial contamination and diseases by contaminated water (Giles and Morgan 1987; Hussain et al. 2012). Furthermore, the plants can be quickly regenerated, and on an industrial scale, there are two elements to success: perfect (constant) quality and the ability to meet industrial demands rapidly. Regardless of the season or weather, hundreds of thousands of plants can be generated in a continuous process using small bits of tissue called explants in a relatively short time and space (Akin-Idowu et al. 2009).

Micropropagation is the rapid vegetative multiplication of virus-free plants in vitro conditions under high light intensity, regulated temperature, and defined nutritional media. Commercial plant production using micropropagation techniques has various advantages over traditional propagation methods like seed, cutting, grafting, and air-layering, among others. However, contamination during in vitro regeneration processes is one of the most serious issues that could thwart the advancement of this method (Da Silva and Kulus 2014; Hesami et al. 2017). Due to their rapid growth properties, a number of infections (microbial contaminants) have imposed a serious challenge to in vitro cultures (Enjalric et al. 1988). Micro-arthropods (mites and their vectors), microorganisms (bacteria, filamentous fungi, and yeasts), viruses, and viroids can all cause contamination in plant tissue cultures (Altan et al. 2010; Da Silva et al. 2016a; Hesami et al. 2018). Contamination could arise as a result of the explants or during the propagation process. Contaminants can be delivered into explants by micro-arthropod vectors (Leifert et al. 1989) or endophytic bacteria during laboratory manipulations (Pereira et al. 2003). Fungi can also come in the form of explants, airborne spores, or a culture. Bacterial and fungal contamination are common in commercial micropropagation laboratories, and they constitute a significant challenge (Reed et al. 1998). According to Obuekwe and Osagie (1989), fungi such as *Aspergillus niger* and *Aspergillus flavus* generate oxalate and aflatoxin toxins, which can kill plant cultures. At all stages of tissue culture, the explants are vulnerable to microbial infection, which lowers their

productivity and can entirely impede successful culture. Plants that have been contaminated may experience variable growth, reduced multiplication, and root rates, or even perish. It is required to remove foreign contaminants from explants and obtaining sterile plant material that is fully free of contamination is quite complex. When dealing with woody plant material, it becomes even more difficult (Niedz and Bausher 2002). In tissue culture, the most prevalent issues are improper techniques and insufficient disinfectant levels. Another issue is explant contamination from contaminated tools, equipment, and people involved in the preparation and culturing of media (Abass 2013). Microbes such as viruses, bacteria, yeast, and fungi, as well as toxins, are found on the surface and inside the plant's body. Microbial contamination wastes time, effort, and materials, resulting in significant financial losses (Hameed and Abass 2006; Omarmor et al. 2007).

2 Overview of the Successful Sterilization Protocol

Although most tissue culture techniques require growing stock plants in ways that prevent infection, disinfecting the plant material and sterilizing tools used for dissection, such as vessels and media in which cultures are produced, will kill surface bacteria (George 1993). There are three main strategies for preventing microbial contamination in plant tissue cultures: preventing their introduction with the initial plant material, avoiding their emergence from the environment during subculturing, and limiting microbial contamination in the cultures at the multiplication and rooting stages. Elimination of bacteria from the initial plant explants placed into the culture is the most efficient means of preventing bacterial contamination in vitro. The use of donor plant explants under stringent sanitary conditions, efficient sterilization of the first explants, and reduction of the size of the original explants to just the apical meristem are all ways of preventing contamination. As a result, sterilization is the one of the important stages in the establishment and maintenance of plants in in vitro cultures. Because of the widespread usage of innovative microwave-based autoclaves, sterilization of equipment should not be a major concern in a modern and well-equipped laboratory (Da Silva et al. 2016a). However, major issues arise during the disinfection of biological materials (e.g., the initial explant), which necessitates more attention and time (Hesami et al. 2018). Furthermore, tissues have the ability to host a variety of bacteria, requiring appropriate and effective sterilizing treatments prior to in vitro culture initiation (Mihaljević et al. 2013). All plant species require the eradication of external and endogenous contaminating microbes, which begins with successful explant sterilization (Constantine 1986; Buckley and Reed 1994; Dodds and Roberts 1985). Explant sterilization is the process of removing contaminants from explants prior to the creation of cultures. Prior to tissue culture, it is critical that the explants be free of pollutants in order to maintain their biological activity (Oyebanji et al. 2009). The size, age, and type of the explant, the conditions of cultivation and physiological state of the stock plant, the time and temperature of exposure, and the type of disinfectant and its

concentration can all affect disinfection efficiency (Da Silva et al. 2016b). Furthermore, the aforementioned conditions may have a negative impact on the survival and regeneration capability of candidate explants, which is critical for maximizing the efficiency of plant tissue culture transformation systems (Da Silva et al. 2016a; Hesami et al. 2018).

Antibiotics, fungicides, and heat and light inactivation are just a few of the ways employed to eliminate fungal and bacterial contaminants (Kneifel and Leonhardt 1992; Leifert et al. 1991; Haldeman et al. 1987). Antibiotics and fungicides have been studied to see how they affect these types of pollutants (George 1993). Contamination must be eliminated without damaging plant cells because these sterilizing chemicals are hazardous to plant tissue. Sterilization procedures vary depending on the plant type and the component (explant) chosen for sterilization. Depending on the growth environment, age, and portion of the plant used for micropropagation, each plant material has a different level of surface contamination. Standard sterilization processes that apply to all plants are difficult to achieve.

According to many studies (Kataky and Handique 2010; Nongalleima et al. 2014; Da Silva et al. 2016a; Hesami et al. 2018), the longer the treatment with higher concentrations of sterilants, the better the asepsis results. However, there is a negative relationship between high sterilant concentrations and explant viability rates, since the latter can be impacted by sterilants, resulting in dehydrated-yellowish explants with low viability. Because the living material should not lose its biological activity and only contaminants should be removed during sterilization, the choice of sterilizing agents (sterilants) and period of exposure is indeed crucial (Tiwari et al. 2012a, b). To save time and effort, accurate and precise sterilization is essential. Tissue responses are influenced by the sterilants employed on explant materials for in vitro multiplication. To minimize explant harm and improve survival rates, the concentration of sterilants, the length of exposing the explants to various sterilants, and the sequence of employing the sterilants must all be standardized (Altan et al. 2010; Da Silva et al. 2016a; Hesami et al. 2018; Rady et al. 2018). In brief, requirements for type, concentration, and time of exposure vary from plant to plant and for different plant tissues depending on morphological characteristics such as tissue softness or hardness (Sathyakumar 2016). Combining multiple sterilants at suitable concentrations is beneficial for sterilization. From the economic standpoint, a cost-effective, easy, efficient, and environmentally friendly sterilizing technique for removing endogenous and surface contamination should be proposed (Purohit et al. 2011; Tiwari et al. 2012a, b). Asepsis has always been a vital feature of successful in vitro plant culturing and mass multiplication to a large extent (Barrett and Casselles 1994; Herman 1996). Thus, the development of effective explant surface sterilization techniques for micropropagation is a critical necessity for in vitro conservation success. Table 1.1 lists the most frequent sterilizing procedures used in plant tissue culture system. These approaches are usually classified as physical or chemical sterilizing methods.

Table 1.1 Sterilization techniques in plant tissue culture

Techniques	Materials sterilized
Steam sterilization/autoclaving (121 °C at 15 psi for 20–40 min)	Nutrient media, culture vessels, glasswares, and plastic wares
Dry heat sterilization (160 °C–180 °C for 3 h)	Instruments, glassware, pipette tips, and other plastic wares
Glass beads sterilization (100–300 °C for 10 s)	Forceps, scissors, scalpels, needles, ring vaccination, and inoculation needles
Infrared (IR) sterilization (1500 °F/815 °C for 5–7 s)	Culture tube and pipette mouths, platinum inoculating hoops, needles, tweezers, and various metal and borosilicate glass instruments
Flame sterilization	Instruments and mouth of culture vessels
Filter sterilization (membrane filter made up of cellulose nitrate or cellulose acetate)	Growth factors, vitamins, amino acids, and enzymes
Alcohol sterilization	Hands of the workers and laminar air flow chamber
Surface sterilization	Explants

3 Sterilization of Instruments and Equipment

Even the laboratory's infrastructure and structural flaws can cause problems with the cultures, potentially contaminating them. For effective tissue culture system, a well-designed and planned laboratory including a washing room, sterile room, growing culture room, storage room, and hardening area is essential. The washroom and storage room are separated, but the sterile and growing rooms must be kept near together to minimize contamination from multiple sources. Dry or steam sterilization methods can be used to disinfect all instruments and equipment used in culture labs. Autoclaving (steam sterilization) is a method of sterilization that uses water vapor under pressure. Exposure to superheated steam in an autoclave for 10–15 min kills nearly all microorganisms. Dry sterilization is the process of sterilizing equipment using an ultrasonic cleaner or a heat sterilization treatment (Misra and Misra 2012).

4 Sterilization of Nutrient Media

To sterilize the culture medium, two procedures are usually employed, i.e., autoclaving and membrane filtration under positive pressure. Autoclaving is a typical process for sterilizing nutrient media for plant tissue cultures with a condition of 15 psi for 30 min (Skirvin et al. 1986; Kyesmu et al. 2004; Ikenganyia et al. 2017). Vitamins, plant extracts, amino acids, and hormones are denatured by autoclaving, and hence the solution of these substances is sterilized using Millipore filter paper with a pore size of 0.2 mm in diameter (Van Bragt 1971). Membrane filters with a nominal pore size of 0.22 μm are commonly used. Yet, sintered filters are utilized for abrasive liquids, viscous fluids, and organic solvents. The titer reduction value, which is the

ratio of the number of organisms confronting the filter under specific conditions to the number of organisms entering it, is one of the parameters that determines the filter's performance. The depth of the membrane, its charge, and the tortuosity of the channels are the other considerations. Before planting the tissue on the nutritional media, it must be fully surface sterilized to avoid infection in the media (Madigan et al. 2018).

Chemical sterilization of the culture medium is a simple and cost-effective alternative to autoclaving. Chlorine dioxide (ClO_2) and sodium hypochlorite (NaOCl) are chlorine-based chemicals that have been found to sterilize culture media effectively (Cardoso 2009; Teixeira et al. 2006). ClO_2 damages bacteria by oxidizing internal components and destroying cell membranes (Huang et al. 1997). NaOCl has been utilized as chemical sterilant of the culture media for in vitro culture of some species, such as *Sequoia sempervirens* (Ribeiro et al. 2011), *Chrysanthemum* (Deein et al. 2013), *Eucalyptus benthamii* (Brondani et al. 2013), *Hyptis leucocephala* and *H. platanifolia* (Nepomuceno et al. 2014). However, using sterilizing chemicals may result in incomplete sterilization of the culture medium and/or phytotoxicity for plantlet production, lowering propagation efficiency (Tiwari et al. 2012a, b; Vargas et al. 2016). Before being used in large-scale micropropagation systems, possible sterilizing chemicals must be investigated for sterilization efficacy as well as phytotoxicity for plant cultivation. Interestingly, autoclaving is still the most common sterilization method in tissue culture labs, owing to its long history of usage in plant tissue culture labs and remarkable repeatability, as well as its successful microorganism sterilization and minimal phytotoxicity (Leelavathy and Sankar 2016).

5 Explant Sterilization

The sterilization of explants is an important stage in the tissue culture process. Surface sterilization of explants is a process that involves the immersion of explants into the appropriate concentration of chemical sterilant(s) or disinfectant(s) for a specified time, resulting in the establishment of a contamination-free culture. Before being inoculated onto the tissue culture medium, the explants' surfaces must be carefully disinfected. The explants taken from the stock plant should first be thoroughly cleaned. Plants can have endophytes, which are microorganisms that live inside plant tissue without causing disease symptoms (Petrini 1991). Endophytic bacteria have been found in plants in vitro cultures in both commercial laboratories and scientific investigations (Leifert et al. 1994), and they frequently impair tree in vitro propagation (Ulrich et al. 2008). Before utilizing the source plants and explants in the culturing process, they are checked for endophytes. Also, in the case of field-grown plant tissues with many microorganisms from the soil and environment, it is necessary to search for alternative protocols to obtain sterile tissues to start a protocol for in vitro plant tissue culture. Two detection methodologies can be used: bacterial strain isolation on growth media (culture-dependent

method) or bacterial sequence amplification from whole plant DNA (culture-independent method) (Quambusch et al. 2014). Because most species of this genus are not cultivable on conventional bacterial growth mediums, the presence of endophytes or other microorganisms in plant tissues is frequently underestimated when culture-dependent approaches are utilized (Koskimäki et al. 2010). Some species, on the other hand, can be isolated from plant material but not discovered using the culture-independent approach. This disparity is common in related research, demonstrating the significance of using both approaches to provide a comprehensive view of the bacterial community (Thomas et al. 2008; Tejesvi et al. 2010).

It is normally not difficult to surface sterilize juvenile material. However, unless the tree produces juvenile sprouts, contamination of explants can be a severe concern when older trees are employed as the starting and fundamental material for tree breeders when selection is done. In rare circumstances, insects deposit spores on field-grown trees. This contamination can be reduced by spraying these trees with insecticides and fungicides, and then wrapping the expended shoots in transparent film bags before collecting the explants to protect them from insects. A vast spectrum of microbial pollutants can be found on the surface of plant parts. Various sterilizing chemicals have been employed to disinfect plant tissues. In most circumstances, hypochlorite treatments have proven to be overly effective. Plant tissues have also been surface sterilized using ethyl and isopropyl alcohol. Explants were cleaned with distilled water to remove dust particles, then washed in a detergent solution before being surface sterilized for 5 min in a 0.1% mercuric chloride, sodium chloride solution. Nodal segments were rinsed with sterile distilled water once more to remove the sterility.

Traditionally, disinfection has been accomplished with sodium hypochlorite (NaOCl), which is a good option for tissue disinfection (Wong 2009; Norton and Skirvin 2001; Ibañez et al. 2005). However, various factors influence this operation, including explant source, mother plant age, cultivar, and genotype (Haissig 1974; Friend et al. 1994; Howard 1994). Several literatures showed that various other types of sterilants are being used, they include ethanol (or isopropyl alcohol), calcium chloride ($Ca(ClO)_2$), hydrogen peroxide (H_2O_2), mercuric chloride ($HgCl_2$), silver nitrate ($AgNO_3$), and bromine water (Da Silva et al. 2016a, b). Surface sterilization of explants should follow a technique that uses the least amount of disinfectant for the shortest amount of time. Take into account that higher concentrations and longer exposure durations can be unfavorable for explant regeneration (Allan 1991; Pierik 1987). Because live materials should not lose their biological activity during sterilizations and only contaminants should be removed, explants are only surface sterilized by treatment with disinfectant solution at appropriate concentrations for a defined time. However, the required type, concentration, and time of exposure of the disinfectant(s) differ for different plant and plant parts (Srivastava et al. 2010) (Table 1.2).

Ethanol is a powerful sterilizing agent but reported to possess phytotoxicity. According to standard protocol, 70% ethanol is used for a few seconds or minutes before being treated with other disinfectants (Abbasi et al. 2016). Hypochlorite is a

Table 1.2 List of sterilants used for the surface sterilization of explants

S. No.	Plant species	Explant	Sterilants	Reference
1	*Setaria italica* L. (Indian foxtail millet)	Seeds	70% (v/v) ethanol and 0.1% (w/v) HgCl2	Satish et al. (2015); Rathinapriya et al. (2019, 2020)
2	*Oryza sativa* L. (rice)	Seeds	70% (v/v) ethanol and 0.1% (w/v) $HgCl_2$	Krishnan et al. (2013); Rameshkumar et al. (2019)
3	*Clitoria ternatea* L. (butterfly pea)	Tender nodal	0.1% Bavistin solution, 70% ethanol, and 0.1% $HgCl_2$	Rency et al. (2018)
4	*Eleusine coracana* L. Gaertn. (finger millet)	Seeds	70% (v/v) ethanol and 0.1% (w/v) $HgCl_2$	Satish et al. (2018)
5	*Bacopa monnieri* (L.) Pennell (water hyssop)	Shoot tips	70% (v/v) ethanol and 0.1% (w/v) $HgCl_2$	Rency et al. (2016)
6	*Nilgirianthus ciliatus* (Nees) Bremek (lesser Kurinji)	Node	0.1% (w/v) Bavistin solution, 0.1% (w/v) $HgCl_2$, and 70% ethanol	Rameshkumar et al. (2016)
7	*Eleusine coracana* L. Gaertn. (finger millet)	Seeds	0.1% (w/v) $HgCl_2$ and 70% (v/v) ethanol	Atul Babu et al. (2017); Atul Babu and Ravindhran (2019)
8	*Couroupita guianensis* Aubl. (cannonball tree)	Seeds	Disinfecting solution containing 1 mL of Tween-20 per 100 mL in 1% (v/v) NaOCl	Shiny et al. (2019)
9	*Citrullus lanatus* Thunb. (watermelon)	Seeds	1% (v/v) NaOCl solution containing 1 mL of Tween-20 per 100 mL	Vinoth and Ravindhran (2015)
10	*Orthosiphon aristatus* (blume) Miq. (kidneys tea plant)	Node	0.2% (w/v) Bavistin and 0.1% (w/v) $HgCl_2$	Swarna and Ravindhran (2013)
11	*Ensete ventricosum* (Welw.) Cheesman (Abyssinian banana)	Shoot tip Leaf sheath	70% (v/v) ethanol, 1, 2, or 3% NaOCl with 3 drops of Tween-20 70% (v/v) ethanol, 0.5, 1, or 2% of NaOCl	Zinabu et al. (2018)
12	*Vitis vinifera* L. (grape)	Buds	1.3% NaOCl and 50 drops per L of triton X-100 solution	Lazo-Javalera et al. (2016)
13	*Magnolia sirindhorniae* Noot. and Chalermglin (*Magnolia sirindhorniae*)	Buds	75% (v/v) ethanol and 0.1% (w/v) $HgCl_2$	Cui et al. (2019)
14	*Solanecio biafrae* (Oliv. & Hiern) C.Jeffrey (Sierra Leone Bologi)	Node	70% ethanol and 10% $Ca(ClO)_2$	Bello et al. (2018)

(continued)

Table 1.2 (continued)

S. No.	Plant species	Explant	Sterilants	Reference
15	*Dianthus caryophyllus* L. (carnation)	Apical and nodal	5% CaOCl	Gautam et al. (2019)
16	*Staurogyne repens* (Nees) Kuntze (mud mat)	Node	5.5% H_2O_2 solution	Köse et al. (2020)
17	*Brassica oleracea* L. (cauliflower)	Curd	1% (v/v) NaOCl, 0.1% (v/v) Tween 20 and 4.5 mM gaseous ClO_2	Bhawana et al. (2015)
18	*Vitis vinifera* L. (grapevine cv. "Flame seedless")	Buds	2.5 mM allyl isothiocyanate	Lazo-Javalera et al. (2016)
19	*Pereskia grandifolia* Haw. (rose cactus)	Leaves	150 mg/L sodium dichloroisocyanurate (NaDCC)	Leng and Chan (2007)
20	*Aerangis ellisii* (B.S.Williams) Schltr. and *Angraecum rutenbergianum* Kraenzl. (orchids)	Seed capsules	0.5% (w/v) NaDCC solution and 2 ml/L Plant Preservative Mixture (PPM™)	Kendon et al. (2017)

highly efficient chemical for removing bacterial contamination, even at micro molar concentrations. When hypochlorite salts (NaOCl, Ca(OCl)2) are diluted in water, they produce HOCl, whose concentration is linked to bactericidal action. At the same time, sodium hypochlorite has a significant phototoxic impact on the explants, emphasizing the usage of calcium hypochlorite as a moderate disinfectant. (Abbasi et al. 2016; Badoni and Chauhan 2009; Cruz-Martínez et al. 2017). The growth of the plant in the tissue culture is hampered by high levels of ethanol and mercuric chloride. Another method for reducing external contamination is to expose the explants to ultraviolet-C radiation for 5 min followed by surface sterilization for around 10 min. The pathogen's DNA can be deactivated by the strength of UV-C light. However, there is a risk of damaging plant molecules, particularly plant DNA (Gangopadhyay et al. 2017). Flow chart showing complete explants sterilization process were provided in Fig. 1.1.

1. Preparation of Stock Plants

   Explant contamination may be reduced if stock plants are adequately cared for beforehand. Plants cultivated outdoors, especially in humid climates like Florida, are generally more "dirty" than those produced in a greenhouse or growth chamber. The contamination of early explants is increased by overhead watering. Similarly, splattering soil on the plant while watering it would exacerbate the initial infection. Fungicides and bacteriocides are occasionally beneficial to stock plants. In clean circumstances, it is occasionally feasible to collect shoots and force buds from them. When the forced shoots are surface sterilized as usual, they should be clear of pollutants. To supply clean material, seeds can be sterilized and grown in vitro. Covering developing shoots for a few days or

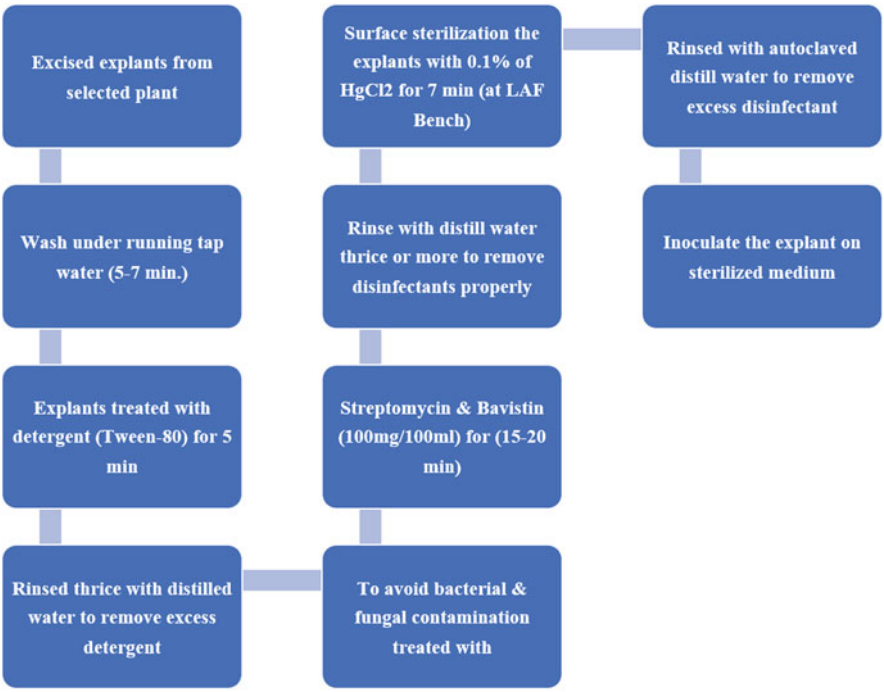

Fig. 1.1 Flow chart showing complete explants sterilization process

weeks before taking tissue for culture might result in cleaner tissue. Explants or material to be cut should be cleaned in soapy water and then put under running water for 1–2 h.

2. Ethanol (or Isopropyl Alcohol)

   Isopropyl alcohol is another name for it. The 70–95% ethanol is widely used in tissue culture labs as a sterilizing agent, but it is also highly phytotoxic. Therefore, explants are only maintained in the chemical for few seconds since prolonged contact with ethanol destroys the delicate explants. The more fragile the tissue, the more it will be harmed by alcohol. Because the tissue that will be explanted or produced is essentially well within the structure that is being surface sterilized, tissues like dormant buds, seeds, or unopened flower buds can be surface sterilized for lengthy periods. Generally, 70% ethanol is used prior to treatment with other compounds. Bello et al. (2018) disinfected *Solanecio biafrae* nodal explants with a mixture of 70% ethanol and 10% Ca $(ClO)_2$ for 15 min, achieving 90% survival and callus development.

3. Calcium Hypochlorite

   Surface sterilization using calcium hypochlorite, either alone or in combination with ethanol (70%), has been used on various explants, including seeds, spadices or spathes, and leaves (Teixeira da Silva et al. 2015). Calcium hypochlorite is used more in Europe than in the United States. It is obtained as a

powder and must be dissolved in water. The concentration that is generally used is 3.25%. The solution must be filtered before use since not the entire compound goes into the solution. Sangwan et al. (1987) effectively sterilized carnation shoots for 10 min using a 5% Calcium Hypochlorite (CaOCl) solution. Similarly, Roest and Bokelmann (1981) surface-sterilized carnation flower pedicels using 5% Calcium Hypochlorite (CaOCl) for 20 min. Assareh and Sardabi (2005) examined the effects of three sterilizing agents ($Ca(ClO)_2$, NaOCl, and $HgCl_2$) on the surface sterilization of *Ziziphus spina-christi* (L.) Desf., and found that $Ca(ClO)_2$ generates disease-free and regenerating explants. Explants of carnation (*Dianthus caryophyllu*s L.) were treated with 5% calcium hypochlorite as a potential substitute for $HgCl_2$. When growing cultures of cv. Parendillo and cv. Yellow Star is treated with 5% Calcium Hypochlorite for 10 min and 15 min, respectively, they are entirely uncontaminated (Gautam et al. 2019). This is because CaOCl has lower toxicity on explant shoot tips, apical meristems, and rhizome discs.

4. Sodium Hypochlorite

   Sodium hypochlorite has a wide antibacterial spectrum and rapidly destroys vegetative spores, bacteria, fungi, viruses, and protozoans (Dychdala 1991) because NaOCl is a strong oxidant of biological components such as protein and nucleic acid (Bloomfield et al. 1991). The most often used agent for surface sterilization is sodium hypochlorite, which is frequently acquired as laundry bleach. It is inexpensive and simple to dilute to the desired concentrations. Commercial laundry bleach contains 5.25% NaOCl. It is typically diluted to a concentration of 10–20% of its original strength, resulting in an NaOCl concentration of 0.5–1%. Generally, plant material is immersed in this solution for 10–20 min. Due to phytotoxicity, an empirical balance between concentration and time must be established for each kind of explant. Yildiz (2002) examined the effect of NaOCl solution concentration and temperature on flax seed germination, seedling growth, and the potential of hypocotyl explant shoots to produce shoots. The study discovered a high correlation between NaOCl concentration and temperature, with 40% disinfectant at 10 °C giving the best results in seedling growth and shoot regeneration. For surface sterilizing of the flaxseed, different concentrations of ethanol and sodium hydroxide solution are being used (Jordan and McHughen, 1988)

5. Mercuric Chloride

   Mercuric chloride is rarely used in labs due to its severe toxicity to both plants and people. Following exposure to mercuric chloride, rinse explants many times with sterile water to remove any mineral residue. According to Naik and Chand (2011), phenolic compound leaching, which produces lethal browning and creates secondary metabolites at the cut surface, substantially influences the proliferation of woody plant in vitro culture. Teak (*Tectona grandis*) explants treated with 0.1% $HgCl_2$ for 15 min exhibited improved bud breaking efficiency, reduced browning, and less microbiological contamination (Antony et al. 2015). After 15 min of exposure to 1.5% $HgCl_2$, the rate of fungal and bacterial contamination was decreased. Taha et al. (2020) looked at using

$HgCl_2$ to surface sterilize mulberry apical shoots and lateral buds. Explants cleaned with 0.2% $HgCl_2$ exhibited the lowest contamination and the highest percentage of shoot regeneration. $HgCl_2$ and/or NaOCl are widely used for the mulberry plant in vitro sterilizing (Damiano and Padro, 2008). Padhi and Singh (2017) observed that 0.1% $HgCl_2$ for 10 min resulted in the maximum explant survival (83.25%) compared to 1% $NaOCl_2$ while examining the effects of $HgCl_2$ and $NaOCl_2$ on the surface sterilization of Lasora explants.

However, due to the peroxidizing action of chlorine atoms and ions that react with proteins, $HgCl_2$ inhibits seed germination, resulting in the death of germinating seedlings (Pauling 1955). Alatar et al. (2017) studied the impact of two surface sterilizing agents, $HgCl_2$ and NaOCl, on the germination of *Solanum lycopersicum*. $HgCl_2$ was shown to totally inhibit seed germination at concentrations ranging from 0.1 to 0.5%, but at a far lower concentration of 0.01%, the seeds were found to be contaminated. Despite being used widely, the United Nations Environment Programme (UNEP) has issued concern about using $HgCl_2$ and NaOCl as it generates toxic substances such as chloroform and chlorophenol.

6. Hydrogen Peroxide

The use of H_2O_2 as a chemical sterilizer in the culture medium, as well as the use of aseptic procedures during explant manipulation, may help to keep the media and the explant free of contamination. H_2O_2 possesses germicidal and fungicidal properties in orchids at low concentrations, without influencing in vitro seed germination or plantlet development (Snow 1985). The activity of plant peroxidases, which work against H_2O_2 by converting it into water and oxygen, has been ascribed to the lack of cellular and tissue damage. Many plant cells include enzymes that break down H_2O_2, such as catalases and peroxidases (Goodwin and Mercer 1972), offering a protective mechanism to shield cells from the harmful effects of peroxides created either by their own metabolism or from an external source. H_2O_2 reduced the growth of moulds in the range of 0.009–0.18% without influencing the germination of orchid seeds (Snow 1985). Chemical sterilization was successful at concentrations of 0.005–0.01% in orchid culture media (Yanagawa et al. 1995). The hydrogen peroxide used for surface sterilization of plant material has a concentration of 30%, which is ten times stronger than what can be acquired at a pharmacy. Hydrogen peroxide has been shown to be beneficial for surface-sterilizing material while in the field by certain studies.

7. Aqueous chlorine dioxide

Chlorine dioxide is an environmental-friendly disinfectant, as this compound produces less toxic compounds and widely used for water disinfection (Lin et al. 2014; Simon et al. 2014). Duan et al. (2016) investigated the surface sterilization potential of chlorine dioxide on two plant species with variable levels of polyphenolic content: Yacon (*Smallanthus sonchifolius*) and Pomegranate (*Punica granatum*). When treating Yacon explants with 1.25 mM chlorine dioxide for 80 min or 1.67 mM for 40 or 80 min, the most significant disinfection rate was obtained. On the other hand, even modest concentrations of

chlorine dioxide (<0.015 mM) severely hindered the survival of pomegranate explants. Despite the fact that chlorine dioxide has a high potential antibacterial action, explant regeneration is dependent on the plant's phenolic content. According to the findings of the study, chlorine dioxide can be used to disinfect plants with low polyphenolic content since polyphenols are quickly oxidized by chlorine dioxide (Ni et al. 1995).

8. Isothiocyanates (ITCS)

   Isothiocyanates are naturally occurring antimicrobial chemicals that are members of the Brassicaceae family and exhibit biocidal activity against a variety of fungal, bacterial, insect, and pest diseases (Baez-Flores et al. 2011). ITCS was used to disinfect "Flame seedless" axillary buds, and it was observed that allyl isothiocyanate (2.5 mM) incubated for 6 h resulted in 50% of buds being free of infection. However, when the grape wine explants were treated for 60 min with 20% clorax and 50 drops/L Triton X-100, they generated 80% healthier buds (Lazo-Javalera et al. 2016).

9. Sodium dichloroisocyanurate (NaDCC)

   Sodium dichloroisocyanurate is a considerably more effective sterilizing agent than sodium hypochlorite because it dissociates to create a solution of hypochlorous acid when employed. Parkinson et al. (1996) successfully sterilized oaktree explants using NaDCC at concentrations ranging from 10 to 300 ppm. Before sterilization, the explants utilized in the study were highly infected with bacteria such as *Xanthomonas* sp. and *Pseudomonas* sp. When NaDCC was used to sterilize the surface, it resulted in explants that were free of contamination and allowed for extended subcultures.

10. Enhancing Effectiveness of Sterilization Procedure:

    Some microbes emerge during the initial phases of tissue culture, while others remain subconscious and are not discovered until later stages of growth (Cassells 1991). As a result, a mild vacuum is used during the procedure. Sodium hypochlorite is commonly mixed with a surfactant (such as Tween 20). Explant solutions are often mixed or constantly swirled. After plant material is sterilized with one of the above chemicals, it must be rinsed thoroughly with sterile water. Typically, three to four separate rinses are done.

11. Use of Antibiotics and Fungicides in vitro

    The use of antibiotics and fungicides in vitro is not very effective in eliminating microorganisms and these chemicals are often quite phytotoxic. Antibiotics and fungicides have been studied in relation to bacterial and fungal contaminants (George 1993). Shields et al. (1984) investigated the impact of fungicides on in vitro fungal contamination as well as their toxicity in tobacco cells. They prescribe carbendazim and fenbendazole (30 g/cm^3) as fungicides. In addition, other fungicides such as imazalil (20 g/cm^3) and captofol (100 g/cm^3) were efficient in preventing fungal contamination, as was a mixture of propiconazole and carbendazim in controlling fungal pollutants. However, at greater concentrations, these disinfectants become toxic, resulting in decreased explant development and viability (George 1993).

12. Plant Preservative Mixture

Plant Preservative Mixture (PPM™) is a broad-spectrum biocide that may be used to control contamination in plant cell cultures during the sterilizing process or as a medium component. PPM™ is available as an acidic liquid solution (pH 3.8). A dosage of 0.5–2.0 mL/L of PPM™ medium is suggested. To treat endogenous contamination and Agrobacterium, higher dosages are necessary. PPM™ according to its creators offers numerous advantages over antibiotics: It works against both bacteria and fungi; therefore, it can be used instead of a combination of antibiotics and fungicides. PPM™ is less costly than antibiotics, making it a viable option for widespread usage. Because PPM™ targets and inhibits numerous enzymes, the development of resistant mutants is extremely rare. Antibiotics have a negative impact on plant materials. PPM™ has no effect on in vitro seed germination, callus proliferation, or callous regeneration when taken as directed. PPM™ dosages of 5–20 mL/L can be used to sterilize seeds and explants with endogenous contamination. When normal surface sterilization is insufficient, this is beneficial.

6 Problems in Surface Sterilization with Chemicals

Some disinfectants (e.g., glutaraldehyde, quaternary ammonium compounds) have improved antibacterial activity when pH rises, while others have decreased antimicrobial activity (e.g., phenols, hypochlorites, and iodine). The disinfectant molecule or the cell surface are affected by pH, which affects antibacterial action. The single most critical element determining the activity of gaseous disinfectants/sterilants like ethanol, chlorine dioxide, and formaldehyde is relative humidity. Because divalent cations (e.g., magnesium, calcium) in the hard water interact with the disinfectant to generate insoluble precipitates, water hardness (i.e., high concentration of divalent cations) lowers the rate of kill of certain disinfectants. Changes in structural and biochemical properties, as well as a residual acidic environment, are caused by peracetic acid. Biochemical properties are affected by iodine. Ethylene oxide is combustible, explosive, and carcinogenic. It causes structural changes and leaves a poisonous residue.

7 Successful Surface Sterilizations with the Crop Plants

Surface sterilization using 70% (v/v) ethanol for 60 s and 0.1% (w/v) $HgCl_2$ for 5 min resulted in successful micropropagation of foxtail millet seeds, with no fungal contamination (Satish et al. 2015). Rameshkumar et al. (2019) reported that surface sterilizing tissue culture produced deep water indica rice cv. TNR1 (Thalainayar 1) seeds with 70% (v/v) ethanol for 1 min and 0.1% (w/v) $HgCl_2$ for 4 min resulted in 84.2% germination percentage. High yield was recorded in the seeds of a finger millet cultivar "CO(Ra)-14" on surface disinfestations with 70% (v/v) ethanol for

1 min and 0.1% $HgCl_2$ solution for 5 min, followed by five washes with sterile distilled water (Satish et al. 2018). Atul Babu and Ravindhran (2019) achieved excellent surface sterilization of finger millet genotype CO9 seeds by using 0.1% (w/v) $HgCl_2$ for 5 min followed by 70% (v/v) ethanol for 25 s.

Rency et al. (2018) demonstrated the effectiveness of a combined surface sterilization process by immersing *Clitoria ternatea* nodal explants in 0.1% Bavistin solution for 30 min, followed by 70% ethanol for 1 min, 0.1% $HgCl_2$ for 4 min, and finally washing with sterile distilled water for three times each 1 min. Surface sterilization of *Nilgirianthus ciliatus* nodal explants in 0.1% (w/v) Bavistin solution for 20 min was accompanied by subjection to 0.1% (w/v) $HgCl_2$ for 4 min, 70% (v/v) ethanol for 1 min, and a final rinse with sterile distilled water (Rameshkumar et al. 2016). The seeds of *Couroupita guianensis* fruits were surface sterilized in a disinfecting solution containing 1 mL of Tween-20 per 100 mL in 1% (v/v) NaClO for 20 min and showed satisfactory plant growth in vitro (Shiny et al. 2019). In sugarcane micropropagation, a concentration of 0.1% NaClO provided the optimum chemical sterilizing treatment, with good microbiological decontamination (96%) and normal growth (Tiwari et al. 2012a, b).

8 Conclusions

Contamination of plant cultures persists despite the most severe application of sterile procedures, resulting in losses ranging from a few cultures to entire batches of culture medium and tissue cultures. Contamination of plant tissue cultures by bacteria and fungi is an insidious process that threatens them throughout the culture period. Even if plant tissue cultures are sterile when they are first started, bacteria can infect them at any time during subsequent tissue culture procedures. As a result, the sterilization of explants, as well as the choice of sterilizing chemicals and exposure period, are crucial for the effectiveness of plant tissue culture.

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Chapter 2
Selection and Preparation of Explants for the Clonal Propagation of Horticultural Plants in Plant Factory Systems

Ahmet Tigrel, Merve Arslan, Beyza Arıcı, and Buhara Yücesan

Abstract Explant preference is a key factor for efficient and sustainable plant propagation under in vitro conditions. Plant genotype and structure must be well observed and identified for the best explant which may differ in the axillary bud breakings using terminal buds on stems located above ground or specialized/underground stems such as bulbs scales, base plates of corms, and the shoot tips of suckers. Since plant factory systems are aimed at uniform and cost-effective propagation systems, determination of explant type and culture conditions are the most critical factors for the establishment of shoot multiplication rate. In this chapter, several horticulture plants including house plants (Monstera, Philodendron, Begonia, etc.), and fruit trees (Aronia, banana, walnut, etc.) used in commercial-scale production in plant factories were investigated for the understanding of the nature of explants as per culture conditions. This phenomenon is also highly correlated with effective surface sterilization. Since plant factories rely on an automation system for particular crops, replenishment of starting material in each cloning cycle prevents the emergence of undesirable traits due to the somaclonal variations. This study reports a comparative and in situ analysis of explant choice for the scalable vitro-plant productions.

Keywords Micropropagation · Ornamental plants · Explant · Regeneration · Plant factory

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1 Nature of Explants

1.1 Basics of Plant Development and Plant Tissue Culture

Plant development can be divided into three major stages including embryogenesis, vegetative development, and reproductive development. The term of embryogenesis in developmental biology describes the process by which a single cell is differentiated into a group of cells having a characteristic but typically rudimentary organization (Mummery et al. 2011). This so-called rudimentary organization in animal embryogenesis shows a dramatic rearrangement or movement of the cells in the blastula stage (a hollow ball of the cells) proceeding with the embryonic tissue layers, tissues, and organ formation (Taiz et al. 2015; Graham et al. 2002). Unlike animals, plant cells are immobile, and the fate of cells are easily predictable since they are effectively packaged during embryonic development within the ovule that forms the seed. Plant, particularly seed-plants have three different tissue types with specialized functions (cortical, vascular, and dermal) with a certain group of cells, namely meristems that enable the elaboration of additional plants structures during growth and development (Taiz et al. 2015). Within this framework, asymmetrical cell division (Fig. 2.1) enables the embryo a variety of complex forms with remarkable developmental plasticity to withstand harsh environmental conditions. This adaptation occurs on a physiological level achieved through developmental

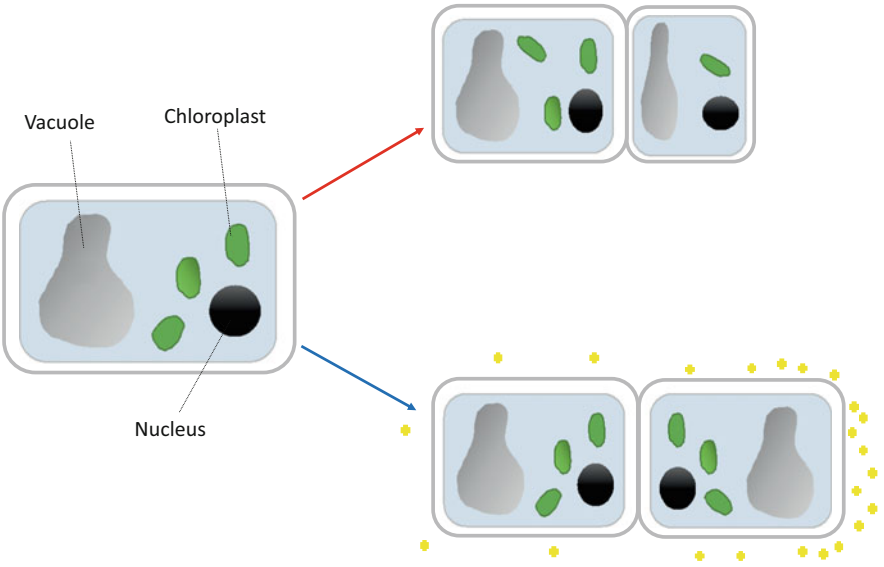

Fig. 2.1 Factors affecting differentiation in plant cells. Red arrow represents asymmetrical cell division, while the blue one shows the cell location effect. This effect can be explained as the exposure of a cell to different chemical or physical factors (yellow signs) as compared to another cell

pathways to recover injuries and/or replace lost parts/organs, which makes the plant tissue and organ culture as a methodology of plant propagation systems long since. Haberlandt had been observed wound tissues in aseptic cultures, the piece of plant parts or explants showed him unlimited cell proliferation, yet more efforts were needed for the experimental confirmation of his concept in 1902 (Haberlandt 1902). After 40 years, aseptic tissue culture methods were much improved with meristematic materials with a wide range of source plants, and the discovery of plant growth regulators such as auxin and cytokinin are both at different combinations and concentrations required in meristematic activity in vitro cultures (Murashige and Skoog 1962). During the process of differentiation, an explant can form a callus, due to the high resemblance of wound-healing tissue that consists of unorganized dividing cells. In the 1960s, those efforts consequently lifted the practical applications under the light of well-documented approaches, such as the single-celled origin of proliferation that mimics zygotic embryo formation over somatic cells from one-step cultures (Sugiyama 2015; Feher 2019). This process was called somatic embryogenesis from single cells on the explant tissues, and all diploid somatic plant cells were widely accepted as totipotent (Steward et al. 1970).

1.2 Explants Under Light of Plasticity and Totipotency

The explant is a piece of plant tissue that enables the transformation of the somatic cells into a callus tissue under certain conditions. These cells are highly differentiated, vary in size with non-isodiametric shapes. Unlike undifferentiated meristematic cells, their nuclei are relatively small and non-centric, vacuoles are larger (Wareing and Phillips 1981). These groups of cells sometimes initiated meristemoids that enable adventitious tissues (Fig. 2.1) or somatic embryos with varying degrees of plasticity depending on signal transductions between explants and growth medium conditions. For instance, potato is an interesting plant for the degree of plasticity with its photoperiod response that results in flowering on the aerials shoot apices; otherwise, reproductive development involves the tuber production via underground metamorphosis in stolon tips in the absence of enough sunlight (Sarkar 2008). As mentioned before, this strategy seems like an important survival mechanism (Van den Berg et al. 1996) and conservation strategy under evolutionary perspective (Sarkar 2008) that resulted in high regeneration capacity, in other words totipotency in plant cells as compared to that of animals. However, recently Feher (2019) reported that plant cells can (re)gain totipotency, but they are not totipotent per se in contrast to the earlier concepts in university textbooks which defines all/most plant cells are totipotent. According to his view, plant tissue culture from a totipotent cell must fulfill two criteria in which totipotency is a cellular term since the cell proliferation is a result of the initiation of single embryonic cell origin and needs to proceed autonomously as a single process. Within this framework, explants need to be induced for an autonomous regeneration in at least three main ways: direct somatic embryogenesis through zygote-like or meristemoids derived from single

cells (1), direct or indirect somatic embryogenesis dependent on various transcription factors (*LEC1*, *LEC2*, *LEC3*, etc.) that promotes overexpression of genes to induce embryo-identity and callus formation (2) and a group of cells dependent on phytohormones levels in meristem centers (i.e., *WUS* and *WOX5* expression; see details in Feher 2019). For the definition of callus, many factors involving hormonal gradients of explants, chromatin regulation in the nucleus, number of cell divisions play a key role in reprogramming of cells or tissues (Wareing and Phillips 1981) that makes the callus as a dedifferentiated tissue involving the least specialized group of cells but allowing high developmental potency. As differentiation results in more specialized cells losing competency for further cell division, the opposite process, dedifferentiation, does the same under in vitro conditions depending on the growth medium formulations, explant selections, explant age, etc. as discussed below.

1.3 Explant Types, Ages, and Location

There are various factors that affect meristematic activity during differentiation in tissue culture systems (Fig. 2.2.). The ontogenic age of the explant is sometimes an important criterion for the regeneration capacity. As a thumb rule, younger tissue makes it easier for surface disinfection to establish aseptic cultures. The newly formed tissues excised from nodal segments, shoot and root tips are widely used micropropagation systems. However, another criterion that is integrated with physiological age is to define the season in which the explant is obtained. The season of the year can be highly correlated with contamination and dormancy in culture. Explants taken from meristematic regions during spring are ready to a flush state at a higher rate of growth and development than the dormant buds collected from the periods between late summer and winter (Smith 2013). To break dormancy, additional pre-treatments can be required such as chilling explants, scarification of seeds, etc. In this case, the quality of the source plant should be considered for the explant selection. Selection of healthy plants as compared to the plants under stress conditions (drought or nutritional) can exhibit disease symptoms which then result in virus contamination after harvesting. Explant selection should be figured out effectively depending on the culture types and the goal of the experiments. In practical terms, any piece of plant can be used as an explant as mentioned before; however, its response may vary in culture medium (Lazzarini et al. 2019). In general, for the rapid multiplication, tissues taken from terminal buds, nodes, and tips are readily effective explants and widely used in culture media with or without phytohormones. In some cases, culture medium and longevity of the medium ingredients are of limited duration period for in vitro cultivation; thus, sub-cultivation or medium replenishment are required accordingly. For instance, if the goal is to induce callus formation, explants from cotyledons, hypocotyls sourced from in vitro germinated seeds, stems, and leaves (lamina or petioles) are widely used (Yücesan et al. 2007; Lazzarini et al. 2019). It is noteworthy to remind that callus induction results in dedifferentiated groups of cells, and those cells may give rise to genetic variation; in some cases, it is

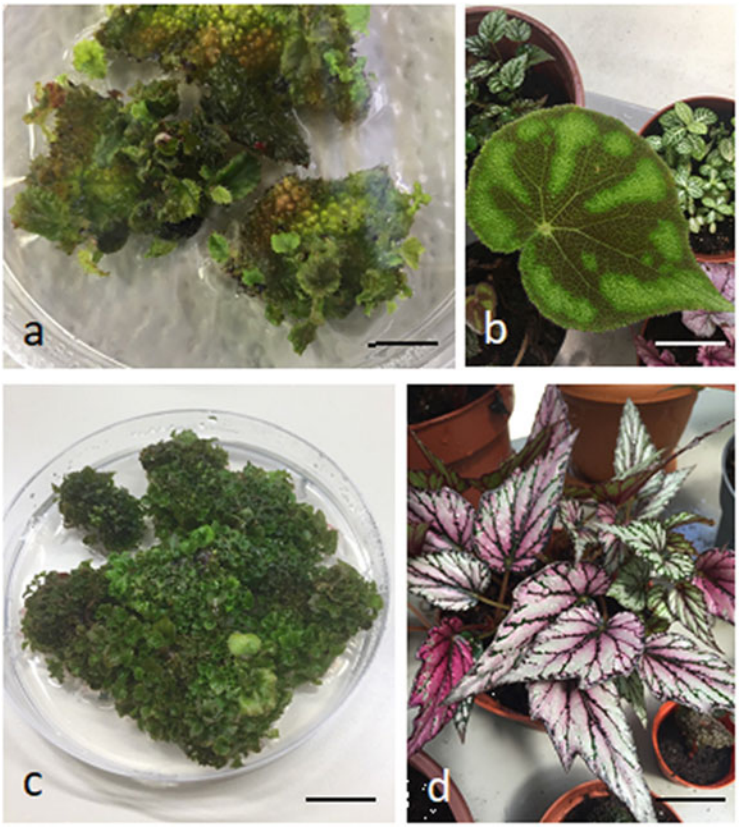

Fig. 2.2 Axillary bud formations followed by cytokinin induction after 6 weeks of leaf lamina explants and healthy plant with well shoot and root systems sourced from Cross Begonia (**a** and **b**), and *Begonia rex* (**c** and **d**). Bar scale represents 0.5 cm for (**a**) and (**c**); 3 cm for (**b**) and (**d**). (Photographs were taken from Xplant Co., Istanbul)

not advisable if the goal is to produce true-to-type plant propagation. For the protoplast isolations, leaf explants excised from aseptically germinated seeds in vitro are recommended, while ovule or anther cultures taken from immature inflorescence are used in haploid plant production (Priyadarshani et al. 2018). Genotypic variations are also very critical to check experimental parameters regarding the establishment of tissue culture protocol for a particular plant species. In some cases, explants cannot respond to the culture medium, instead, they perform tissue death after a couple of days or weeks. Since the plant genotypes display large inter- and intraspecific differences in response to the culture, some may remain recalcitrant or nonresponsive, while others may exhibit (de)differentiation activity on the contrary (Verma et al. 2016).

As mentioned above physiological age of explant, local concentrations of endogenous auxin and/or cytokinin, etc. are important criteria for the explant preferences.

Very recently, Raspor et al. (2021) mentioned a very complex developmental program regulated by auxin and cytokinin level for de novo shoot organogenesis. In that, they reported a comprehensive explanation of the hormonal regulations addressing all possible factors during the physiological development of in vitro plants. However, people usually consider the concentration of phytohormones in the medium, while what makes an actual difference in the regeneration capacity, is often the endogenous hormones within the explant itself. Therefore, in addition to Atilla Feher's conclusion (Feher 2019), the exogenous plant growth regulators reflect on endogenous hormone levels in two ways: the signal uptake from the media into the plant tissues becomes endogenous; (1) enabling hormonal homeostasis which might trigger further biosynthesis/metabolism or degradation of the phytohormones that perform a similar function(s). (2) In literature, there is a limited number of studies regarding hormone uptake through certain tissue and organ cultures. Local hormone concentration of cytokinin or particularly auxin can vary between plant parts, regardless of exogenous hormones, and variations between tissues in local hormone content are an important factor for regeneration capacity.

2 Preparation of Explants

2.1 Excision of Explant from Source Plant

Plant tissue culture techniques for the horticultural crops are mainly applicable through stem, leaf, and roots. However, this is often not as easy as it seems. Plant genotype and structure must be well observed and identified for the best explant. In general, as seen in Table 2.1, the crops differ in the axillary bud breakings using terminal buds on stems located above ground or specialized/underground stems such as bulbs scales, base plates of corms, and the shoot tips of suckers. For the banana micropropagation, shoot-tip explants were prepared by removing the outer layers of tissue from suckers with a clean knife. Since banana needs progressive surface sterilization (see below in Sect. 2.3.1), the dead tissue of each sterilized block was cut off to leave a certain portion containing an intact apex with one or more pairs of leaf primordia together with a thin rhizomatous base in contact with regeneration media. Wong (1986) reported that in the absence of apical domes from the explants resulted in two to four shoot productions only from leaf axils of Cavendish genotypes. However, Swamy et al. (1983) reported multiple shoot formations in the presence of apical domes of cultivar Robusta. The rate of shoot multiplication is not only related to explant preparation but also cytokinin use in regeneration media. In the case of banana production in vitro, benzyl adenine is the most common growth regulator for the shoot multiplication as compared to other cytokinins, while kinetin induced shoot elongation and root vigorous root formation for all cultivars tested (Wong 1986).

Explant preparation in woody plants depends on the proper time in which new growth is forced by pruning or grafting in rootstocks. For instance, walnut (*Juglans*

Table 2.1 Explant preparation addressing plant structure, plant source and explant type from some selected ornamental crops (Data obtained from Xplant Co., Istanbul)

Plant name	Plant structure	Explant source	Explant type	Tissue culture technique	Goal of the study
Monstera sp.	Underground stem	Rhizome	Segments with newest shoot tips	Micropropagation	Multiplication
Musa sp.	Underground stem	Rhizome	Shoot tips from young suckers	Micropropagation Callus culture, Embryogenesis	Multiplication
Begonia sp.	Leaf	Leaf lamina	Scarified midveins	Direct shoot organogenesis	Multiplication
Aloe sp.	Underground stem	Crown	White-creamy steam detached leaves.	Embryogenesis Organogenesis	Multiplication
Ficus sp..	Stem	Terminal buds, shoot tips	Stem segments with 2–3 nodes	Micropropagation Callus cultures	Multiplication
Phalaneopsis sp.	Fruits	Seeds	Seeds with embryos	Germination	Multiple germination of vigor seeds in vitro
Kalanchoe sp.	Leaf	Lamina	Whole or piece of scarified lamina, in some cases petioles attached with it	Direct or indirect organogenesis	Multiplication
Calathea sp.	Underground stem	Rhizome/ suckers	Shoot tip per sucker	Micropropagation	Multiplication
Aronia sp.	Stem	Terminal buds, shoot tips	Stem segment with at least 1 node	Shoot organogenesis	Multiplication
Calissia sp.	Steam	Nodes	Stem segments with at least one node	Micropropagation	Multiplication
Physalis sp.	Stem and fruits	Nodes	Stem segments from aseptic seedlings	Micropropagation	Multiplication
Crocus sp.	Underground stem	Corms	Corms with alive embryonic axis	Callus cultures Somatic embryogenesis	Callus formation Adventitious bulb production

(continued)

Table 2.1 (continued)

Plant name	Plant structure	Explant source	Explant type	Tissue culture technique	Goal of the study
Muscari sp.	Underground stem	Bulbs	Fleshy young scale leaves	Direct or indirect embryogenesis	Adventitious bulb formations
Zingiber sp.	Underground stem	Rhizome	Rhizome segments with buds	Callus culture	Multiplication

nigra) shoot culture in vitro was achieved by two types of explants from nodal cuttings (up to 1.0 cm with one to three buds each) and shoot tip (1–2 cm long) explants (Bosela and Michler 2008). Shoot-tip explants excised from walnut enables the direct elongation of the axillary shoots, while nodal cuttings typically induce shoot development through a rosette prior to the internodal elongation. Licea-Moreno et al. (2015) reported that the use of epicormic branches as protrusions out of walnut trunks are effective explant source for the walnut multiplication in timber production. Since the phenolization causes most of the fails during in vitro establishment of in many woody plants (Thomas 2008; Stevens and Pijut 2018; Bhatia and Sharma 2015), direct use of explants that are newly sprouted from sticks collected from grafted plants minimizes the phenolization under in vitro conditions (Licea-Moreno et al. 2015; Yegizbayeva et al. 2020).

Seeds are commonly used in tissue culture systems after proper surface sterilization. Seed germination under in vitro conditions provides stock plants materials as much as possible. Direct use of seeds in in vitro cultures applies to large-scale production of *Phalaenopsis* sp. Many commercial labs use in vitro germination of orchid plants (Utami and Hariyanto 2019). Since the seeds of *Phalaenopsis* are tiny, the fruits after surface sterilization (see Table 2.1) apply to commercial production of orchids through in vitro germination. Young and fresh shoots followed by germination contain cotyledonary leaves that can be a useful explant for tissue culture. For instance, Gurel et al. (2011) reported flamingo-bill type explants excised from 2 weeks old seedlings of *Digitalis davisiana*, an endemic foxglove species after germination. For the preparation of flamingo-bill explants, newly formed root tissue, one cotyledon and the shoot-tip meristem of the seedling were removed; the remaining hypocotyl with one cotyledon was used as an explant. They reported different callus types (friable, creamy, regenerative) within 2 weeks depending on culture media, and successful shoot organogenesis afterward. It was mentioned above that leaf tissue is the most important explant source in terms of showing meristematic activity in many horticultural crops (Table 2.1).

Numerous leafy plants such as exotic Begonias, Peperomias, Kalanchoes, etc. can be propagated directly on the leaf explants. However, in monocots, such as Liliaceae, Iridaceae, Amaryllidaceae, Asparagaceae, a variety of explants excised from bulb scales, base plates of corms and bulbs and inflorescence are used (Chen and Chang 2006 for *Phalenopsis* sp.; Sanikhani et al. 2006 for *Kalanchoe* sp.; Khurana-Kaul et al. 2010 for *Jatropha* sp., Yücesan et al. 2014 for *Muscari* sp.).

Due to its high regeneration capacity, *Begonia* sp. can be propagated easily by shoot-tip cutting, by rhizome and leaf cuttings as well (Toogood 1999). In vitro conditions provide more productive and alternative solutions to the clonal propagation over conventional techniques wherein the warmth and moisture promote growth at the damaged parts of the veins. The use of cytokinin, i.e., benzyl adenine, with or without an auxin combination encourages the regeneration of axillary buds of leaves in *Begonia rex and B. masoniana* (Fig. 2.2). Nhut et al. (2010) reported a large-scale production of begonia from petiole explants using a thin cell layer as a model for micropropagation from axillary buds of one plant with five petioles within 32 months. The thin cell layer was first described in tobacco and the technique was proven to be effective in many crops of monocots and dicots. The thickness of the plant tissue for the preparation of explants affects the shoot regeneration, 3 mm of TLC is recommended for the shoot formation. In the same report, BA and NAA are recommended at low concentrations (0.5 mg/L BA and 0.1 mg/L NAA) for an efficient shoot formation over 75%. Verma et al. (2016) reported various plant regeneration protocols in several endemic crocus species from corm-derived callus cultures. For the callus cultures, they investigated different explant sources including corms, leaves, and petioles, and more embryonic calli were obtained from the corm explants. In recalcitrant plants, juvenile parts of the tissue, in the case of *Crocus* sp., basal part of corms are responsive to the somatic embryogenesis and plant regeneration. Since auxins and cytokinins are also key to determine the embryogenic response due to their pervasive participation in the cell cycle regulation and cell division (Verma et al. 2016), the medium preparation and cleaning process of explants are such issues that need to be addressed.

2.2 Surface Sterilization and Contamination

Explants are required to be surface disinfected before the cultivation on the growth medium. This is widely achieved by commercial chlorine bleaches containing less than 5% (v/v) sodium hypochlorite, and the application of chemical sterilization is widely dependent on explant type. Very small seeds can be surface disinfected in microcentrifuges or other conical tubes and may require centrifugation to precipitate the seeds followed by decanting off the chemical solution with a micropipette or else. In Table 2.2, surface disinfection of some selected horticultural crops is given and the length of exposure time during sterilization depends on the explant source accordingly. In addition to commercial bleach, mercuric chloride is widely utilized at varying concentrations between 0.1 and 0.5% (w/v) for 5–10 min as a last chance for stubborn microbes. As seen in Table 2.2, where the mercuric chloride is shown as a disinfectant agent, soil-borne, and epiphytic fungi can easily be eliminated from explants. However, it is highly toxic to nearly all organisms and quickly volatiles even at significant low quantities with its vapor might be inhaled in research labs (Risher and Amler 2005). Tween-20 solution is a wetting agent added to disinfectants to reduce surface tension allowing better surface contact. When the commercial

Table 2.2 Chemical sterilization of explants sourced from some selected horticultural crops (Data obtained from Xplant, Co., Istanbul)

	Plant species	Explant type	Chemical solution	Duration of sterilization	% of successful sterilization
Ornamental plants	*Calissia repens* (pink lady)	Nodal segments	0.1% of mercuric chloride;	5 min	More than 90%
	Tradescantia zebrina (Inchplant)	Nodal segments	20% of commercial bleach (Domestos®)	10 min	More than 80%
	Fittonia albivenis (mini white)	Shoot tips, nodal segments	0.1% of mercuric chloride	4 min	More than 90%
	Calathea sp.	Rhizome; underground stem	10% Domestos® for 60 min + 5% of hydrogen peroxide for 5 min	65 min	Less than 50% (for many varieties).
	Caladium candidum	Leaf lamina	70% (v/v) of ethanol for 1 min + 10% of commercial bleach for 10 min	15 min	More than 80%
	Ficus lyrata	Leaf lamina	70% (v/v) of ethanol for 2 min + 15% of commercial bleach for 10 min	17 min	Between 70 and 80%
	Ficus benjamin	Young and fresh nodes	0.2 (w/v) % of mercuric chloride	5 min	Above 70%
	Monstera deliciosa	Rhizome nodal segments	Mix with few drops of liquid soaps for 15 min + 10% of commercial bleach for 60 min + 5% of hydrogen peroxide and 5% of white vinegar mix for 3 min	78 min	Above 70%
	Saintpaula ionantha (African violet)	Leaf lamina and petioles	0.1% mercuric chloride for min	5 min	Above 80%
	Phaleneopsis (orchid)	Seed	Hydrogen peroxide 5% for 10 min	10 min	Above 70%
	Aloe vera (and various succulents)	Stem	White vinegar and water solution (1:1) for 20 min; 20–30% of commercial bleach for	40 min	Between 60- and 80%

(continued)

Table 2.2 (continued)

	Plant species	Explant type	Chemical solution	Duration of sterilization	% of successful sterilization
			20 min with few drops of liquid soap or Tween-20		
Fruits and rootstocks	*Juglans nigra* (walnut)	Sticks	Sticks in culture room conditions pretreated with fungicide 0.1% of captain for 5 min + 15% of commercial bleach for 10 min or 0.2% mercuric chloride for 10 min	15 min	Between 60- and 80%
	Aronia melanocarpa	Nodal segments	Fungicide pretreatment for 5 min + 0.1% of mercuric chloride for 10 min	15 min	Between 50- and 60%
	Musa sp. (banana)	Suckers	Three steps pf sterilization, 50% of bleach 20 min, 25% of bleach for 15 min; 10% of bleach 10 min	45 min	Between 50 and 70%
	Various rootstocks of almonds, apricot, cherry	Shoot tips and nodes	Fungicide pretreatment for 5 min + 0.1% of mercuric chloride for 10 min, or 30–40% of commercial bleach with few drops of surfactant for 25–30 min	15–35 min	Above 80%

bleach is used in sterilization, few drops of Tween-20 make the solution soapy on the surface, and this way of sterilization decreases the contamination risks for the tissues. In the absence of Tween-20, explant can be washed with few drops of liquid soaps alone or in combination with chlorine bleach solutions. Hydrogen peroxide contains several accounts of germicidal effectiveness including its bactericidal, viricidal, sporicidal, and fungicidal properties producing destructive hydroxyl-free radicals that can attack membrane lipids, DNA, and other essential components present in pathogens (Abdollahi and Hosseini 2014).

Pretreatment of some chemical agents makes the contamination risks minimal in some cases. For instance, 70% (v/v) of ethanol or isopropyl alcohol is widely used prior to the bleach or mercuric chloride application. Ethanol is acting on coagulation on the bacteria cell wall, *Streptococcus aureus* and *Streptococcus pyogenes* were killed at a contact time of 15 s (Kampf and Hollingsworth 2008). For the avoidance from soil-borne pathogens, various underground stems, rhizomes, and suckers, etc. need intense surface disinfection when the cultivation is done in greenhouse conditions where they need high water, high humidity, rich and fertile soils. In general, explant excised from active growth stage such as spring flush tips is generally cleaner than dormant tissues. Similarly, taking plant material out of the greenhouse and placing it in relatively dry conditions a few weeks earlier than the initiation of the experiment through excision of explants from the plant may reduce the contamination risk (Smith 2013). Direct exposure of gibberellic acid (100 mg/L) onto multimodal segments of *Monstera* sp. before surface sterilization may increase bud induction at nodal segments (observed data). Explant sterilization with fungicide was observed to be effective in bulbous plants (Altan et al. 2010). For instance, *Lilium* bulb-scale explants were treated with Nystatin and Benomyl at 10 and 50 µg/g, and results were found to be effective on successful surface sterilization (Altan et al. 2010). Similarly, Haldeman et al. (1987) reported combination of fungicide and antibiotics could be used for the control of microorganisms and reduction of plant damage. The least plant damage and clean explant are desirable for a sustainable tissue culture system. To achieve this, the least concentrated disinfectant solution (s) and exposure time based on the trial-and-error method or earlier literature are highly recommended.

2.3 Medium Selection for Culture Initiation

Explant sterilization as mentioned above is the most critical stage of plant tissue culture studies. Before testing the efficacy of growth medium conditions, researchers must ensure that the risks of contamination have been completely disappeared at culture initiation. To achieve this, there are some strategies that differed from one lab to another. For instance, preparation of basal medium without growth regulators but including sugar is a useful tool to check contamination risks within the first week of culture initiation. At this stage, control of the surface sterilization provides an insight into the outcomes of culture in future efforts. In some cases, endophytic contaminations are such a big problem and need to be recovered after the addition of several chemical agents into the culture medium. The culture medium is divided into two categories including growth medium in which seed germination and seedling growth are performed, and regeneration medium is to perform shoot multiplication and root formation with or without intervening callus formation. After ensuring that there is no risk of contamination raised from explants and growth media, explant types (i.e., leaf, petiole, stem, shoot tips) should be investigated in terms of regeneration capacity for the respective regeneration medium. In literature, there are a plethora

of works in which researchers tested the regeneration capacity of different explants with respect to the culture media. For the horticultural crops, various permutations and combinations can be used for the assessment of the regeneration capacity of explants. Since the auxin and cytokinin combinations are key factors to determine the physiological responses in the cell cycle regulation and division (Gurel and Wren 1995), several combinations of auxin (NAA or IAA) and cytokinin (BAP or TDZ) efficiently induced callogenesis on the explants (Yücesan 2018). In general, BAP concentrations ranging between 0.5 and 2.0 mg/L with or without auxin (NAA, IBA, or IAA) at ranges between 0.1 and 0.5 mg/L can be available to run a tissue culture system depending on explant type and plant genotype (Beyl 2010). Since there is no rule for the way of somatic embryogenesis and organogenesis, a researcher should be very circumspect in their experimental setup taking heed of the shelf life of the culture media. Xplant Biotech company (İstanbul, TR) recommends maximum shelf life of up to 10 weeks for the culture media depending on the explant size and number for the disposable Petri plates containing 25 mL of culture media. The longer term storage may cause more root formation which makes the clones difficult to single out increasing the risk of contamination possibly raised by the worker in front of laminar flows. On the other hand, prepared stock media can be stored frozen for 24 weeks without any problem, i.e., no precipitation or reduced growth responses appear after transplanting the explants. Prepared media should be utilized within 2–3 weeks of storage at room temperature (Hall 1999). Cloning media in which cytokinin level is kept high may tend to the somaclonal variation, yet this scenario can deteriorate the uniformity of the plant lines desirable for the market. Another problem is to be faced with a vitrification problem in culture media. This rather insidious problem may suddenly appear for the horticultural crops, and what can be done in this case is indeed extremely complex and costly. Regeneration media is generally expected to produce multiple clones from axillary buds or somatic embryos through the dedifferentiation stage of explant tissues; therefore, culture room conditions (temperature, light, etc.) and culture media selection can play a key factor in the vitrification. In this sense, an increase in culture tubes, jars, or Petri plates in culture rooms will also raise up the utilities, especially electricity for the light sources, and that may cause an increase the actual temperature of the culture vessels resulting in water droplets under the lids. Unlike an efficient regeneration, as expected, this phenomenon can adversely and progressively affect plant growth and development (see Fig. 2.3). In literature, there are many reports for vitrification problems addressing the medium selection and the high number of sub-cultivations (Gaspar 1991; Reed et al. 2010). The authors recommend reducing the cytokinin concentration to a significantly low amount, or even not use it at all to avoid vitrification. Once culture medium is optimized and explants that are available for the clonal multiplication with high regeneration capacity, synseed technology favors a cost-effective approach for germplasm conservation (Yücesan 2019). In that, embryonic and non-embryonic tissues can be encapsulated in a hydrogel matrix; thus, protecting the plant materials in a gel matrix from the environment. Selected growth or regeneration media for the synseed provides a nursery for the germination

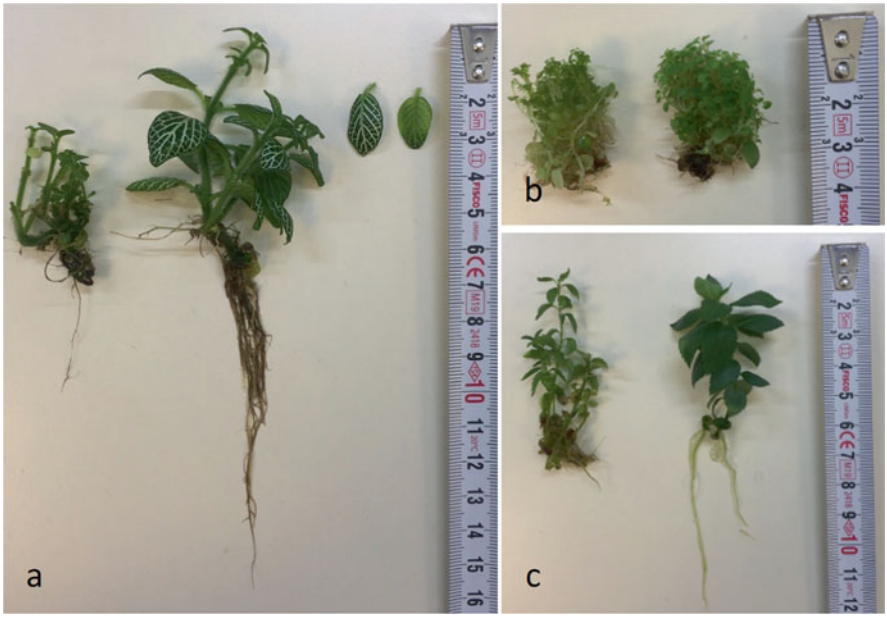

Fig. 2.3 Vitrification problem (the left ones in each figure) after subsequent sub-cultivations of Fittonia *White Nerve* plant (**a**), *Peperomia obtusifolia* (**b**) and *Aronia melanocarpa* (**c**) in a commercial plant propagation system. The plants at right position are of healthy appearance with a well-rooted system and a high survival rate during acclimatization at greenhouse conditions. (Photographs were taken from Xplant Co., Istanbul)

of the encapsulated material, and those of synseeds can be handled properly in terms of storage and transportation in a cost-effective way.

3 Practical Information and Applications on Explant Selection and Preparation for Horticultural Crops

3.1 Explant Preparation for Some Selected Ornamental Crops

Ornamental crops are propagated widely for their aesthetic value; thus, the propagation and improvement of quality flower color and fragrance, type of leaves, general plant shape and architecture, and the creation of novel variations through chimeric- or somaclonal variations can be aimed at ornamental plant market (Debergh 1994; Thorpe and Harry 1997). Micropropagation provides a great potential for large-scale plant multiplication that can be minimized the production expenses in a wide scope of low-cost tissue culture applications including medium

and explant selection with high adopting practices and low labor costs from undeveloped countries. In addition, there is no doubt that micropropagation is the only tool that maintains competitiveness among the growers for the market preferences since conventional techniques require long-term production for the hardwood cuttings that need to be woody available dipping the source material into layer beds for budding or shooting under greenhouse conditions. The adaptation of these studies according to in vitro conditions is of great importance.

3.1.1 *Sansevieria trifasciata* (Snake Plant)

Sansevieria is a very popular ornamental foliage with different colors varying from dark pale green to grayish green as variegated forms. It is widely used as an air-cleaner since the plants absorb the hazardous pollutant from the atmosphere (Yusnita et al. 2011). For the explant preparation, half or three-fourth of the young fully expanded leaves towards the base can be used and the leaves can be divided into 4- or 5 equal pieces prior to surface disinfection. Surface sterilization can be done using mercuric chloride at 0.1% (v/v) for 5 min, and 70% of ethanol (v/v) can be treated for 1 min. Kaur and Mugdal (2021) very recently reported the use of an aqueous solution of Dettol (an antiseptic detergent) and leaf explants can be further excised to small pieces (1 cm^2). Unless otherwise stated, *Sansevieria* sp. with a large number of leaf segments is of greater regeneration capacity of 90–95% within 4 months (Yusnita et al. 2011; Kaur and Mugdal 2021) depending on regeneration medium in vitro and weaning steps in greenhouse conditions.

3.1.2 *Ficus elastica* (Rubber Fig)

Ficus elastica is one of the very popular ornamental *Ficus* sp. with shiny oval leaves (10–40 cm) along the midvein. *Ficus elastica* contains a milky white latex, a chemical compound that separates from its sap and is carried and stored in different cells. For the explant preparation, young and fresh shoot tips in 4–6 cm can be excised from the mother plant. For more explants, nodal segments with dormant buds can be treated with warm and soapy water prior to disinfection in 10% of chlorine bleach for 15–20 min. All outer leaves and stems that have been exposed to bleach should be completely cut off and discarded. Since the leaves are very low regeneration capacity (observed data), stem segments, and shoot tips can be used as an explant source for regeneration. Mokshin et al. (2008) reported upper young leaves as an explant source derived from 18-month-old greenhouse samples of *Ficus elastica*. They also reported the shoot-tip had the highest regeneration capacity producing 24 shoots per explant in 1.5 cm length within 3 months.

3.1.3 *Philodendron* sp. (Love Tree)

Philodendrons have been used for interior home design as of 1880, and they grow very smoothly for long periods without intense care. They are the second largest member of the Araceae family and require very little water that makes the plants easy maintenance for the breeders (Mayo et al. 1997). Due to their beautiful foliage, philodendrons are popular in market demand with an increasing number of new hybrids varying red, yellow, or orange foliage (Chen et al. 2002). Explant preparation for the philodendron can be achieved after defoliation, shoot cuttings of 5–10 cm can be washed under running tap water for 15 min, and nodal explants with a later bud can be surface disinfected with 70% of ethanol for 1 min prior to the exposure to 1% of sodium hypochlorite (v/v) containing two or three drops of Tween-20 for 20 min on a rotary shaker (Chen et al. 2012). This protocol is effective with stem nodal segments as shoots formed directly from TDZ treatments with a frequency of regeneration 17–42% depending on genotype with 3 months.

3.1.4 *Kalanchoe blossfeldiana* (Christmas Kalanchoe)

Kalanchoe is an herbaceous, perennial succulent and popular house plant of the genus Kalanchoe which is native to Madagascar. Based on our experiences on kalanchoe in vitro propagation, regeneration capacity differs from one cultivar to another. Therefore, it is strongly recommended that one should compare several cultivars as much as possible. The general principle of explant preparation is to wash all green parts in warm and soapy water to remove soil particles. Surface disinfection for all green parts preferably leaf parts and shoot tips can be achieved in containers filled with 20% of commercial bleach for 20 min. After rinsing five times in sterile water, white areas of explants where the bleach makes white-creamy color at the edges should be discarded. Leaf lamina (15 × 15 mm), stem (max 10–20 mm), and shoot tips with primordial leaves can be placed onto regeneration medium (Smith 2013).

3.1.5 *Begonia* sp. (Indoor *Begonia rex*)

Begonia is one the largest flowering plant genus with its 2002 species around the world (Frodin 2004). The American Begonia Society classifies the begonias into several major groups, and Rex is one of the popular plants in house decors (Plants of the world 2021). Following the surface sterilization, *Begonia rex* leaves can be dissected into 4 or 5 pieces (5 cm × 1 cm) and petioles (0.5 cm) longitudinally. Each leaf explant can be placed onto a regeneration medium where the incubation takes 2 months for multiple shoots. Regeneration capacity depends genotype selection and medium selection in *Begonia* species. Tissue culture techniques can be efficiently applicable for the *Begonia* sp. as compared to its conventional

propagation techniques. For the surface disinfection, leaf explants 70% of ethanol for 45 s, and 15% of commercial bleach for 15 min can be used. Both sides of the leaf explants (abaxial and adaxial sides) can effectively be used in multiple cloning of *Begonia rex* varieties.

3.1.6 *Monstera deliciosa* (Monstera; Swiss Cheese)

With its 22 species, the genus Monstera is one of the popular ornamental crops grown for their leaves. Due to the presence of holes, the leaves can be gigantic but looks like a Swiss cheese that makes the plant famous worldwide. Since it's a vine plant that climbs up trunks with the aerial roots that help in nutrition and may completely cover the tree with its wide holed leaves. For the explant preparation, fresh and young shoots should be identified first. Then, soil particles must be brushed carefully under running tap water. Monstera is a rhizome plant that sends out aerial roots and shoots from its nodes. Thus, a number of nodes should be identified for the selection of explant. Each node discs excised from the rhizome can surface disinfected using Tween 20, ethanol 20% (v/v) and 2.5% of sodium hypochlorite solution according to the protocol reported by Palomeque et al. (2019). Explant can be placed into MS basal medium for the bud growth and development in vitro (Fig. 2.4).

3.2 Explant Preparation for the Fruits: Examples for Some Selected Cultivars

3.2.1 *Musa* sp. (Banana)

Banana is a globally important crop with over 100 million tons of production. Tissue culture techniques are a prerequisite for the clonal propagation of the crop. For the explant preparation, sword-like suckers weighing 300–1000 g can be used, and each sucker provides only one explant for the trials. Therefore, many suckers are needed to proceed with the tissue culture application for the banana (Fig. 2.4). As mentioned in Sect. 2.2.1, underground body parts may be infected by several pathogens, and banana plants grown in greenhouse conditions make it difficult to obtain healthy and clean plant materials for a sustainable tissue culture propagation. To achieve clean stock materials, shoot tips from young suckers of 20–50 cm in length can be used as an explant source. Each sucker must be trimmed into small cubes (about 1 cm^3). The optimal size of the explants depends on the culture vessels and the purpose of the experiment. The cut edges of the explants that are transferred to the culture medium can be blackened over time, and those black edges should be observed in parallel to the shoot tip growth. Otherwise, necrosis may deteriorate meristematic tissues suppressing (de)differentiation. At this stage, initial growth may be slow with a high mortality rate. For surface disinfection, progressive chemical sterilization can

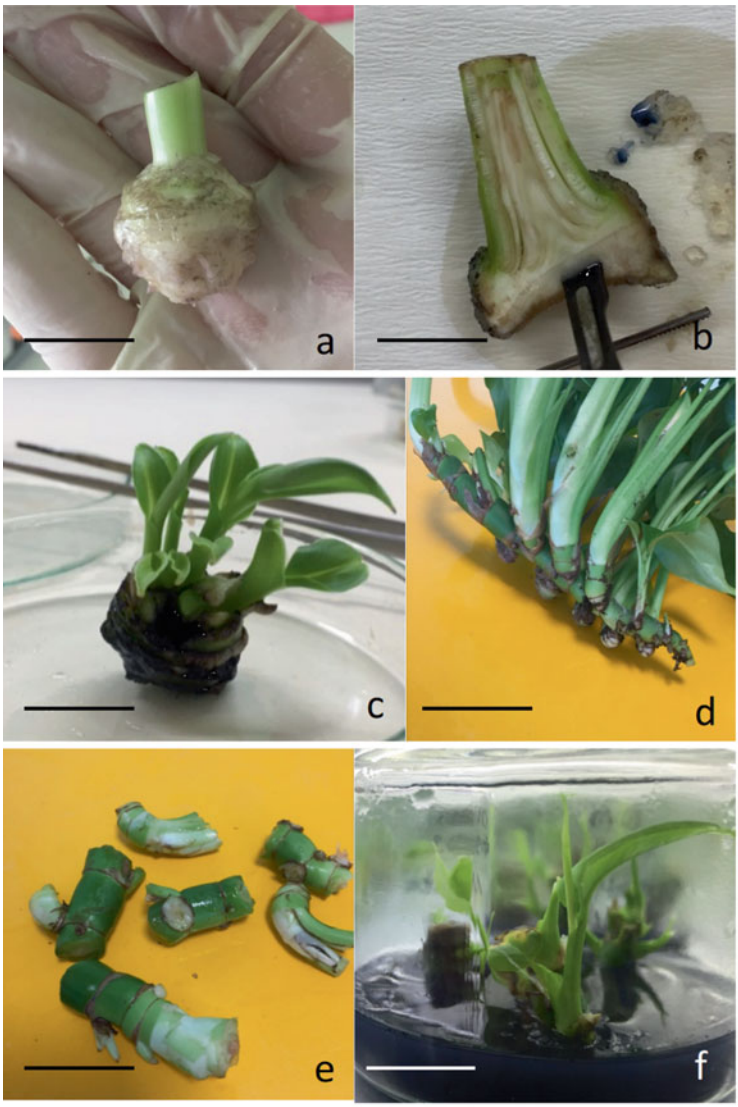

Fig. 2.4 Steps of explant preparation in dwarf Cavendish banana, a ball-like sucker ready for surface sterilization (**a**), anatomical dissection of the banana sucker with the leaf sheaths for the continuation of the stem that may induce shoot formation at the basal meristematic regions (**b**), multiple shoot formation successfully achieved after 4 weeks of culture initiation of the sucker (**c**); a rhizome structure of Monkey Monstera before surface sterilization (**d**), nodal segments after surface sterilization (**e**), rooting stage of the Monstera after 8 weeks of culture incubation. (Photographs were taken from Xplant Co., Istanbul)

be recommended for each layer of the sucker. Every step can be treated with 0.1% (w/v) of mercuric chloride for 2 min removing the outermost layer in each cycle. In total, three layers can be peeled off and preparation of cubic explant can be done thereafter. To maintain a high number of plant material, the first layer may contain some eyes which may send out young shoots over time. These layers containing eyes before trimming carefully can be transplanted into the pots and maintained under greenhouse conditions in case of scarcity of stock plant material.

3.2.2 *Aronia melanocarpa* (Black Chokeberry)

Aronia melanocarpa is one the richest antioxidant berry plants belonging to the Rosacea family. The micropropagation technique is a useful tool for the propagation of the crop since most cultivars are also nearly seedless (Almokar and Pırlak 2018). Semi-woody branches of *Aronia* sp. can be obtained from the mature plants in the spring period. Branches containing 2–3 nodes with shoot buds that are ready for shooting can be kept in a growth chamber in a dipping solution of Hoagland solution containing 0.1% (w/v) of fungicide (i.e., Captan). Following the sprouting from the nodes in each branch, fresh shoot explants were dipped into commercial bleach solution containing <5% of sodium hypochlorite for 20 min prior to dipping into 70% of ethanol (v/v) for 1 min. After rinsing the explants with the autoclaved water, nodal explants can be transferred to the growth medium.

3.2.3 *Juglans regia* (The Common Walnut)

The common walnut is a large dedicious tree and very popular economically important ornamental crop with its edible fruits. Micropropagation has been evaluated as an alternative to conventional vegetative propagation but shoots culture protocols for many cultivars have not yet been developed (Bosela and Michler 2008). For the explant preparation, Leal et al. (2007) reported an experimental protocol using plant materials showing glabrous shoot tips with 4 weeks growth, 0.5–0.8 cm in diameter with internodes in 2 to 5 cm in lengths and vegetative leaf buds that have been already present from the mature trees during the spring period. For sterilization, vegetative leaf buds (3 cm) can be excised from the branches. Surface disinfection is carried out in commercial bleach in continuous stirring for 15 min. Antioxidant solution (i.e., 100 mg/L polyvinylpyrrolidone or ascorbic acid) is sometimes used in woody plants.

4 Plant Factories: Industrial Scale Crop Production with Artificial Light

4.1 Energy Saving Solutions for the Commercial-Scale Plant Productions

In horticultural crop propagation, there are two dynamic factors including a selection of cultivar and the marketplace where the customers can afford it. As seen in dress fashion design, one should follow up what customers want, when they buy it. Over the past 30 years, the horticultural crop trade has found ever-increasing ways to propagate valuable crops in practical and cost-effective way with commercial laboratory production. A well-equipped lab system, skilled workers for plant propagation in vitro and well-established protocol addressing the best explant choice for sustainable production is sometimes not enough for all that is needed for commercial production in a timely fashion. What to grow? is a right question at the initiation of work since all outcomes needs a certain period that the entrepreneurs should be persistent, and those products are of great value to convince potential customers for their preference. In this sense, Suttle (2011) reported two risks raised from the most common failures of commercial laboratories over the last decade addressing the growing plants that the market either does not want or need, and as a second approach is to business with the customer on which contractual basis with the solving problems what they need.

As to the commercial lab systems, Kozai et al. (2016) reported an interesting point of view that people are facing a trilemma in which shortage supply of food, limitations of resources, and degradation of environments at the global level as well as national level. In this sense, plant factories play a critical for solving this trilemma using methodologies based on new concepts that need to be developed with substantially improved with less resource consumption. Plant factories with artificial light systems can be one such systems to use the resources efficiently in every step of plant tissue culture system with high productivity in unit area and production of high-quality plants without using excessive chemicals (Kozai et al. 2015). Micropropagation in commercial-scale laboratories is faced with high initial investments, electricity, and labor costs. Therefore, next-generation smart plant factories with LED (light-emitting diode array) light technologies may significantly reduce the initials and operations costs. For the horticultural crops at stage 3 size with a well-rooted system, in vitro plants can be propagated after intense research starting from the best explant choice to medium selection with the highest regeneration rate. In this sense, plant factories integrated with LED lighting may maximize the cost performance for the light quality, photosynthetic photon flux density, and lightning direction for the improvement of the micropropagation system (Fig. 2.5). Runkle (2016) reported that efficacy values of LEDs, which refers to the photosynthetic photon converted by electric energy increase within a decade, and during this period durability, longevity, and other costs can be innovated through the market demands with higher efficiency. Similarly, Lan-lan et al. (2020) reported that the effectiveness

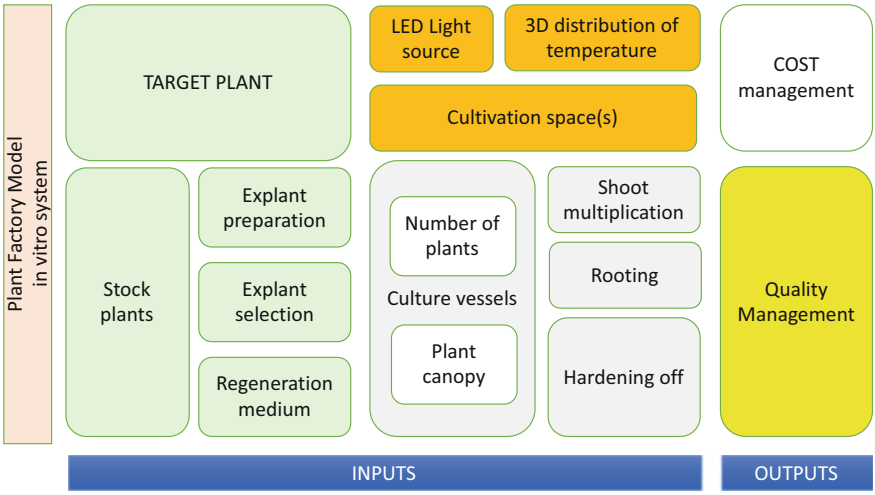

Fig. 2.5 Improvement of micropropagation systems in plant factories. The green box represents plant selection and protocol establishment. Orange boxes represent the place where the cultivation takes place with culture vessels in gray boxes. Outputs as a consequence of well-established inputs provides an insight towards the cost and quality management

of LED is due to consistent with the absorption spectra of the clones during plant regeneration, growth, and development. On the contrary, many tissue culture labs are still using fluorescent lambs. Fluorescent lamps have many disadvantages (i.e., high energy consumption due to the high calorific values) despite its developing technology over time, and LEDs may substitute the fluorescent lamps with its energy saving, and environmentally friendly technology for plant tissue culture.

4.2 Problems and Limitations from Explant to the Market

As discussed in the previous title (Sect. 2.4.1), two dynamic factors of cultivar selection and the market demands are key points to the plant propagation in commercial labs or plant factories. For the horticultural crops, ornamental plants and fruit trees have different scenarios for large-scale production in commercial labs. Fruits, in general, do have an exact time for the planting, thus appropriate scheduling for the vitro plants is a prerequisite for sustainable propagation, while many ornamental crops do not. In this case, ornamental crops, especially indoor plants (succulents, leafy plants, orchids, etc.) should be introduced into a production cycle, as it is subject to planning for production throughout the year and is shaped with different inter- or intraspecific varieties according to market demands. Yet, the latter point can be very costly in terms of the workforce. Regardless of the approach, propagation should be done by considering every plant within a suitable micropropagation

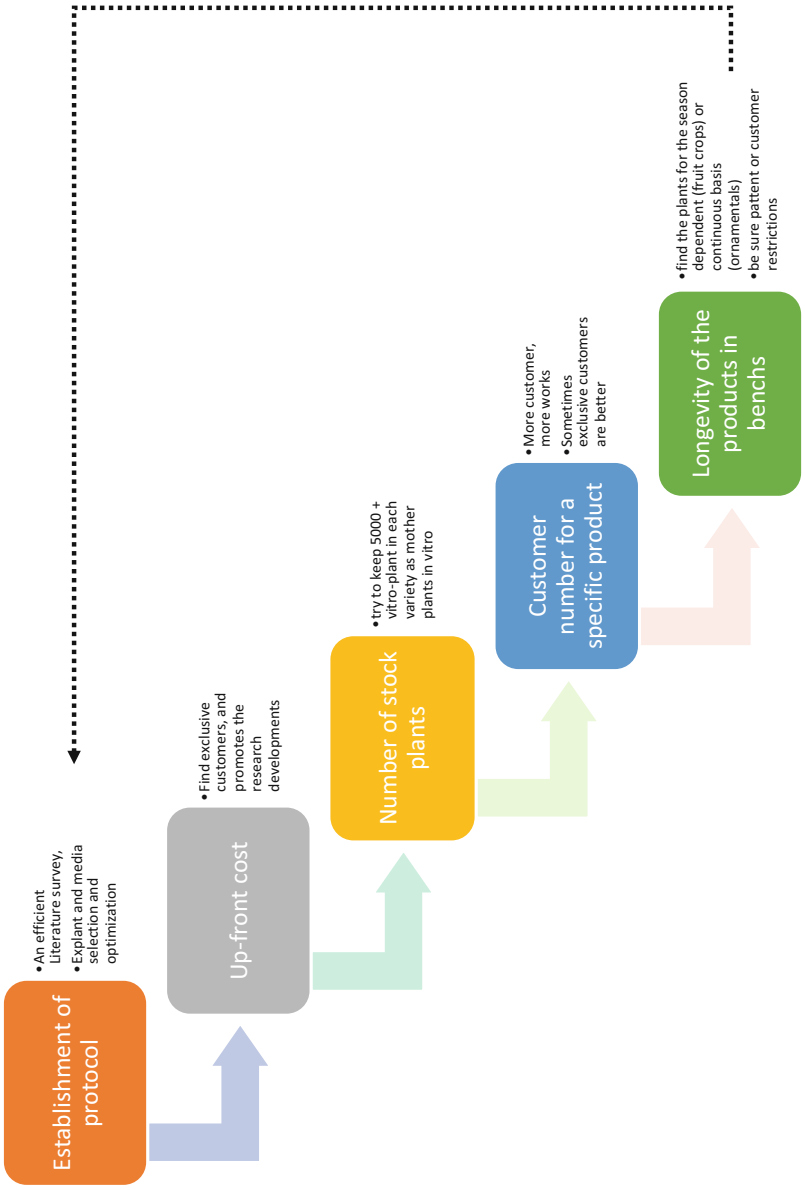

Fig. 2.6 Some steps of commercial horticultural plant propagation in vitro. Each step in the box offer a proceedng strategy for the commercial plant propagation in vitro. Dotted arrow represents research and development strategy, once needed by the potential customers starting from protocol re-establishment

strategy. Often the price of vitro plants is yet to be defined until considerable scientific research has been carried out on the protocol optimization for a given plant. For a sustainable micropropagation system, the lab team with all co-workers should be integrated into each step of plant propagation, labor numbers and their works should be identified clearly. The steps in Fig. 2.6 offer some safer routes to show the process for arranging customers with their needs in advance. In this system, estimation of the cost is highly dependent on the customer's preferences for the horticultural crops. Labor costs play a key role in the cost analysis, and it also depends on the financial status of the countries wherein the commercial labs are located. Apart from the technical staff who are responsible with literature surveys and scientific research, the number and speed of plant cutters, relatively non-technical but physically able to cut the plant consecutively are of great value for the processing. In many Asian countries, especially in China, Thailand, and India, tissue culture labs produce value-added horticultural crops within a price range between 30 and 50 US dollar cents per plant depending on the genotype and finish product volume. However, in the post-pandemic period, we witness that many vitro plants varieties as TC clones (tissue culture) are auctioned through social media (Tissue Culture, Thailand 2021) that some rare products find buyers with very high prices (over 30 US $ per culture vessel). This seems a remarkable development as a sales strategy today, and therefore, the importance of micropropagation in plant production is increasing day by day.

5 Conclusions

Plant tissue culture is an excellent and easy way to copy millions of single plants within a certain period. However, modeling an efficient system is of great importance starting from explant to the pots. In commercial production, knowledge and experience are extremely decisive as the system must be customer oriented. New plant introductions, patent activities, protocol developments and improvements are such defining roles for a sustainable propagation system. In this sense, explant selection and preparation takes its role at the first place of the work, and the knowledge behind the successive propagation with a cost-effective automation system enables the entrepreneurs to critical thinking for the improvements system for scalable productions integrated with novel approaches in which explant cutting is done by robotic systems at high clean environments, a monitoring system for the detection of early-bound microbial contaminations in culture vessels, improvement of artificial light systems used in the system, etc. Understanding the nature of explant as presented in this chapter is a key to success in commercial production in several ways including the evaluation of regeneration capacity of the given plant at given protocol in which regeneration has already been reported. If not, the whole system should be optimized starting from genotype selection to growth/regeneration medium formula with the trial-and-error method. Considering all needs for the explant is of great importance for the commercial production of horticultural crops.

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Chapter 3
Use of Alternative Components in Cost-Effective Media for Mass Production of Clonal Plants

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Abstract The demand for economically important plants, particularly medicinal and horticultural crops has increased drastically. On a large scale, the availability of propagules for the cultivation of these crops remains a challenge since plant parts such as seeds, rhizomes, and corms needed for propagation are often the economically important parts in high demand. For decades, tissue culture techniques have been used to bridge the supply versus demand gap for both medicinal plants and horticultural crops. However, mass propagation of plants in vitro is hampered by the costs associated with such in vitro techniques. In tissue culture protocols, the cost of prepared growing media, amongst other inputs, can be a limitation. Murashige and Skoog (MS), Linsmaier and Skoog (LS), Gamborg (B5), Lloyd and McCown (LM) Woody Plant, Driver and Kuniyuki Woody (DKW), BDS, BABI as well as Nitsch and Nitsch (NN) media have been used in tissue culture as basic media for decades. Main components of these media include micro- and macronutrients, vitamins, amino acids or nitrogen supplements, source (s) of carbon, and undefined organic supplements in some cases, which are solidified by gelling or solidifying agents. To attain cost-effective protocols for mass propagation, media containing different low-cost components can be used as alternatives. However, mass propagation protocols are often species-specific or vary amongst species due to differences in nutrient requirements and plant physiology. Optimization of components is imperative for achieving low-cost high multiplication and survival rates during

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acclimatization. This chapter aims to explore low-cost media for mass propagation of clonal plants to sustain the agricultural industry and counteract food insecurity.

Keywords Tissue culture · MS media · Media substitutes · Clonal plants · Mass propagation

1 Introduction

Plants are vital as crops for cosmetics, traditional and modern medicines. Amongst other factors, the demand for plants is strongly influenced by the human population and the demand in different industries such as the food, cosmetics, and the pharmaceutical industries. There are inconsistencies between plants available for disposal/accessible plants and the rate at which those plants are harvested, thus there is poor plant supply from both natural and/or cultivated plantations (Sahu and Sahu 2013). For public demand to be met and for mass and sufficient production of quality plants to be successfully provided, an array of low-cost propagation techniques should be practiced.

Micropropagation or in vitro propagation is an conservation method, which produces new organisms from protoplasts, cells, callus, small pieces of tissue (shoot tip, leaf, lateral bud, stem, or root), and/or excised organs (Idowu et al. 2009; Nkere and Mbanaso 2009; Chandra et al. 2010; Chawla et al. 2020). The technique is characterized by rapid cell proliferation, and once the culture is established, the periodic subculturing eliminates the need to generate plants from seeds (Idowu et al. 2009). An important benefit of the technique is the potential to multiply plants from small pieces of the stock plant in a relatively short period of time, regardless of the season (Ngomuo et al. 2014). Therefore, it is considered as the most suitable tool in commercial production, resulting in plant uniformity and high yields that improve productivity (Moyo et al. 2011). Micropropagation is done in closed culture vessels with plants grown in culture media supplemented with essential nutrients and plant growth regulators. The growing environment is controlled and has limited challenges such as diseases, seasonality, and deficiency of nutrients (Shahzad et al. 2017). Micropropagation as a biotechnology tool has many applications in both research and commercial industries as it provides a controlled incubation environment that can be precisely modified to achieve desired and novel developmental paths through trait selection (Goodger and Woodrow 2010). These include controlled production of secondary metabolites, protein levels, and overall plant quality. Consequently, this leads to improved product consistency (Idowu et al. 2009).

A number of successful micropropagation protocols have been developed, mostly for endangered plant species and plants that produce recalcitrant seeds, with germplasms that are difficult to store using conventional methods. These protocols are also valuable for plant species that are heterozygous, sexually incompatible and with sterile genotypes, as found in many ornamental, vegetable, and fruit crops (Conger 2018). There are many examples of commercially developed

micropropagation protocols used in the agriculture industry for food sustainability and conservation of plants or crops such as cassava, potato, artichoke, asparagus, garlic, sugar cane and many soft (small) fruits, fruit trees, various herbaceous and woody ornamental plants (George et al. 2008; Previati and Benelli 2009). One of the major demerits in the application of plant tissue culture is that it is capital and labor intensive leading to relatively high production costs, thus limiting the technique to be maximized on a commercial level.

Different plants have different genotypes. Thus, for optimum development, they need customized growing media for their specific developmental requirements. In the past, it was easy to manipulate both macro- and micronutrients in growing media as they were individually prepared as stock solutions and combined to make media (Phillips and Garda 2019). However, nowadays pre-prepared and ready-to-be-used media are available in the market for mass propagation. This makes it hard to manipulate the nutrients in the media for optimum growth and development of specific plant species or genotypes (Phillips and Garda 2019). Therefore, the efficiency of micropropagation protocols requires investigation of their suitability for application at a commercial level. This chapter aims at giving insights on the low-cost media useful for mass propagation of horticultural crops for both commercial and research purposes.

2 Components of Plant Tissue Culture Growing Media

Plant tissue culture media play an imperative role in the mass propagation of plants for both conservation and commercial production purposes. Although, the routine use of plant tissue culture techniques has been practiced for decades (Ubalua et al. 2014), large-scale micropropagation of horticultural plants has been hampered by high costs encountered by most small or emerging laboratories, as they do not have capital to procure the ever evolving high-tech machinery, their operating costs and maintenance for mass production (Kodym and Zapata-Arias 2001). Plant tissue culture media components normally include source(s) of carbon, amino acids or nitrogen source, macro- and micronutrients, vitamins, undefined organic supplements, plant growth regulators, buffers, and solidifying agents (Saad and Elshahed 2012). Sucrose is commonly used as a carbon source and agar as a common gelling agent. Inorganic nutrients or elements are considered essential for plant growth because plants fail to complete their life cycle without them; their action is specific and cannot be replaced completely by any other element, their effect on the organism is direct; and are constituents of essential plant metabolic or physiological processes (George and de Klerk 2008). For healthy and vigorous growth, inorganic nutrients are specifically optimized for different plant species. Generally, inorganic nutrients are defined according to their required concentration in plants, into macronutrients and micronutrients, with concentrations greater and less than 0.5 mM, respectively (Sharifi et al. 2010; Saad and Elshahed 2012). Besides carbon (C), hydrogen (H), and oxygen (O), macronutrients include ions of nitrogen (N), potassium (K), calcium

(Ca), phosphorus (P), magnesium (Mg), and sulfur (S); whereas, micronutrients or trace elements are iron (Fe), nickel (Ni), chlorine (Cl), manganese (Mn), zinc (Zn), boron (B), copper (Cu), and molybdenum (Mo). Cobalt (Co), aluminum (Al), sodium (Na), and iodine (I) are essential or beneficial for some species but their specific role in cell growth has not been clearly established (George and de Klerk 2008; Khan and Khan 2010; Saad and Elshahed 2012). Organic compounds, notably vitamins and plant growth regulators are incorporated to improve plant growth. Undefined organic supplements are sometimes used as substitutes for defined vitamins or amino acids and/or as additional nutrients. They include fruit juices, coconut milk, banana homogenate, yeast extracts, and protein hydrolysates (George and de Klerk 2008). All the described components contribute to the growth of plants and development, thus positively affecting tissue culture and in vitro propagation.

Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962) has been used as a basis for growing plants in tissue culture for years. MS basal medium is known to be the most frequently cited medium. Following MS medium and its modifications, Woody plant medium and Driver and Kuniyuki woody plant medium were specially developed for woody plant application (Phillips and Garda 2019). Moreover, they are both composed of potassium sulfate (K_2SO_4) and calcium nitrate ($Ca(NO_3)_2{\cdot}4H_2O$) as sources of phosphate and nitrogen, respectively, compared to MS and its modifications (Table 3.1). Though MS medium is widely used for successful regeneration of several plant species, it is not always the best medium for optimum growth for some plants (Greenway et al. 2012). As a result, other media including modified MS (MMS), B5 growing media and BABI (B5 medium as modified at Arkansas Biosciences Institute), amongst others, were introduced in plant tissue culture to provide the plants with all the essential nutrients, energy, and water necessary for growth and development (Greenway et al. 2012). Moreover, BABI basal medium provides a different macronutrient composition in terms of salts in comparison to MS (Table 3.1). The different macronutrients found in BABI basal medium makes it an effective basal medium for optimum growth for certain plant species. Table 3.1 shows different tissue culture media and their nutrient compositions that set them apart from one another. The concentrations of macronutrients in MS medium, for example, was double the concentration of the same macronutrients in LS medium, while the concentrations of the constituent micronutrients were the same in both media. There were two nitrate sources (i.e., KNO_3 and NH_4NO_3) in MS medium when compared to a single source of nitrate (KNO_3) in B5 medium. A comparison between WPM and DKW media indicates higher concentrations of macronutrients in DKW medium. These clearly show that differences in media components such as macro- and micronutrients, vitamins, and carbon sources can affect plant growth and development. Therefore, component optimization is crucial for in vitro propagation and successful micropropagation protocols.

Table 3.1 Macro- and micronutrient composition of some most frequently used growing media in plant tissue culture (Nadirah et al. 2019; Phillips and Garda 2019)

Growing media	MS	LS	B5	BDS	BABI	WPM	DKW	NN
Macronutrients (mg L^{-1})								
KNO_3	1900	950	2500	2500	2500	–	–	950
K_2SO_4	–	–	–	–	–	990	1559	–
NH_4NO_3	1650	825	–	320	320	400	1416	720
$Ca(NO_3)_2{\cdot}4H_2O$	–	–	–	–	–	556	1948	–
$NH_4H_2PO_4$	–	–	–	230	230	–	–	–
$NaH_2PO_4{\cdot}H_2O$	–	–	150	150	150	–	–	–
$(NH_4)_2SO_4$	–	–	134	134	134	–	–	–
$MgSO_4{\cdot}7H_2O$	370	185	250	250	250	370	740	185
KH_2PO_4	170	85	–	–	–	170	265	68
$CaCl_2{\cdot}2H_2O$	440	220	150	150	440	96	149	166
Micronutrients (mg L^{-1})								
H_3BO_3	6.2	6.2	3	3	3	6.2	4.8	10
KI	0.83	0.83	0.75	0.75	0.75	–	–	–
$MnSO_4{\cdot}H_2O$	16.9	16.9	10	10	10	22.3	33.5	–
$ZnSO_4{\cdot}7H_2O$	10.6	10.6	2	2	2	8.6	–	10
$Zn(NO_3)_2{\cdot}6H_2O$	–	–	–	–	–	–	17	–
$CuSO_4{\cdot}5H_2O$	0.025	0.025	0.039	0.039	0.039	0.25	0.25	0.025
$Na_2MoO_4{\cdot}2H_2O$	0.25	0.25	0.25	0.25	0.25	0.25	0.39	0.25
$CoCl_2{\cdot}6H_2O$	0.025	0.025	0.025	0.025	0.025	–	–	–
$NiSO_4{\cdot}6H_2O$	–	–	–	–	–	–	0.005	–
$FeSO_4{\cdot}7H_2O$	27.8	27.8	27.8	27.8	27.8	27.8	33.8	27.8
Na_2EDTA	37.3	37.3	37.3	37.3	37.3	37.3	45.4	37.3
Vitamins and organics (mg L^{-1})								
Myo-inositol	100	100	100	100	100	100	100	100
Nicotinic acid	0.5	1	1	1	1	0.5	1	5
Pyridoxine HCl	0.5	1	1	1	1	–	0.5	0.5
Thiamine HCl	0.1	10	10	10	10	1.6	2	0.2
Glycine	2	–	–	–	–	–	20	2
L-glutamine	–	–	–	–	–	–	250	–
Sucrose (g L^{-1})	30	30	20	30	30	20	30	30
pH	5.8	5.8	5.5	5.8	5.8	5.6	5.5	5.8

MS Murashige and Skoog, *LS* Linsmaier and Skoog, *B5* Gamborgs B5, *BDS* modification of B5 by Dunstan and Short, *BABI* modification of B5 by the Arkansas Biosciences Institute, *WPM* woody plant medium, *DKW* driver and Kuniyuki Woody Medium, *NN* Nitsch and Nitsch

3 Substitutes for Main Components of Media to Reduce Costs

Carbon source(s) and gelling agents are some of the factors that make tissue culture media expensive as they can be costly, leading to high cost of micropropagation (Purohit et al. 2011). Micropropagation or media costs can be reduced by increasing multiplication rate and/or substituting expensive media components such as agar with liquid culture using temporary immersion bioreactors (TIB) or other cheaper alternatives (Ayenew et al. 2012). For many tissue culture laboratories, the major objectives are successful multiplication and cost-effectiveness (Mohamed et al. 2010; Vorpsi et al. 2012). The growth and development of plantlets is influenced by several factors, including tissue culture conditions, concentrations of micro- and macronutrients, and physical viscosity of plant growth media (Mohamed et al. 2010). Agar contributes above 70% of the total production costs of media, thus making it the most expensive component of growing media (Agrawal et al. 2010; Mohamed et al. 2010), followed by sucrose which accounts for 15–20% of total production costs (Kodym and Zapata-Arias 2001; Sahu and Sahu 2013). Although there are several components making up plant tissue culture media, only the most expensive substitutes will be discussed, which can significantly reduce media expense.

3.1 Solidifying Agent Substitutes

For decades, agar and gelrite have been utilized as conventional gelling agents in tissue culture media to grow microbial strains and plants globally (Sharifi et al. 2010). Moreover, research and commercial industries have largely employed gelling agents like phytagel, gelatin and gelrite although they are all known to possess certain impurities. Phytagel and gelrite are said to be clearer with minimum impurities, especially when used in smaller quantities per liter compared to agar (Ubalua et al. 2014). In African countries, the price of both agar and gelrite is greatly increased by currency exchange rate and import fees (Ubalua et al. 2014). Agar is popular for its stability and resistance to metabolism, however, its affordability arising from its high cost, and over-exploration of its sources remains an important concern (Deb and Pongener 2010). High levels of agar in media may also have inhibitory effects (Mohamed et al. 2010). Table 3.2 provides a list of some alternative gelling agents that have been reported to be as favorable as agar and gelrite in tissue culture media. These include sago, isubgol, corn and cassava starch, guar gum, and ispaghol.

Henderson and Kinnersley (1988) reported that starch-gelled media had higher embryo formation and plantlet production from anthers compared to agar-gelled media. However, non-toxicity (Babbar and Jain 1998), inertness (Ubalua et al. 2014), embryo abortion (Kohlenbach and Wernicke 1978), and incidences of

Table 3.2 Alternative gelling agents for agar and gelrite

Solidifying agent	Plant	Results	Reference
Sago	*Solanum tuberosum* L. (potato)	No significant differences were observed in plant growth between sago-gelled media and agar	Naik and Sarkar (2001)
Locust bean gum (LBG)	*Ceratonia silique* L. (carob tree)	No significant differences in agar (0.9%) and combination of agar (0.6%) together with LBG (0.3%)	Gonçalves and Romano (2005)
Locust bean gum	*Rhododendron ponticum* (iberian rose)	Shoot regeneration was higher in media mixed with LBG and agar (0.4% and 0.5%, respectively) compared to media gelled with agar alone. Generally, there were no significant differences	Gonçalves and Romano (2005)
Guar gum	*Crataeva nurvala* (three leaved caper)	Shoot proliferation was better on guar gum gelled rather than agar-gelled media	Babbar et al. (2005)
Isubgol	*Isatis tinctoria* (woad)	Better shoot regeneration on isubgol-gelled media than agar-gelled media	Saglam and Cifici (2010)
Isubgol	*Musa* spp. (banana)	A significantly higher shoot and survival rate (100%) was observed on isubgol-gelled media compared to agar (79–83%)	Agrawal et al. (2010)
Isubgol	*Syzygium cuminni* (Indian blackberry/ jamun)	No significant differences in shoot regeneration between isubgol and agar-gelled media	Babbar and Jain (1998)
Ispaghol	*Solanum tuberosum* L. (potato)	Good gelling capacity and healthy plantlets were obtained on ispaghol (10, 12, and 14 g L^{-1})-gelled media. No significant differences in regeneration in media gelled with agar and ispaghol	Shah et al. (2003)
Gum katira	*Syzygium cuminni* (Indian blackberry/ jamun)	No significant differences in regeneration between gum katira and agar-gelled media	Jain and Babbar (2002)
Cassava starch	*Ipomoea batatas* L. (sweet potato)	Starch-gelled medium enhanced regeneration and propagation after 8 weeks and 3 years storage. However, gelrite and agar were most efficient	Ubalua et al. (2014)
Corn starch	*Prunus domestica* (plum)	There were no significant differences in the number of shoots obtained from corn starch-gelled medium (50 g L^{-1}) and agar (6 g L^{-1})	Vorpsi et al. (2012)

hyperhydricity (Pasqualetto et al. 1988) on starch-gelled media are indeterminate. Improved shoot regeneration was observed when a combination of starch, semolina, and potato powder was used as a gelling agent while growing African violet (Sharifi et al. 2010). Moreover, starch/gelrite mixtures containing household brand corn starch, potato starch, rice flour, cassava flour, and pounded yam were also investigated (Zimmerman et al. 1995; Kodym and Zapata-Arias 2001; Smykalova et al. 2001). Combinations of corn starch and gelrite (Zimmerman et al. 1995), as well as corn and potato starch with agar (Mohamed et al. 2010) at low concentrations, have

been used for propagation of different species. Rice, cassava, and yam flours resulted in poor quality plants (Kodym and Zapata-Arias 2001). A gelling mixture with 5% corn starch and 0.05% gelrite for fruit crops, apple and red raspberry promoted shoot regeneration (Zimmerman et al. 1995). For optimum growth and development of plants, 13% of potato and 10% of corn starch are the recommended concentrations (Kodym and Zapata-Arias 2001). However, at those concentrations, dissolving, dispensing, and purification are not easily attainable. It is therefore highly advisable to mix 6% of starch with 0.1% agar or 5% of starch with 0.05% gelrite. This is far less than the quantity needed for both agar and gelrite individually as gelling agents. Most importantly, the price of starch is 1% of agar and 1.3% of gelrite (Mohamed et al. 2010).

Higher micropropagation rates were obtained due to cell differentiation and rapid growth in *Humulus lupulus* plants cultured in media with corn starch compared to those in media with agar (Smykalova et al. 2001). There were no significant differences in results obtained from corn (50 g L^{-1}) media-grown shoots and agar media-grown shoots. Additionally, Smykalova et al. 2001 and Mohamed et al. (2010) reported more shoots obtained from explants cultured in media with 50 g L^{-1} potato and 60 g L^{-1} corn starch. Moreover, the price of corn starch was 99% lower than that of agar (Mohamed et al. 2010). Aged cassava starch (3 years old) was tested by Ubalua et al. (2014) on sweet potato regeneration against fresh cassava starch, agar, and gelrite. There were no significant differences in frequency for plants cultured in aged cassava-gelled media, agar, and gelrite. This means that aged cassava starch can be a good gelling alternative for both agar and gelrite in in vitro propagation of sweet potato (Ubalua et al. 2014).

3.2 Carbon/Sucrose Substitutes

Sucrose is another expensive constituent in plant tissue culture media (Kodym and Zapata-Arias 2001; Sahu and Sahu 2013). Commercial sugar is a good substitute for sucrose. Commercially, there are two main sources of sugar mainly cane and beet, which produce either white or brown sugar (Kodym and Zapata-Arias 2001; Sahu and Sahu 2013). In order to achieve optimum micropropagation rates, the recommended electrical conductivity (EC) for sugar should be below 150 μScm^{-1} (Kodym and Zapata-Arias 2001). The use of sugars with the EC of 440 μScm^{-1} and above can result in lower micropropagation rates. Sugars with higher EC contain inhibitors and hinder proper growth and development of plants. An experiment on banana using commercial sugar as a substitute/replacement for sucrose conducted by Kodym and Zapata-Arias (2001) revealed that commercial sugars; white and light brown to be specific, with lower EC had the highest shoot regeneration rates. The study also revealed that beet and cane sugar with low EC may have the same properties as purified sucrose from Sigma® (Kodym and Zapata-Arias 2001). Statistically, there were no significant differences in shoot regeneration rates from subcultures incorporating beet and cane sugars with low EC (<150 μScm^{-1}) when

compared to purified sucrose. According to Kodym and Zapata-Arias (2001), commercial sugar is 10% cheaper than purified sucrose and does not compromise the quality of tissue culture growing media, which leads to good quality plantlets.

3.3 Distilled Water

The major component in growing media preparation is either distilled or de-ionized water, which is generated through distillation (Santana et al. 2009; Sahu and Sahu 2013). Costs associated with distillation in order to eliminate ions and possible microbial contamination are high. Thus, autoclaved tap water and filtered water (aquaguard) can be a good alternative for distilled water in tissue culture (Prabhuling et al. 2013; Saraswathi et al. 2016). An experiment on the use of tap water and filtered water as a substitute for distilled water was conducted on *Fragaria ananassa*, *Musa* spp., and *Musa acuminata*. Bud proliferation, shoot regeneration, and growth were not compromised by the use of tap and/or filtered water (Kaur et al. 2003; Prabhuling et al. 2013; Saraswathi et al. 2016). Another study by Sunandakumari et al. (2004) on *Mentha piperita* shoot proliferation showed no significant difference between the use of tap water and double distilled water. Thus, utilization of tap and filtered water in plant tissue culture may not necessarily compromise plant quality and is much cheaper compared to distilled water (Saraswathi et al. 2016).

4 Efficiency of Mass Propagation in Cost-Effective Media

There are a number of factors affecting the success of in vitro propagation. These include media type, explant type, light, temperature, culture vessel type and headspace gaseous composition, explant polarity, subculturing, genotype, and the season of explant collection (Conger 2018). The ability of plant cells to regenerate into whole plants have proven to be a reliable basis for in vitro propagation. Propagation through plant tissue culture technique generally includes four stages: culture initiation, bud/shoot multiplication, plantlet regeneration (including rooting), and acclimatization (hardening or weaning); which are crucial for the growth of healthy new clonal plants. Mass propagation through tissue culture provides new plants containing the genetic material of only one parent (they are essentially clones of the parent plant, true-to-type plants); clonal plants also bypass the immature seedling phase and therefore reach the maturity phase faster, have optimal yield, uniformity and disease-free planting material of high quality (Ngomuo et al. 2014). Most importantly, mass propagation of economically important crops contributes to a sustainable production of raw materials in a number of commercial industries, such as the food, forestry, horticulture, and other agriculturally related industries.

Thus, the use of plant tissue culture technique can contribute towards the amelioration of the growing concern of food insecurity especially in the developing countries.

A lot of research has been done to minimize the cost of mass propagation through tissue culture by substituting expensive media components and equipment with low-cost alternatives and by increasing the rate of multiplication (Ayenew et al. 2012). Cost-effective alternatives include substitutes such as cheaper and effective gelling agents (Gitonga et al. 2010; Ayenew et al. 2012; Ranaweera et al. 2012), plant growth regulators (Gitonga et al. 2010), and basal media (Santana et al. 2009). Other cost-effective strategies include designing special equipment to improve commercial micropropagation (Etienne et al. 2013), such as using chemical sterilization (Alina et al. 2020) rather than heat sterilization and ex vitro rooting (Ranaweera et al. 2012; Etienne et al. 2013) (Table 3.3). The use of liquid media, direct shoot regeneration, and ex vitro rooting noticeably reduced the cost of micropropagation of some plant species (Kaur and Arora 2015).

A number of factors should be taken into consideration when searching for low-cost in vitro propagation protocols that can be efficiently applied commercially. An ideal medium should be able to provide nutrients required for plant growth and development and sustain a homogenous growth rate (Santana et al. 2009). A sterile environment must be sustained in in vitro propagation for disease-free plants and better plant recovery when transferred into the field. A highly efficient micropropagation protocol must ascertain high quality and well-developed plants that can easily adapt to greenhouse and field conditions. Several plant species that have been successfully produced commercially through in vitro propagation allows for availability of horticultural plants, propagules, or seedlings for plantations and sustainable production throughout the year; however, the population demand of some plants need to be met sustainably at a low cost of production for profitability. Though micropropagation is a promising tool in agro-technology, there is still a lot of work to be done to improve its efficiency at a low input cost for it to benefit small-scale farmers and the commercial industry at large.

5 Conclusion

Micropropagation is a biotechnology tool that has gained popularity in both research and commercial industries. The technique has extensive application in agriculture, food, cosmetics, and the pharmaceutical industry. It provides multiple plants from small pieces of the stock plant in a relatively short period of time, regardless of the season, resulting in plant uniformity and high yields that improve productivity. Though it is a highly suitable tool in commercial production, micropropagation can be expensive. A lot of research has been done to find alternative cost-effective mass propagation techniques. These include exploring alternative, cheaper main media, and/or media components such as basal media, gelling agents, carbon source, and distilled water. These media components contribute to the high cost of micropropagation and can be manipulated to reduce production costs. Other than

Table 3.3 Examples of horticultural and plantation crops commercially in vitro propagated using cost-effective media

Plant species	Horticultural/ plantation	Micropropagation method	Media composition		Survival (%)	References
			Shoot formation	Rooting		
Camellia sinensis (L.) O. Kuntze	Plantation	Zygotic embryos	MS basal medium (Murashige and Skoog 1962) supplemented with 3 mg L^{-1} BAP, 0.5 mg L IBA, 30 g L^{-1} sucrose, and 150 g L^{-1} sago	Pulse-treated in 50 mg L^{-1} IBA for 3 h grown in coir dust:top-soil:sand at the ratio of 1:1:1	100%	Ranaweera et al. (2012); Ranaweera et al. (2013)
Coffea arabica L.	Plantation	Somatic embryogenesis	(MS/2), 4.52 μM, 2,4-D, and 4.65 μM kinetin-(embryogenic aggregates)	–	91%	Etienne (2005); Etienne et al. (2013)
			MS/2,17.8 μM 6-BA (somatic embryogenesis)			
			MS, 1.33 μM 6-BA, 234 mM sucrose (germinated embryos)			
			Substrate surface, 3:1 peat moss-based media and coffee pulp (embryo plantlets to seedlings)		55%	
		Horticulturally rooted mini-cuttings	Somatic seedlings obtained as described above	Mixture of 30% sand and 70% commercial peat of the peat moss type	90%	Georget et al. (2017)
Ipomoea batatas (L.) Lam.	Horticultural	Nodal cuttings	2 g Easygro® vegetative fertilizer, 30 g L^{-1} of table sugar, and 9 g L^{-1} of agar	Same media was used without auxins	Kemb-36 66.7% Tainurey 67%	Ogero et al. (2012)
Malus domestica Borkh.	Horticultural	Nodal segments	20 mL PGR-free liquid MS medium with 2% sucrose	Same media was used	MM106—89% B9—87%	Mehta et al. (2014)
	Horticultural	Stem cuttings	0.2% (w/v) of Hydro Agri's fertilizer-EDTA, 2% (w/v) table	–		Santana et al. (2009)

(continued)

Table 3.3 (continued)

Plant species	Horticultural/ plantation	Micropropagation method	Media composition		Survival (%)	References
			Shoot formation	Rooting		
Manihot esculenta Crantz.			sugar, 0.25% (w/v) molasses, 0.027 µM NAA, 0.011 µM IBA, 0.027 µM IAA, 0.023 µM GA3 (from Radixone and Activol), and 10% (w/v) cassava modified starch		60% in all cultivars tested	
Musa acuminata Colla	Horticultural	Sucker cuttings	POME solution (1%), 30 g/L^{-1} sucrose, 2.00 g L^{-1} agar, 1 g L^{-1} activated charcoal, and 2 mg L^{-1} BA	–	Above 50%	Nadirah et al. (2019)
Musa spp.	Horticultural	Shoot meristems (direct organogenesis)	MS, 6 mg L^{-1} BA, 3% sugar, glass beads	MS, 3% sugar, 1 mg L^{-1} Anatone (low-cost growth regulator), and 3% gelrite	65%	Gitonga et al. (2010)
Saccharum officinarum L. cv. Nayana	Plantation	Shoots	0.3 mg L^{-1} BA, 1 mg L^{-1} PBZ, 30 g L^{-1} sucrose and 116 mg L^{-1} Vitrofural®	MS, 40 g L^{-1} sucrose and 1 mg L^{-1} NAA	95%	Alina et al. (2020)
		Spindle leaf roll segments	MS, 0.5 mg L^{-1} BA, 0.5 mg L^{-1} Kin, 0.5 mg L^{-1} IAA, 100 mg L^{-1} Myo-inositol, 3% (w/v) Sucrose, and 0.8% (w/v) Agar Shoot elongation: Liquid MS, 0.5 mg L^{-1} BA, 0.5 mg L^{-1} GA_3, 100 mg L^{-1} Myo-inositol, 3% (w/v) Sucrose	Liquid MS, 3 mg L^{-1} IBA, 3 mg L^{-1} NAA, 200 mg L^{-1} Myo-inositol, 3% (w/v) Sucrose	97%	Kaur and Sandhu (2015)
Ananas comosus var. comosus	Horticultural	Slips	MS, 1 mg L^{-1} BA, 80 g L^{-1} Enset flour "'Bulla"	MS free of hormones	95%	Ayenew et al. (2012)

Benzyladenine or 6-benzylaminopurine (BA), indole-3-butyric acid (IBA), indole acetic acid, (IAA), α-naphthaleneacetic acid (NAA), gibberellic acid (GA_3), 2,4-dichlorophenoxyacetic acid (2,4-D), Kinetin (Kin), Paclobutrazol (PBZ), Palm Oil Mill Effluent (POME), Murashige and Skoog basal medium (MS)

the substitution of expensive media components, costs can be reduced by using alternative low-cost equipment and by increasing multiplication rate. MS basal medium (full strength or half-strength) and its modification (MMS medium) are known to be the most frequently used plant tissue culture media and are generally recommended for herbaceous plant tissue culture. The use of low-cost alternatives to replace agar, distilled water, and other expensive media components can lower input cost associated with micropropagation protocols.

Agar or solidifying agents play a crucial role in plant growth and development in tissue culture. They have biochemical and structural qualities that lead to nutrient diffusion, thus impacting shoot induction in plants. Plant growth and development is a result of the media constituents, gelling agents, and explant interaction. Media gelled with gelrite and agar are easily dispensed and clear, allowing easy identification of contamination presence, compared to starch-gelled media. Moreover, cultures solidified with gelrite and agar have a longer life span compared to those with starches. Even though starches are cheaper, always available, and good agar and gelrite alternatives, they are not pure, not clear, not as inert, and are very labor intensive. Therefore, the combination of starches with agar and/or gelrite has been recorded to be a better, alternative gelling agent. Some researchers use starches individually such as sago and isubgol; however, all these require optimization per species or micropropagation protocol. Working with precision when processing starch is very important and that may lead to reduced tissue culture production costs of around 80–90%. Commercial sugar is widely used as a substitute for purified sucrose, is 10% cheaper, and does not compromise the quality of plantlets. Autoclaved tap water or filtered water is used as a substitute for distilled water, contributing to the reduction of micropropagation cost. The efficiency of the technique does not only rely on the quality of plants and regeneration rate, but also on its cost-effectiveness and simplicity to be employed by different producers from varied backgrounds in order to sustainably produce required seedlings for farmers, nurseries, and other stakeholders in the industry.

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Chapter 4
Novel Plant Growth Regulators in In Vitro Establishment of Horticulture and Plantation Crops

Priya Chugh and Arun Kumar

Abstract Several integrated signals and molecules control growth and development in plants. Plant growth regulators are such molecules, which are either present in natural form or in synthetic compounds. The former are called plant hormones and the latter are known as plant growth regulators (PGRs). These molecules have a wide range of applications in horticulture and plantation crops. The prior research evident the success obtained by the use of PGRs in plant processes such as floral induction and fruit formation, harvesting and post-harvesting of horticulture crops. The micropropagation using meristem and shoot culture produced a large number of identical plants. This technique has been commercially used in horticulture crops. Recently, several novel PGRs have been exploited with their dynamics role in horticulture and plantation crops such as jasmonate (JA), chlormequat chloride (CC), brassinosteroids (BR), salicylic acid (SA), nitric oxide (NO), strigolactone (SL), and polyamines like putrescine (Put), spermidine, and spermine. Karrikins, EDHA (a PGR containing 27% ethephon and 3% DA-6) and paclobutrazol. This chapter provides us the insight information of novel plant growth regulators, which are used in establishment for in vitro growing horticulture and plantation crops.

Keywords PGRs · Horticulture crops · Plantation crops · Plant hormones

1 Introduction

Plant hormones are the naturally producing metabolites that regulate various metabolic processes in plants especially in cell morphology, i.e., cell divisions, size, structure, and function (Ferguson and Grafton-Cardwell 2014). Hormones are

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produced at various locations and move to target sites (Rademacher 2015) and are strongly influenced by the developmental stage of plants, target tissue, nutrient availability, and environmental stress (Ferguson and Grafton-Cardwell 2014). The production of these hormones is controlled by several endogenous signals, intrinsic genetic programs together with the crosstalk between different hormones (Dias 2019). The plant hormones exist in a very low concentration, resulting in major challenges in their isolation (Rademacher 2015). So to overcome this, synthetic hormone analogs were produced which have similar functions. This conclusion results in the production of synthetic hormones at a commercial scale and is termed plant growth regulator (PGRs). PGRs have also been frequently used in various agriculture practices to improve growth under harsh conditions, enhance crop yield, and prevent post-harvest damage (Fahad et al. 2016; Bergstrand 2017). PGRs become great associates for finding the elevating production with better phytotechnical, phytosanitary, and commercial quality (Dias 2019). Plant tissue culture is the technique, which uses nutritive media as a substituent for soil for the growth of the plants under aseptic conditions. This technique evolved on a large scale in basic and applied research. There are several advantages to plants growing through in vitro techniques like disease resistance, rapid multiplication of rare plant genotypes, genome transformation, etc. (Altpeter et al. 2016). Micropropagation is a technique, which becomes a commercial enterprise and provides an advantage over the conventional propagation method (Debnath et al. 2006). As amorphous literature is available for defining the role of traditionally presented five hormones viz., auxins, cytokinins, gibberellic acid, abscisic acid, and ethylene in tissue culture. Certain novel growth regulators that are discovered and their role needs to be explored further in in vitro conditions. These novel PGRs are jasmonates, salicylic acid, brassinosteroids, strigolactone, nitric oxide, polyamines (putrescine, spermidine, and spermine), karrikins, EDAH (a PGRs containing 27% ethephon and 3% DA-6), paclobutrazol.

2 Jasmonates (JAs)

Jasmonates (JAs) are lipid-derived compounds with a wide application as signal molecules and combating abiotic stress. Jasmonates are broadly distributed in plants as a natural growth regulator. It does not work independently but forms a network of crosstalk with other phytohormones (Kaminska 2021). They are dispersed in plants tissues usually at young tips, leaves and flowers. The endogenous level of JAs increased in response to exogenous stimuli such as osmotic stress, mechanical damage, and pathogen attack.

Jasmonates along with their methyl esters are the linolenic acid-derived cyclopentanone-based compound. The α-linolenic acid is the initiating molecule released from chloroplast membranes and undergoes several steps of reaction ignited by various enzymes present in plastids, peroxisomes, and cytoplasm (Ghasemi Pirbalouti et al. 2014; Sharma and Laxmi 2016). The active form of jasmonic acid

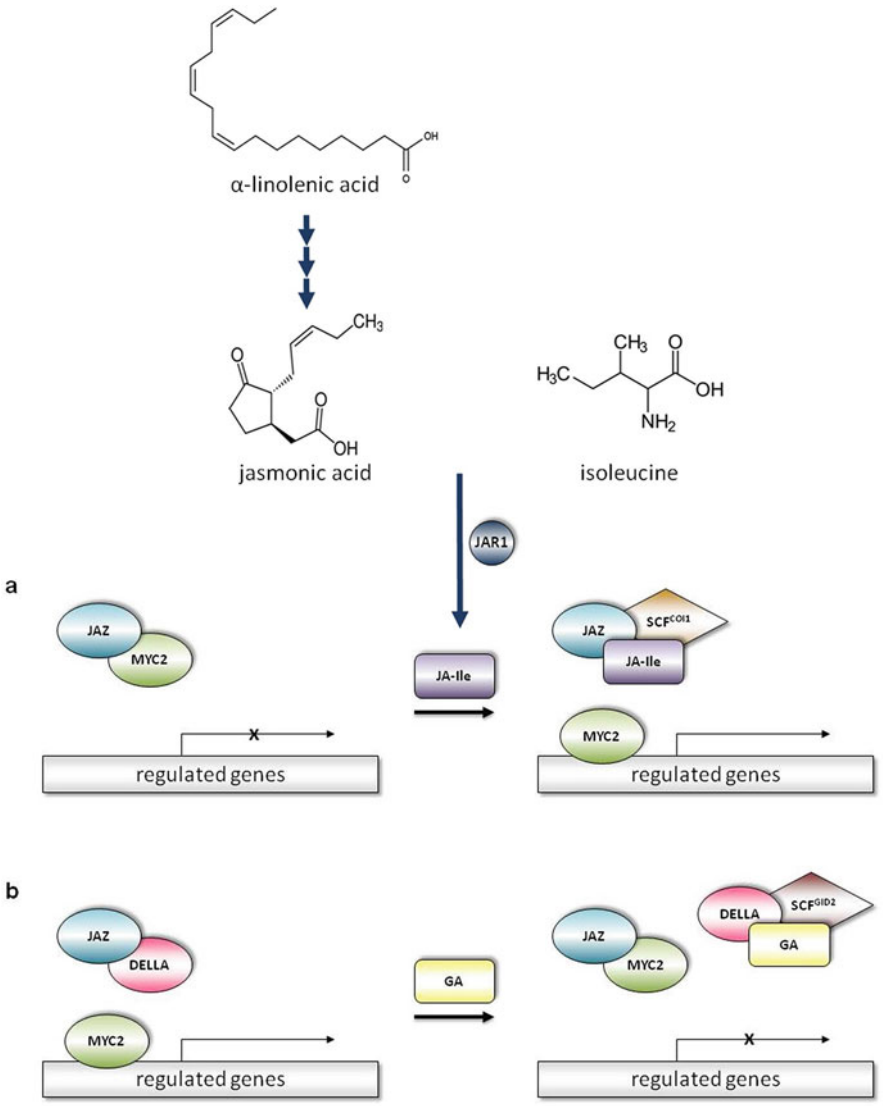

Fig. 4.1 Representation of jasmonic acid biosynthetic pathway. (Source: Kaminska 2021)

synthesized by JAR1 (Jasmonyl-L-amino acid synthetase) is JA-Ile perceived by the COI1 receptor (the F-box protein CORONATINE INSENSITIVE 1) (Fig. 4.1) (Wasternack and Hause 2013; Ueda et al. 2020). Staswick (2009) reported in chemical analysis that OsJAR1 encodes enzyme-conjugated JAs with isoleucine, tryptophan, valine, methionine, and phenylalanine. In the absence of JAs, the transcriptional repressor JAG (JASMONATES ZIM DOMAIN) binds and inhibits the MYC members of the transcription factors, which leads to the leaf's growth. The

JAZ-MYC interaction also takes part in plant growth during plant defense responses (Major et al. 2017; Guo et al. 2018). JA-Ile binds to the SCFCOI1 (Skp1-Cullin-F-box) ubiquitin ligase complex and promotes degradation of JAZs thus releasing MYC2 to trigger the expression of JA-responsive genes.

3 Role of Jasmonates in Tissue Culture

Jasmonic acid and its derivative inclusively called jasmonates (JAs) are mainly lipid-deduced signaling composites. The α-linolenic acid (α-LeA) of chloroplast membranes led to the formation of jasmonates by oxidative/reductive processes through the lipoxygenase pathway. Accordingly, JAs are members of the oxylipins family (Wasternack and Song 2017). The role of JAs is related to several alterations in plant structural processes. The first JA derivative is the methyl ester of JA. Numerous studies have shown that JAs and MeJA (methyl ester of JA) is involved in plant senescence by stimulating chlorophyll degradation. They have also been constituent in signal transduction pathways that control defense responses in plants and have shown efficiency to enhance the secondary metabolites products in cell culture (Wasternack 2015). The role of MeJA is to increase cellular respiratory, proteolytic and peroxidase inside the leaves (Liu et al. 2016). Additionally, Rohwer and Erwin (2008) followed by Hummel et al. (2009) reported that JAs also are involved in defense responses to herbivore attacks, promotes shooting and storage organ formation. They even have an extensive operation in plant propagation through micropropagation approaches in tissue culture (Fig. 4.2). Successful micropropagation rigorously relies upon the relevant PGRs and their suitable concentration. Exogenously JAs can affect numerous morpho-physiological responses in the plant life cycle. Jasmonates do not work singly, however, involved in a complex signaling pathway (Yang et al. 2019). Exogenous JAs restrict plant growth and development by repression of cell growth and expansion (Patil et al. 2014; Cipollini 2005; Major et al. 2020). The high level of endogenous production of JAs was observed in young organs with a high rate of cell division. The growth-promoting ability of JAs cannot be barred and evidence reported that JAs-intermediated response might be the result of changes in internally produced cytokinins level, which affects and regulates cell cycle (Avalbaev et al. 2016). JAs also act as growth inhibitors as observed in *Vicia faba* pericarp under ex vivo conditions due to cell cycle disorder (Dathe et al. 1981). JA and MeJA are the potent inhibitors of kinetin as well as N-phenyl-N′-(2-chloro-4-pyridyl)urea-prompted callus growth in *Glycine max* (Ueda and Kato 1982). JAs caused disturbance in cell cycle progression in *Nicotiana tabacum* BY-2 cell lines which was due to prevention of DNA replication (Swiatek et al. 2002). The molecular techniques such as gene expression studies showed that MeJA arrested G2 phase of the cell cycle in Arabidopsis genome by repressing the activation of the M phase (Pauwels et al. 2008).

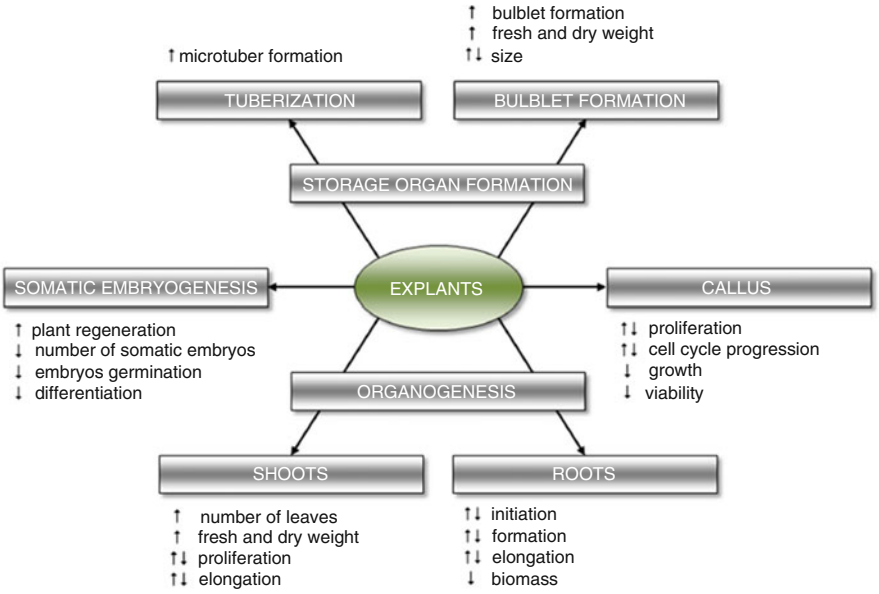

Fig. 4.2 The influence of jasmonic acid under in vitro conditions. (Source: Kaminska 2021)

Andrys et al. (2018) while propagating Lavender (*Lavandula angustifolia* Mill.) used MS media supplemented with JA with various concentration levels to influence the growth, antimicrobial and antioxidant activity. The results from these studies revealed that high JA concentration (1–1.5 mg dm3) in media results in a decrease in the number of secretory trichomes while low JA concentration (0.5 mg dm3) causes an increase in the number of trichomes. Furthermore, the antioxidative and antimicrobial activity increased at higher JA concentrations (Andrys et al. 2018). JA in concentrations of 0.5–2.0 μM reduced the time for shoot initiation, amplified shoot and root length, leaves number, nodes, and leaves of *S. tuberosum* growing in MS medium (Kumlay 2016). Ruduś et al. (2001) reported the inhibition in callus growth by JA and MeJA in *Medicago sativa* during the differentiation stage at concentrations varying from 5 to 50 μM. For numerous horticulture species, JA at a concentration up to 10 μM promoted shoot proliferation when added to a medium. Media supplemented with JA and N6-(2-Isopentenyl) adenine (2-iP) augmented shoot multiplication in onion (*Allium cepa*, 0.1–10 μM JA) and *Narcissus triandrus* (4.8 μM JA) (Santos and Salema 2000). The increase in shoot number of *Dioscorea cayenensis* and *D. rotundata* was observed using 10 μM JA combined with kinetin (Ovono et al. 2007). The addition of 1–10 μM of JAs into culture medium supplemented with IBA or NAA increased leaf morphological growth, fresh and dry weight of shoots of *Pyrus communis* and *P. cerasus* × *P. canescens* shoots (Ruzic et al. 2013). In *S. tuberosum, B. oleracea* and *Lycopersicon esculentum* root formation was stimulated by JAs at low concentrations (up to 1.0 μM) (Ravnikar et al. 1992; Tung et al. 1996; Toro et al. 2003; Zhang et al. 2006). JAs is also

responsible for inducing changes in the cell division marked by cortical microtubules reorientation and radial expansion at the initiation stage of storage organs conformation (Podwyszyńska et al. 2015). JAs involvement in tuber formation not only restrict to stolon apex in plants (Cenzano et al. 2003) but also during further cell expansion of medullary tissue in *S. tuberosum* micro tuber discs cultured in vitro (Takahashi et al. 1994).

4 Thidiazuron

Thidiazuron possesses cytokinin-like activity with molecular weight of 220.25 and formula-$C_9H_8N_4OS$. It does not contain the nitrogenous base ring as observed in other adenine-type cytokinins such as benzyl-aminopurine (BA), zeatin and kinetin (Kin). It is the synthetic PGR, earlier used as a cotton defoliant and an effective synthetic PGR for organogenesis, reformation, and various developmental pathways, including shoot promotion, somatic embryogenesis, and in vitro flowering induction (Guo et al. 2011). TDZ has enabled the in vitro cultures practice for woody and recalcitrant species, which led to their genetic transformation and improvement. There are some drawbacks of TDZ related to its usage in plant tissue culture (Dewir et al. 2018). Matand et al. (2020) reported the high proliferation of daylilies under in vitro conditions using TDZ. Generally, TDZ used at low concentrations to initiates extracellular signal that were perceived and transduced into cell development and metabolic process under in vitro plant propagation (Khan et al. 2014; Makenzi et al. 2018). For inducing shoot organogenesis, TDZ concentration less than 1 mM has proven to be effective (Giridhar et al. 2018). One report states that being a phenylurea compound it has cytokinin-like potency. The application of TDZ induce cytokinin response. TDZ analysis for organogenesis at low concentration (0.0–0.005 μmol L^{-1}) induces ZR (Zeatin ribosides), while high concentrations (0.5 μmol L^{-1}) its result were similar to isopentenyl adenine (iP), caused rapid cell division and stimulation of shoot organogenesis in *Dianthus* spp. (Casanova et al. 2004). Matand et al. (2020) used thidiazuron on stem tissue and reported successful induction of multiple shoots as compared to the traditional protocol on daylilies. The various concentrations of TDZ were used in several banana (*Musa* spp.) species to induce direct organogenesis, where 1 ppm TDZ was suitable and resulted in the highest number of plantlets, shoots, and increased plant height (Rai et al. 2019).

5 Brassinosteroids

The naturally occurring steroidal compounds that were recently added to the category of traditional hormones and considered as the sixth novel plant growth hormones are brassinosteroids (BRs). BRs control numerous important developmental and growth processes such as initiation and termination of flowering, plant canopy

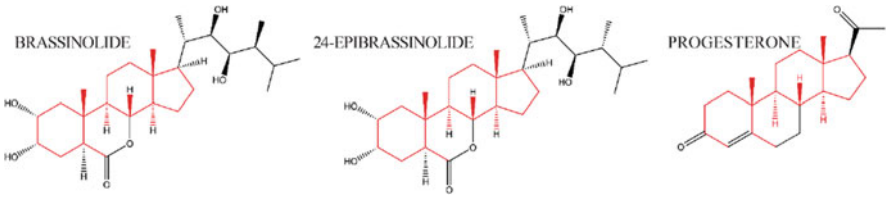

Fig. 4.3 The structure of brassinosteroids: brassinolide,24-epibrassinolide, and progesterone. (Source: Oh et al. 2020)

structure, micropropagation, cell morphology, vegetative growth, flowering, fruit set and ripening, quality, and yield. BRs are also effective in improving resistance/tolerance to harsh biotic and abiotic stresses (Baghel et al. 2019). These are also well known to augment post-harvest fruit management. BRs collectively referred to as "pleiotropic phytohormones" due to multiple and diverse physiological roles in plant development (Kanwar et al. 2017). The structures of various brassinosteroids are shown in Fig. 4.3.

The position of BR biosynthesis is confined to the endoplasmic reticulum (ER) and the receptors are present at the cell surface. The enzymes involved in BR biosynthesis in *Arabidopsis* have been localized in ER (Northey et al. 2016). From the ER, brassinosteroids moved into the apoplast, where they are straightforwardly attached to BR INSENSITIVE1 (BRI1)-a plasma membrane (PM) receptors (Fig. 4.4) and its homologs, BRI1-LIKE1 (BRL1) and BRL3 (Nolan et al. 2020). In Fig. 4.4, when BRs are absent (left), plasma membrane-receptors BRI1 and BAK1 are repressed by BKI1 and BIR3. Furthermore, BIN2 kinase capacities as a negative controller and phosphorylates BES1 and BZR1 (transcription factors) hinder their movement via numerous systems. BSS1 structures a complex with BES1 and BZR1 in the cytoplasm, and THXh5 lessens BZR1 in the core, further inactivating these TFs. This prompts generally a low expression of BR-initiated genes and higher expression of BR-stifled genes. At the point when BRs, are available, they bind to the receptor BRI1 and co-receptor BAK1 and initiate BR signaling (right). BKI1 and BIR3 separate from the receptor complex, permitting BRI1 and BAK1 to become phosphorylated and actuated. BSKs/CDGs are phosphorylated and actuate BSU1 phosphatase to restrain BIN2. Dephosphorylation by PP2A permits BES1 and BZR1 to work with other TFs and cofactors to promote BR-prompted gene expression and restrain BR-stifled gene expression (Table 4.1).

Pacholczak et al. (2021) concentrated on the impact of BRs on root cuttings of *Berberis thunbergii*. The cuttings were splashed with a water solution of the growth regulator. BRs impact emphatically on the level of establishing and root length. The physiological and biochemical boundaries, i.e., chlorophyll content, total soluble sugar, free amino acid, and hydrogen peroxide and catalase activity were also elevated. BRs also play role in xylem differentiation in culture medium, as auxin and cytokinin promote differentiation of tuber explant into the tracheary component (TEs). The nanomolar concentration of brassinolide expanded the rate of tracheary development by ten times in *Helianthus tubersosus* (Iwasaki and Shibaoka 1991)

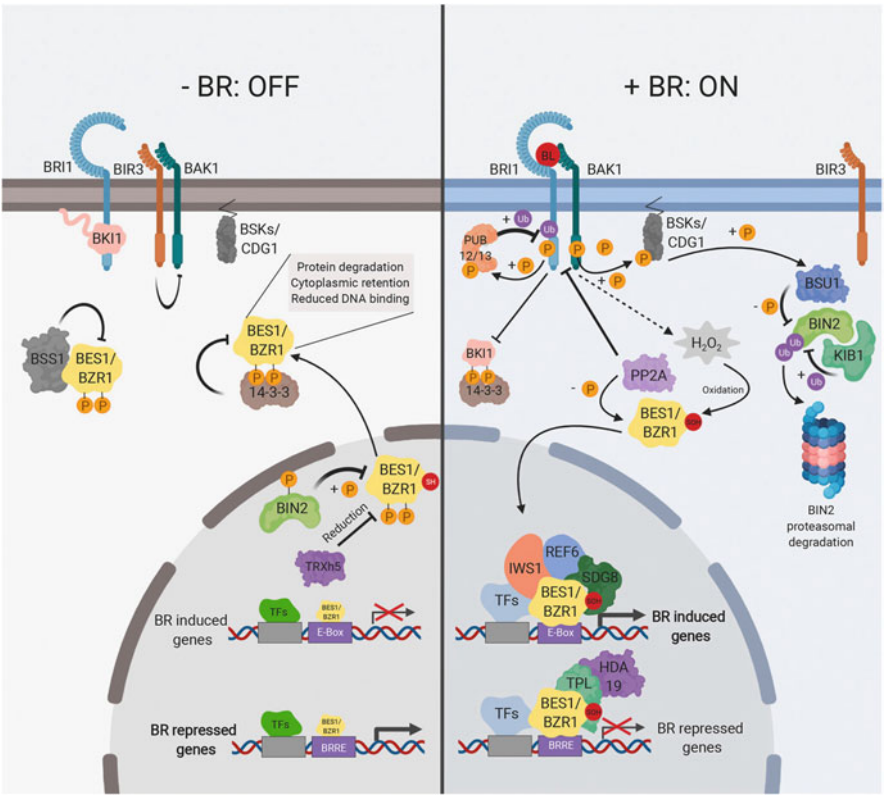

Fig. 4.4 BR signal perception pathway. (Source: Nolan et al. 2020)

Table 4.1 Abbreviation used in BR signaling pathway

Abbreviation	Meaning
BRRE	*BR response element*
BSU1	*BRI1 SUPPRESSOR1*
P	*Phosphorylation*
PUB12/13	*PLANT U-BOX12/13*
SDG8	*SET DOMAIN GROUP8*
SH	*Reduced Cys residue*
SOH	*Oxidized Cys residue*
TPL	*TOPLESS*
TRXh5	*THIOREDOXIN H-TYPE5*
Ub	*Ubiquitination*

Source: Nolan et al. (2020)

Likewise, there were more tracheary elements and a greater number of total cells showing that BRs promote cell division in the culture medium (Oh et al. 2020). The role of BR has been exploited on countless horticulture species, for example, cassava (*Manihot esculenta*), yam (*Dioscorea alata* L.), and pineapple (*Ananas comosus*)

and have been improved by the utilization of 28 homocastasterone as recommended by Bieberach et al. (2000).

Further examinations recommended the utilization of 5F-HCTS (28-homoethylcastasterone) branch elongation in in vitro developed shoot of *Malus prunifolia* along with the release of ethylene (Pereira-Netto et al. 2006). 24-epibrassinolide was used in the micropropagation of orchids by Malabadi and Nataraja (2007a, b), who revealed effective initiation of protocorm-like bodies and in vitro recovery of *Cymbidium elegans* utilizing shoot tip area. The in vitro impact of brassinosteroids was noticed depending on the various alteration in shoots number, chlorophyll content, hill reaction activity, antioxidative enzymes, polyphenol oxidases (PPX), and ascorbate peroxidases (APX) in *Arachis hypogea* (L.). The outcome uncovered that in vitro shoot augmentation was viewed as best in 1 mL L^{-1} BR concentration. The antioxidative enzyme activity was also increased in the presence of BR (Verma et al. 2012).

5.1 Salicylic Acid

Salicylic acid (2-hydroxy benzoic acid) is a water-dissolvable auxiliary metabolite and phenolic compound. SA has been utilized in various plant species both in vivo and in vitro to investigate its role in synthesis and accumulation (Ali 2020; Singh and Gautam 2013). SA initially was proposed to be synthesized from phenylalanine by means of cinnamic acid (Dempsey et al. 2011). In some bacteria, the *Arabidopsis* SA-*deficient* 2 (*sid2*) mutant uncovered that pathogen-induced SA synthesized through the isochorismate pathway. Wildermuth et al. (2001) explained that enzyme phenylalanine ammonia-lyase (PAL) changes phenylalanine over to trans-cinnamic acid, which acts as an alternative precursor of SA in the phenylpropanoid biosynthesis pathway.

The exogenous use of SA (0.5 mM) was advantageous in vitro shoot development and multiple shoot regeneration, root arrangement and prolongation, survival rate of plant and proline content (Sakhanokho and Kelley 2009). The expansion of salicylic acid to the supplemented medium solution of hydroponically cultured *Silybum marianum* at various concentrations (100, 200, and 400 M) revealed more production of flavonolignans as examined by proton nuclear magnetic resonance. Alvarado et al. (2019) reported that SA act as an elicitor in numerous medicinal plants under in vitro conditions and increases the concentrations of bioactive compounds. It has been accounted that the root culture of *Withania somnifera*, when elicited by JA and SA, upgraded the development of the bioactive molecules Withanolide A, Withanone, and Withaferin A (Sivanandhan et al. 2013). Mozafar et al. (2017) concentrated on the role of SA and Fe-nanoparticles in the tissue culture of strawberries to conquer the effect of dry spell. SA makes up for the adverse consequence of moisture stress on strawberry plants. The analysis concluded that Fe-nanoparticles in combination with SA can be a useful technique for giving higher amounts and quality in the culture of strawberries and helpful in adjusting plants to

drought conditions prior to transplanting in the field conditions. The addition of SA with silver nanoparticles on the culture media leads to the raised level of glycosides as well as alleviates the callus growth (Golkar et al. 2019). MS media supplemented with 100 mg L^{-1} led to an increase in leaf number and area, shoot and root number, length in potato (*Solanum tuberosum*), (Alutbi et al. 2017). Treatment of MeJA and SA increased the production of saponins in adventitious roots of Javanese ginseng (*Talinum paniculatum*). The adventitious roots were sub-cultured in the medium with various concentrations of MeJA or SA and incubated for 5, 10, and 15 days. The results revealed that the saponin production level was elevated by 1.5- and 1.3-fold upon addition of 0.2 mM MeJA and SA for 15 days (Faizal and Sari 2019). The exogenous supplementation of salicylic acid in culture media became effective in stimulation of secondary metabolites production, which could be partially attributed to the increase in photosynthetic pigment contents and ROS (reactive oxygen species) scavenging capability in addition to improved plant growth and biomass in *Withania coagulans*. Furthermore, the exogenous supplementation of salicylic acid also regulated the mRNA stage of genes concerned in secondary metabolite biosynthetic pathway for enhancing the production of the secondary metabolite, photosynthetic efficiency, improved plant bloom and increased plant biomass of tissue culture raised seedlings of *W. coagulans* (Maurya et al. 2019).

6 Polyamines

Polyamines (PA) are molecules constituents of bending carbon (C) chains with amino groups that are anionically charged at neutral pH. In eukaryotes, there are three biogenic molecules viz., putrescine (Put), spermidine (Spd), and spermine (Spm) considered to be polyamines and they are created through various pathways. Diamine putrescine is synthesized with the use of the arginine decarboxylase (ADC) pathway or ornithine decarboxylase (ODC) pathway (Rakesh et al. 2021). The additions of aminopropyl groups to Put synthesizes Spd and Spm. Methionine, which is common precursor of ethylene and PAs is converted into S-adenosylmethionine (dSAM) which undergoes decarboxylation reaction in the presence of enzyme SAM decarboxylase (SAMDC) and releases aminopropyl groups (Kaur-Sawhney et al. 2003). Spermidine is produced when spermidine synthase (SPDS) transfers aminopropyl group to Put. Similarly, spermine is produced when spermine synthase (SPMS) transfers the aminopropyl moiety to Put. Spd and Spm are found in cell wall attached to pectic polysaccharides and controls lignification, cell wall pH etc., while Put is found in cytoplasmic extracts (Bais et al. 2000) (Fig. 4.5).

1 mM L^{-1} polyamines [spermine (Spm) and putrescine (Put)] were tested for callus growth of *Pinus sylvestris* in Krogstrup medium (K) and Murashige and Skoog medium (MS). Spm (spermine) had growth inhibitory response in both media putrescine downregulated the arginine decarboxylase (ADC) and ornithine decarboxylase (ODC) activity in K medium. While in M medium, Put upregulated

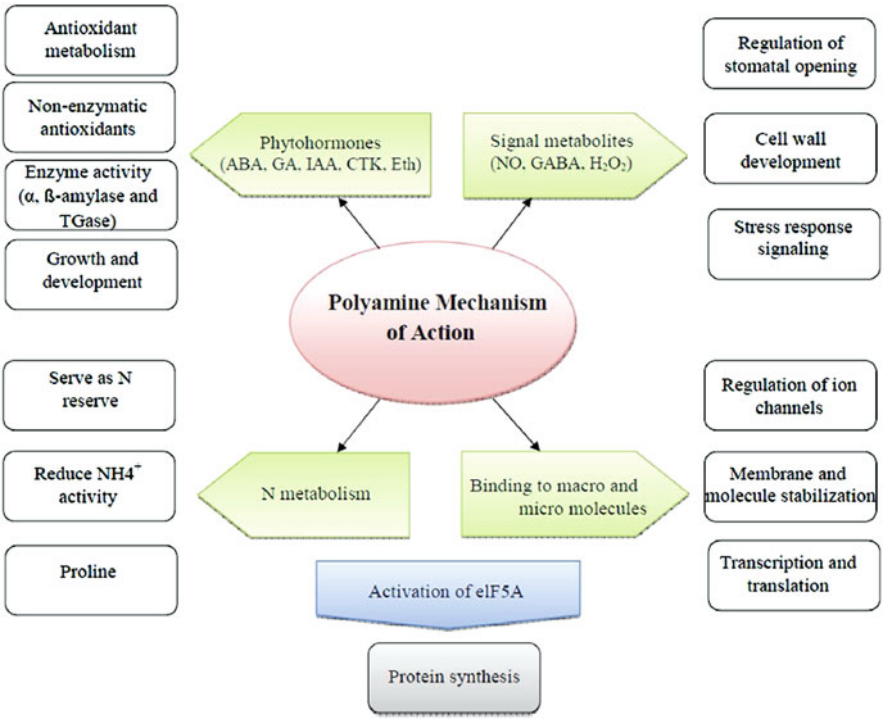

Fig. 4.5 Various roles of polyamines in plants. (Source: Mustafavi et al. 2018)

dry weight via two fold as compared to K medium and additionally improved ADC and ODC activities (Rakesh et al., 2021). Tang et al. (2005) studied the recovering of brown tissues into ordinary callus in *Pinus virginiana* and showed that Put, Spd, and Spm at concentration 1.5 mM individually confirmed 19.4, 18.9, and 1.4% recovering rate, respectively, and there was an upregulation in the total antioxidative activity and metabolites. However, a mixture or combination of these polyamine led to a lower retrieval rate in recovering brown tissue in *Pinus*. The role of PAs along with PGR in tissue culture enhanced multiple shoot induction and rooting in pea plant regeneration (Ajithan et al. 2020).

100 μM Spm and 1000 μM Put were found to be best for embryogenic callus expansion, meristemoid formation, and rhizogenesis in coconut (Rajesh et al. 2014). PAs proved to recover the quality of in vitro cultures of *Bacopa monnieri* and findings suggest that the polyamines enhanced the phenolic, flavonoid, and antioxidative content. Further Spd at 1 mM concentration along with BAP at 2.0 mg L^{-1} showed best result for shoot improvement (Dey et al. 2019). In *Stevia*, the positive effect of polyamines and plant growth regulators was studied and observed that 2 mg L^{-1} each of 2, 4-D and BA along with 2 mg L^{-1} Spd showed high-quality callus performance. Whereas media supplemented with 1 mg L^{-1} BA and 1 mg L^{-1} Spd displayed best result for shoot regeneration. However, media with

2 mg L^{-1} Put showed better outcomes. Culture media having 1 mg L^{-1} NAA in combination with 1 mg L^{-1} Spd showed best rooting performance (Khalil et al. 2016). The effect of polyamines was also studied in horticultural plants such as *Acacia, Lycopersicon, Sulcorebutia,* and *Syringa.* In *Acacia* and *Sulcorebutia*, 0.3 mM and 1 mM Put, respectively, provided a satisfactory response in shoot length, fresh weight, and rooting percent. While in *Lycopersicon*, 0.5 mM Spd confirmed the excellent effects. In *Syringa,* 0.5 mM Put followed by using 0.2 mM Put gave the best result (Scholten 1998). Kim et al. (2016) revealed that 50 mg L^{-1} Put in the culture medium fortified with 2 mg L^{-1} BAP showed in vitro shoot initiation and development in *Polygonum tinctorium*. Spm and Put have been analyzed for their impact on shoot initiation in *Ruta graveolens*, and it was recorded that 80 μM Spm expanded shoot capacity by 2.5 fold. The furanocoumarin content, a primary natural compound found in *Ruta graveolens,* was enhanced by Spm (Diwan and Malpathak 2008). In *Pisum sativum*, Spd at 20 mg L^{-1} showed the best result for shoot regeneration (Alvarado et al. 2019).

In another study on date palm, Put and Spm (at 100 mg L^{-1} concentration) and 200 mg L^{-1} glutamine in culture media showed high number of somatic embryos. On the other hand, high embryonic callus fresh mass was produced by explants containing spermidine alone and a combination of glutamine and spermidine in solid media. Glutamine in combination with putrescine and spermidine was significantly better in the maturation of embryonic callus to promote somatic embryogenesis of date palm (El-Dawayati et al. 2018).

7 Karrikins

Karrikins are compounds isolated from plant-derived smoke (Van Staden et al. 2004; Gupta et al. 2021; Kulkarni et al. 2021). Six karrikins have been determined, namely KAR1, KAR2, KAR3, KAR4, KAR5, and KAR6 (Nair et al. 2014). KAR1 and KAR4 are the most active form of karrikins (Nelson et al. 2012). Pyrolysis of cellulose and sugar produce karrikins (Flematti et al. 2011; Van Staden et al. 2004). Karrikins negatively modify hypocotyl elongation in plants but stimulate seed germination and photomorphogenesis (Nelson et al. 2010; Waters et al. 2017; Gupta et al. 2020). The chemical structure of karrikins is somewhat similar to phytohormones strigolactones (SLs) as butenolide ring of KARs and lactone D ring of SLs are closely associated (Waters et al. 2012; Zwanenburg et al. 2016). The KARs structure combines a six-membered pyran ring with a five-membered butenolide ring (Nair et al. 2014). The difference among their structure is based on methyl groups (Fig. 4.6).

Smoke water has been used to increase the embryo formation in *Pelargonium hortorum* hypocotyl explant. Treating the hypocotyl with smoke water during the induction phase doubles the number of embryo from per explant (Senaratna et al. 1999). In another study, smoke water was also used to increase the somatic embryogenesis of *Pinus* wallichiana genotypes (Malabadi and Nataraja 2007a, b). The

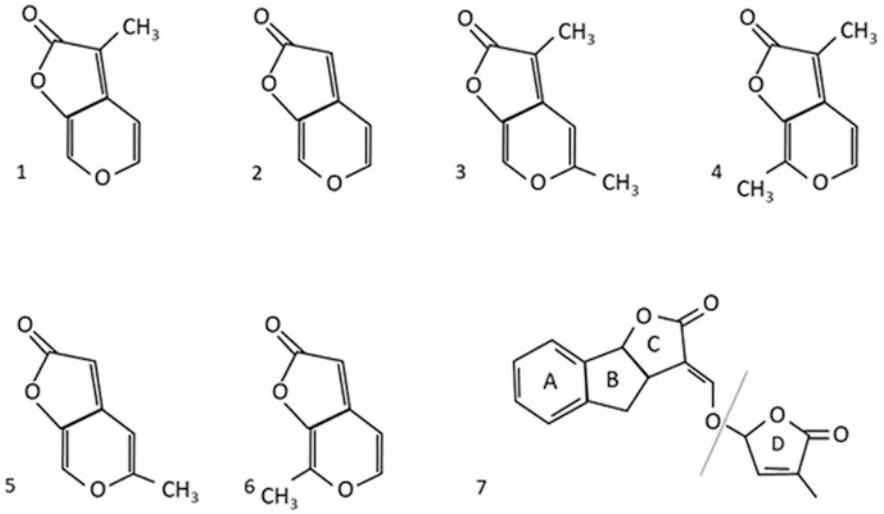

Fig. 4.6 The known chemical structures of karrikin family representatives and strigolactone analog. 1. KAR_1 2. KAR_2 3. KAR_3 4. KAR_4, 5. KAR_5 6. KAR_6 7. Strigolactone analog GR-24

in vitro culture of *Baloskion tetraphyllum* revealed the effect of KAR1 on somatic embryogenesis induction in coleoptile, shoot and leaf explants. The results showed that KAR1 accelerate the emergence of torpedo embryos (Ma et al. 2006). The stimulation of cell division caused an increase in fresh and dry callus weight in soybean seeds using KAR1 (Jain et al. 2008). In *Brassica napus*, use of smoke water improves the regeneration rate, root and shoot length in the plantlets obtained from microspore-derived embryos (Ghazanfari et al. 2012). The smoke water is also proved to be affecting the increase in the number of germinating embryos of *Pinus wallichiana* (Malabadi and Nataraja 2007a, b). Smoke water in the culture medium stimulates the seed germination of epiphytic orchids. *Vanda parviflora* and *Xenikophyton smeeanum* (Malabadi et al. 2008, 2011). Both SW and KAR1 were applied to improve in vitro propagation of another epiphytic orchid, *Ansellia africana* (Papenfus et al. 2016).

8 Paclobutrazol

Triazole compounds have growth-regulating properties and collectively they are called plant multi-stress protectants due to their ability to increase antioxidant enzymes (Jaleel et al. 2007). Paclobutrazol function is mediated by altering the level of classic hormones such as gibberellins, abscisic acid (ABA), and cytokinins (Fletcher and Hofstra 1990). It also influenced the isoprenoid pathway and changes the status of phytohormones by inhibiting gibberellin synthesis, decreasing ethylene production, and enhancing cytokinin and ABA contents (Kamountsis and Sereli

1999). Due to its interference in biosynthetic pathways of classic hormones, it is mainly used as growth retardants and stress protectants (Gopi and Jaleel 2009). Indrayanti et al. (2019) studied the effect of paclobutrazol for in vitro medium-term storage of banana germplasm. The MS media was supplemented with BAP and IAA, with paclobutrazol (PBZ) at concentrations of 0, 2.5, and 5.0 ppm as a growth retardant. In vitro cultures were maintained for 6 months without subculturing at 18–22 °C in an incubation room. The effect of different concentrations of PBZ on vegetative growth showed that PBZ at concentrations of 2.5 and 5 ppm could significantly slow the growth of plant height, the number of leaves, ratio of leaf length to width of all banana variants and reduce the number of shoots of banana variants. Nowello et al. (1992) observed that 1 ppm paclobutrazol applied to cultured shoots of *Vitis vinifera* caused plantlets to have leaves with a reduced area and bearing smaller stomata than usual. Stem length was also reduced. When roots were formed, they were thicker and more numerous than those on the control shoots. Smith et al. (1990) found that *Chrysanthemum* sp. plantlets rooted in a medium containing 0.5 to 2.0 ppm paclobutrazol were less liable to wilt on being transplanted to soil than the control. Research into growth retardants like paclobutrazol could prove useful to the horticultural industry by enabling the production of previously unavailable and/or unusual species such as ornamental plants or fruit trees (Kepenek and Karoglu 2011). Ziv (1992) also discovered that adding paclobutrazol to liquid media to induce the proliferation of compact bud clusters or meristemoids results ultimately to enhanced wax formation and normal stomatal function in the plantlets during the hardening stage.

9 Conclusions and Future Prospects

The use of plant growth regulators is an alternative way to achieve sustainability as these synthetic compounds improve the plant architecture without hampering the nutritional aspect. The micro quantity of PGRs leads to alter several morphological and physiological behavior. The coordination and integration of complex signaling events during their application will help to understand the new loops in plant metabolism. The use of PGRs in horticulture crops provide better result concerning yield. The flowers, fruits, and vegetables are economically valuable species. In vitro culturing techniques using PGRs will provide better understanding of growth regulation mechanism. The future prospect of horticulture and plantation crop will also rely on molecular and biotechnological approaches. Through tissue culture, novel technologies like gene editing may assist to understand the regulation at genetic level. The manipulation or altered combination of synthetic growth regulators can explored further in tissue culture. Lacunae of knowledge is there regarding use of karrikins and paclobutrazol under in vitro studies in horticulture crops.

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Chapter 5
Potential Role and Utilization of *Piriformospora indica*: Fungal Endophytes in Commercial Plant Tissue Culture

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Abstract The techniques in plant tissue culture have become pioneered and made manifold significant advances during the last couple of decades. Several researchers have directed to standardize the growth parameters in vitro micropropagation techniques in tissue culture previously but relatively the ex vitro techniques are still under exploration. In in vitro techniques, plantlets are raised under the sterile and controlled growth conditions viz., high humidity with a low light intensity ultimately makes the plantlets with altered morphology, anatomy, and physiology. The plantlets must be gradually hardened to field conditions. This progression assists to avoid the shock produced during a transient as well as to get a high survival rate of robust, vigorous, and healthy plants after transplantation. *Piriformospora indica* is a recently described and immensely versatile root endophytic fungus. This fungus is cultivable on artificial media, possesses plant growth promotional ability and can be colonized with a wide range of host plants. The *P. indica* significantly stimulates revival capacity, helps to escape the transplant shock, and acts as an efficient bio-hardening agent in various micropropagated plantlets. The maximum commercial exploitation of tissue culture plants does not only depend on the quality of plants but also the high survival rates of the plants. The significance and methods of utilization of the *P. indica* in the biological hardening techniques of the micropropagated plants have been summarized in this chapter. Thus, this chapter emphasizes the utilization of *P. indica* as a novel biological hardening agent for the establishment and development of in vitro raised plants.

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1 Introduction

Plant tissue culture utilizes the basic ability of the plant cell, i.e., cellular totipotency for the expeditious mass multiplication of the commercially important plants. The plant tissue culture is considered as the significant approach for conservation, improvement and propagation of numerous elite genotypes in agriculture and horticulture (Debnarh et al. 2006). The commercial success of tissue cultured plants not only depends upon the mass production of disease-free, true to type, and uniform plants but also on the rate of survival during the transfer from lab to land (Saxena and Dhawan 1999). Approximately 60% of the total production cost in tissue culture is needed to spend on labor, rooting, and acclimatization (Hazarika 2003). The plant tissue culture has surpassed the mere stage of in vitro micropropagation but the reestablishment process of the tissue cultured plants still bears the scope for considerable research and study. There are numerous significant justifications that can be taken into account to understand the reasons for the higher mortality in in vitro raised plantlets during transplantation. The most important reason among all is controlled growth conditions provided to the plants during in vitro culturing viz., high humidity, aseptic handling, and exogenous sugars supplemented media and accumulation of ethylene (Jeong et al. 1995). The inability of tissue cultured grown plants to survive and flourish after transplantation is linked to their struggle for managing water loss, ineffectual stomata, underdeveloped cuticle, expanded mesophyll air spaces in leaves, fragile root system, and the transition from heterotrophic to photoautotrophic nutrition (Pierik 1987; Ziv 1991). By considering the difference between the in vitro and in vivo plant growth conditions and its potential impact on the commercial production of micropropagated plants, hardening is considered as a crucial step in the plant tissue culture. Biological hardening methods using microbial inoculants offer an alternative to other hardening methods (use of chemical pesticides and/or growth promoters) in contemporary tissue culture, as they are eco-friendly and safer for micropropagated plantlets and soil (Nowak and Shulaev 2003). In vitro bacterization, ex vitro bacterization, mycorrhization, and priming with microbial derivatives can benefit the tissue cultured plants to improve plant nutritional status, water management, and to develop resistance against various biotic as well as abiotic stresses (Conrath et al. 2002; Fortin et al. 2002; Hussain et al. 1994). The recently reported endophyte *Piriformospora indica* was found very efficient to overcome the growth penalty imposed by transplantation shock during the acclimatization process (Sahay and Varma 1999, 2000); hence, in light of its significance, the present review outlined the utilization of *P. indica* in biological hardening of micropropagated plants.

2 Strategies Used in the Plant Hardening Process

The perusal of literature manifests that numerous strategies used by several researchers to minimize the loss of plants to improve the acclimatization of the tissue cultured plant in field conditions. The key approaches are described in the following subheading.

2.1 Optimization of the Propagule Size Before Transplantation

The various research studies indicate that there is an impact of the size of the propagule on the survival rate of the plantlets under the hardening process. The loblolly pine (*Pinus taeda*) propagules with an average shoot height of 2.6 cm survived efficiently and developed vigorously as compared to 1.4 cm propagules (Leach 1979). The propagule size must be greater than a specified standard for vigorous plant growth with increased survival possibility. The initial bamboo (*Bambusa vulgaris*) plantlet height ($\geq$ 5.0 cm) affected positively post-transplantation survival and growth of plantlets (Vale et al. 2019).

2.2 Use of Culture Vessels

The development of miniature plantlets in the laboratory relies on the available microclimate inside the culture vessel viz., air, temperature, and light. The quality, quantity, and distribution of these factors depend upon the physical characteristics of the culture vessel. The hermetic closures retain the optimum humidity (Bottcher et al. 1988) and also reduce the risk of contamination but such sealed culture vessels hinder the proper gracious exchange and disturb the concentration balance of CO_2 and ethylene gas. This disproportionate gaseous exchange produces irregularities and anomalies in the physiology and structure of plants (Perl et al. 1988). The lid transmittance is not uniform in the culture vessels; moreover, the reach of irradiance to plantlet is also dependent upon the culture vessel.

The plantlets of *Prunus avium* (wild cherry) and *Prunus insititia* (damson) were pre-acclimatized to greenhouse light and temperature before transplantation; by retaining culture vessels for a few days even after transferring to the greenhouse (Jones and Hopgood 1979). However, there is a possibility of a double greenhouse effect in such practices, due to heat buildup in the vessels.

In recent years, various scientists are putting efforts to improve the vessel with proper ventilation. Huang and Chen (2005) optimized the important physical properties required for culture vessels such as air exchange rate, transmittance distribution, and spectral irradiance.

The gas and light-permeable culture vessel was designed and used to encourage photoautotrophic growth in the in vitro developing rose gum (*Eucalyptus urograndis*) plantlets which can increase the survival rate of plantlets in the hardening stage (Tanaka 1991). The culture vessels developed from TPX (4-methyl-1-pentane polymer) and CPP (a polypropylene), which provided ventilation through its air-permeable film resulted in enhanced photosynthetic efficiency, fresh weight and dry weight of shoot as well as root in crucifix orchid (*Epidendrum*) (Teixeira da Silva et al. 2005). The rooted *Phillyrea* plantlets were successfully acclimatized under in vitro conditions by utilizing vented culture vessels (Lucchesini and Mensuali-Sodi 2004). Similar significant results were reported in potato (*Solanum tuberosum*) (Zobayed et al. 2001), black oil plant (*Celastrus paniculatus*) (Rao 2007), and beleric myrobalan (*Terminalia bellirica*) (Suthar et al. 2009).

2.3 Use of Chemicals

The application of plant growth retardants can encourage reduced internode, short leaves with dark green coloration, and thickened roots (Graebe 1987). The growth retardants can overcome the wilting damages in micropropagules (Smith et al. 1991). The application of plant growth retardants triggered reduced plant growth in citrus which ultimately survived the plant during the acclimatization stage (Hazariki et al. 2002). Plant retardant ancymidol improved shoots and roots in garden asparagus (*Asparagus officinalis*) (Chin 1982); in addition, it intensified green leaves with shorter height is observed in *Prunus avium* (Snir 1988). Three triazole assisted plant to diminish the water stress effect and improved water relation capacity in domesticated apple (*Malus* x *domestica*) (Swietlik et al. 1983). For obtaining the supraoptimal results in plantlets against various biotic, abiotic stresses and improved growth under in vivo hardening stages jasmonates used in potato (Pruski et al. 2002), salicylic acid in Arabidopsis plants (Ton et al. 2002), chlorocholine chloride in false daisy (*Eclipta alba*) (Ray and Bhattacharya 2008), abscisic acid in tomato (*Solanum lycopersicum*) (Hooker and Thorpe 1998). The survival rate of the micropropagated plants during transplantation was increased by using polyvinyl resin in cabbage (*Brassica oleracea*) L. (Wardle et al. 1979), phenylmercuric acetate in tomato (Srinivasa Rao 1985), silicone rubber in white bullace (*Prunus instititia*) (Fuchigami et al. 1981) and acrylic latex polymer in walnut (*Juglans regia*) (Voyiatzis and McGranahan 1994).

2.4 Use of Alternative for Solid Media

Hammerschlag et al. (1987) observed a greater number of shoots in peach (*Prunus persica* L.); however, elongated shoots were reported in Caribbean pine (*Pinus caribaea*) (Skidmore et al. 1988) after culturing in liquid media. Due to liquid

media quicker absorption of nutrients and growth regulators is possible which promotes the development of new buds and shoot elongation. The growth-retardant phenolics secreted by in vitro growing propagules get quickly dispersed to harmless levels in liquid media (Purohit et al. 2015).

2.5 Photoautotrophic In Vitro Culturing

In the convention culture media, sugar is supplemented as a source of carbon as the in vitro growing plantlet in the culture vessel gets CO_2 concentration below the required level therefore the growing plantlets become heterotrophic or partial heterotrophic (Kozai et al. 1992). The insufficient CO_2 concentration can create anadverse impact on the photosynthetic efficiency of the plant when transferred to in vivo conditions (Nguyen and Kozai 2005). The externally sublimated sugar reduces the water potential of media and the growing plantlets can be prone to various microbial infections (Pospíšilová et al. 2016). The laggy plants with reduced water potential perform poorly in the acclimatization stage, due to the presence of sugar in growing media. Therefore, in the last decades, the sugar-free media is altered by providing aerial CO_2 to the plants.

Purohit et al. (2004) tried to induce photoautotrophic ability in pala indigo (*Wrightia tomentosa*) plantlets in in vitro growth conditions by providing optimum CO_2 concentration; however, plants also showed enhanced multiplication rate, biomass, and leaf area. Further CO_2-enriched ambience provided during in vivo condition formed robust plantlets that exhibited rapid acclimatization in sapota (*Achras sapota*) (Dave and Purohit 2004) and beleric myrobalan (*Terminalia bellirica*) (Suthar et al. 2009; Purohit and Habibi 2010).

3 Constrains in Non-biological Hardening

Every method employed to improve the strength of the plant during the hardening process has its own set of strengths and limitations. Optimizing the size of the propagule for increasing the survival rate of the plantlets is a time-consuming and laborious task; likewise, developing a proper culture vessel for micropropagule will be specific for that particular plant as every plant's requirement for CO_2, ethylene, light, and ventilation is different. These vents can pave way for contaminants to multiply in the culture media. Plantlets cultured in stationary liquid conditions can create hyperhydricity and such stationary liquid media require a support matrix to prevent shear injuries and proper root development. The establishment of controlled CO_2 growth chambers is a costly affair and required sophisticated laboratory structures. The use of chemicals for priming of plantlets can increase the cost of production, and these can also scorch the juvenile plants under the weaning stage. Therefore, we need to look for an efficient hardening method and research revealed

that biological hardening is more compatible for interaction with the plant compared to chemicals.

4 Biological Hardening: An Avenue to Overcome Transplantation Shock

The dearth of favorable bacteria around the micropropagated plants produces weak physiological adaptability to natural surroundings and an acclimatization process (Borkowska 2002). In recent decades, the biological approach is utilized to alleviate the adverse effects produced during the state of acclimatization of in vitro plants in the field condition. The growth-promoting potential of several microorganisms is utilized under in vitro as well as in vivo conditions to pre-adapt the micro-cloned plants during the acclimatization phase (Rai 2001; Rai et al. 2001). Bio-hardening is a relatively novel feature of tissue culture science (Srivastava et al. 2002). Several successful attempts were tried to co-cultivate various microbial agents with young in vitro and in vivo grown plants with viz., safed musli (*Chlorophytum borivilianum*) (Dave et al. 2003), spider plant (*Chlorophytum* sp.) (Gosal et al. 2010; Mathur and Mathur 2010), horticultural plant species (Rai et al. 2001), and medicinal plant species (Yadav and Singh 2011). Mycorrhiza imparts growth promotional activities (Parkash et al. 2005), protection from infections (Reddy et al. 2006), and better nutrient uptake (Lovato et al. 1996) in the host plant; therefore, the mycorrhizal technology indirectly assists the plant to overcome transplantation shock (Estrada-Luna et al. 2000).

4.1 Piriformospora indica, *a Potential Endophyte for Bio-hardening*

The fungus *P. indica* is recently characterized and delineated as a basidiomycete endophyte of the order Sebacinales. It was isolated from the rhizospheric soil of the mesquite (*Prosopis juliflora*) and wild jujube (*Ziziphus nummularia*) which are typical inhabitants of the Thar Desert of India (Verma et al. 1998). The fungus was considered as a mutualistic endophyte due to nonpathogenic conduct towards the host plant (Peškan-Berghöfer et al. 2004).

The *P. indica* can act as a plant growth promoter (Varma 1999, Varma et al. 2000; Shahollari et al. 2005), bioregulator, biofertilizer, phytoremediater, biocontrol agent, and biotic as well as abiotic stress protector (Oelmüller et al. 2009).

In recent decades, the endophytic fungus *P. indica* (*Serendipita indica*) is grabbing the attention of researchers in plant tissue culture, by considering its tremendous potential to benefit the host plant in various aspects. The *P. indica* mimics several beneficial activities of vesicular arbuscular mycorrhizal (VAM) at the

hardening stage of micropropagated plants. Though mycorrhiza assists to comfort the young, transplanted plants while exploiting them practically, it has some shortcomings. The commercial applicability of AMF is hampered by the unavailability of an authentic axenic culture. Moreover, mycorrhiza is an obligate biotroph that requires a live host to survive (Newman and Reddell 1987), so it is an additional constrain for laboratory research. Some ericoid and ectomycorrhizal fungi can be cultured on the artificial complex media, but their host range is very limited like Ericaceae or woody plants.

The *P. indica* can ameliorate the shortcomings of AMF as it can be cultivated on artificial media and interact with a wide spectrum of the host as compared to the AMF (Schäfer and Kogel 2009; Tsimilli-Michael and Strasser 2008), therefore, is considered as a promising endophytic candidate as a bio-hardening agent in tissue culture techniques. The maize plant (*Zea mays* L.) was used as the host plant in the first co-cultivation trial of the *P. indica*; furthermore, numerous agronomically important crops, medicinally important plants, forest trees were also analyzed in various studies of co-cultivation with this fungus (Ansari et al. 2014).

P. indica can function harmoniously with other plant's beneficial microorganisms such as *Azotobacter chroococcum* and Rhizobium (Kumar Bhuyan et al. 2015; Ray and Valsalakumar 2010), so the single *P. indica* endophyte can be used as hardening procedure or it can be applied in combination with other microorganisms. The ability of *P. indica* to co-culture under in vitro and in vivo conditions again favors the use of *P. indica* as a potent biological hardening agent.

The endophyte *P. indica* and *Sebacina vermifera* inoculated with micropropagated transgenic strawberry plant resulted in better acclimatization efficiency in the host plant (Husaini et al. 2012). The survival rate observed during the transplantation process in *P. indica* inoculated strawberry (*Fragaria ananassa*) and tobacco (*Nicotiana tabacum* L.) plantlets were 79% and 90%, respectively (Sahay and Varma 1999, 2000). The *P. indica* improved adventitious roots of pelargonium and poinsettia (*Euphorbia pulcherrima*) quantitatively as well as qualitatively (Druege et al. 2007). In the microbial *biotization,* experiment the combination of *P. indica* and *Pseudomonas fluorescens* enhanced the secondary metabolite content (saponin), the rate of survival, nutrient absorption, and field performance of the *Chlorophytum* sp. (Gosal et al. 2010). The percentage of survival of plantlets that could survive after transplantation was found more in the case of in vitro banana (*Musa* spp.) seedlings inoculated with *P. indica* (Li et al. 2019). The effect of various bioagents and antitranspirants on the rate of survival after transplantation was studied in tomato plantlets, among all the treatments lowest plantlet mortality was exhibited by *P. indica* (Gupta et al. 2017). In vitro inoculation of *P. indica* with apple plant propagules turned to sturdy, healthy, and vigorous plantlets at acclimation stage compared to control plants (Masoudi et al. 2020), similar kind of plant establishment was reported in coleus barbatus (*Coleus forskohlii)* (Das et al. 2014) and aloe-leafed cymbidium (*Cymbidium aloifolium*) (Shah et al. 2019). An endangered plant picrorhiza (*Picrorhiza kurroa*) micropropagated and biologically hardened with *P. indica* endophyte by in vitro inoculation (Das et al. 2017a, b). The rate of survival of the plantlets in in vivo conditions was raised by 1.3 folds; moreover,

the number of roots per plantlets was increased by 1.25 fold within 4 weeks and hence *P. indica* improved the performance of plants under greenhouse conditions (Das et al. 2017a, b). In vitro plantlets of medically important plant *C. forskohlii* inoculated with *P. indica,* adjusted and habituated better to the external environment than uninoculated plants (Das et al. 2012). Trzewik et al. (2020) treated the in vitro *Rhododendron* hybrids plantlets with *P. indica* prior to shifting to the hardening stage, the shoot length, leaf number, and chlorophyll content were increased in plants after 15 days.

4.2 Taxonomy and Molecular Identification of P. indica

The position of *P. indica* in the basidiomycetes division and order sebacinales was confirmed by molecular phylogenetic analyses (Weiss et al. 2004; Qiang et al. 2012). The phylogenetic position of sebacinales under Agaricomycetes is not yet determined, but they are classified into two major clades viz., group A and group B. The anamorphic *P. indica* is categorized into group B. Further phylogenetic studies revealed that *Piriformospora* is taxonomically similar to *Serendipita,* but *Serendipita* is a teleomorph and *Piriformospora* is anamorph (Weiss et al. 2016).

According to Zuccaro et al. (2009), the *P. indica* contains a minimum of six chromosomes of 15.4–24 Mb genome size. The sequence analysis of *P. indica* has revealed that the fungus has some biotroph-related genomic adaptations (Varma et al. 2012a, b). On the converse, *P. indica* also exhibits some genomic similarities with saprotrophic and hemibiotrophic phytopathogenic fungi like enzyme arsenal associated genes, but these are poorly expressed in the primary biotrophic phase. Thus, *P. indica* is symbolized as a missing link between saprotrophs and mutualistic biotrophs (Zuccaro et al. 2011).

4.3 Morphology of P. indica

The detailed morphology and ultrastructure of *P. indica* were explained by Kost and Rexer (2013). The mycelium appears white, mostly hyaline, homogeneous with regular ramification pattern, septated, multinucleate, heterokaryotic (Zuccaro et al. 2009, 2011). Mycelium prominently grows within the media and aerial hyphae are merely present on the media surface. Hyphae are thin, intertangled, and often show H-connection and anastomosis. (Singh et al. 2003). In the shaking broth, the mycelia aggregate appears in small clumps. The structure of hyphae is dependent upon media composition.

Chlamydospores are pear-shaped, smooth, multinucleated, and filled with granular material. The young developing spores produced from swollen hyphae are colorless clavate and get thick light yellowish upon maturity, mostly present in

clusters. The spore germination and the branching of newly producing hyphae are dependent upon the water and nutrient content of the media.

4.4 Root Colonization with Crops and Orchids

Root colonization of *P. indica* studied in various agriculturally important crops and orchids. The rate of colonization, as the reciprocal benefits of both the interacting partners, may get hampered (Sherameti et al. 2008a, b).

In the majority of the host plants, the *P. indica* commences the colonization by direct penetration (Jacobs et al. 2011) typically with biotrophic mode, later this establishment is shifted to the necrosis phase, in which the fungus kills the host root cells for proper colonization in the host root (Varma et al. 2013). The *P. indica* exhibits a unique feature of mutualism with barley plant root that it can colonize and proliferate in the dead root cells of the host; such colonization is not reported in obligate biographic AMF (Deshmukh et al. 2006). However, with few host species necrotrophic interaction or host cell damage not executed by *P. indica.* Schäfer and Kogel (2009) revealed that only biotrophic mode of infection exists in the *P. indica* while interacting with barley (*Hordeum vulgare*) and arabidopsis (*Arabidopsis thaliana*).

The colonization is restricted to specific regions (rhizodermal and cortical) of the root system, excluding the endodermis region and aerial plant parts (Khatabi 2009). The mycelium found encompassed the root surface of the arabidopsis*;* moreover, hyphae and the chlamydospores could observe in roots hair and epidermal cells (Peškan-Berghöfer et al. 2004). The rate of *P. indica* colonization increases along with the root maturation and the maximum fungal biomass was observed at the differentiation stage especially in the root hairs region. The AM fungi favorably colonize only the young host roots, which are not observed in *P. indica* (Schäfer and Kogel 2009).

The voluminous fungal hyphal network developed by the endophyte stimulates nutrient absorption in the host plant (Das et al. 2013); however, the proper exchange of nutrients depends upon the physiological activity of the plant host cell (Schäfer and Kogel 2009).

The *P. indica* interaction was also investigated in greasewort (*Aneura pinguis*) of the family Aneuraceae, the hyphae penetrated the moss thalli, produced a hyphal bundle under the epidermal root cells and completely occupied the living moss cells (Kottke et al. 2003). The *P. indica* also showed significant interaction with Ericaceae candidates (Selosse et al. 2007). Several members of the Brassicaceae have not shown any interaction with Glomerales but with such species *P. indica* colonized successfully (Peškan-Berghöfer et al. 2004). In the co-cultivation study of *P. indica* with ectomycorrhiza-forming plant viz., pedunculate oak (*Quercus robur*), only spindly intercellular hyphae were observed but Hartig net and palmetto were not detected (Kost and Rexer 2013).

Deshmukh et al. (2006) investigated the stage-wise colonization of *P. indica* in barley roots, during the initial days of inoculation 1–2 dai (days after inoculation) the fungal chlamydospores get germinated and gets established in the root epidermis. The invasion on single rhizodermal cells with the formation of intercellular hyphae, occupation of the major root zone by inter- and intra-cellular hyphae and emergence of sporulation was reported at 3, 7, and 10 dai (days after inoculation). The existence of fungal was not observed in vascular tissue, however, colonized prominently in root hair than elongation and apical meristem.

4.5 Interaction with Other Soil Microbes

The endophyte *P. indica* interact differentially with different soil microorganisms (Ansari et al. 2014) in order to *attune* and coexist with them in the rhizosphere of plants (Varma et al. 2012a, b). *The P. indica* remain neural to few bacteria viz., *Azospirillum brasilense* Sp245, can be favorably co-habited with some rhizosphere bacteria (Varma et al. 2014) like *Pseudomonas putida*. However, it works antagonistically with some in the soil matrix (Varma et al. 2014) viz., *Serratia liquefaciens* MG1, *P. fluorescens* WS5, and *Burkholderia cepacia* LA3. The studies indicate that the *P. indica* is a more efficient symbiont for the host compared to the bacteria (Gosal et al. 2010), but the more adequate and satisfactory impact is produced in the host plant upon using the combinations of bioagents (Gosal et al. 2010; Franken 2012). Various studies indicate that the establishment and spread of the plant pathogenic fungi viz., *Aspergillus sydowii, Rhizopus stolonifer,* and *Aspergillus niger* inhibited by *P. indica;* however, *P. indica* harmonize with algae in plant growth promotional activity (Ansari et al. 2014).

The recent molecular investigations of *P. indica* revealed the close link with a *Rhizobium radiobacter* in endosymbiotic associations. The original culture was deposited at German Resource Centre for Biological Material, Braun-schweig but the presence of any other bacteria is not reported in the culture filtrates of *P. indica* (Whipps 2001). In the hyphal walls of the originally deposited *P. indica* culture, *R. radiobacter* was found and it was confirmed by 16S rRNA and PCR assay. The existence of *R. radiobacter* was only observed after lysing the fungal mycelium. The biological activities of the *R. radiobacter* were studied on barley and those were showing resemblance with *P. indica* in terms of growth promotion and abiotic stress resistance. This endosymbiotic association of the fungus and bacteria is not disengaged to date (Glaeser et al. 2015). The existence of endosymbiotic association was previously also reported in VA mycorrhizal fungi belonging to Glomeromycota (e.g., *Gigaspora, Geosiphon pyriforme, Glomus margarita*) (Bianciotto et al. 1996) and Basidiomycota (*Laccaria bicolor*) which enhanced the rate of fungal spore germination. These endogenous bacteria influence the associated fungus in various ways such as toxin production (Partida-Martinez and Hertweck 2005), vegetative reproduction, and development of sporangia and spores.

4.6 *Cultivation of* P. indica

The fungus, *P. indica* can grow axenically, preferably utilizes soluble starch and glucose as a carbon source (Serfling et al. 2007; Varma et al. 2014); moreover, it is compatible to grow on a variety of culture media conditions and compositions. *P. indica* can be cultured on both solid and liquid media. In the initial studies, *P. indica* cultured on regular MYP and MA media, further Singh et al. (2003) opined that numerous synthetic and complex media viz., MMN 1/10 medium (Herrmann et al. 1998), modified aspergillus medium (Varma et al. 2001a, b), M4 N medium, MMNC medium (Kottke et al. 1987), MS medium (Murashige and Skoog 1962), WPM medium (Ahuja 1986), MMN medium (Johnson et al. 1957), Malt extract medium (Galloway and Burgess 1952), PDA medium (Martin 1950), and Aspergillus medium (Kaefer 1977).

The morphology of the mycelium varies with the media compositions, but some typical morphological characteristics distinguish the fungus from other types of fungi viz., coaxial growth with rhythmic circles (Singh et al. 2003). In some *P. indica*–plant interaction studies, the media composition was found to affect fungal behavior response to the host plant. The nitrogen in media influenced the actions of *P. indica* on *Populus* plantlets; the fungus vandalized the shoots of the plant similar to phytopathogenic fungus (Kaldorf et al. 2005).

The growth conditions and media provided for the culturing of any bioagent create an impact on the secretion of antimicrobial compounds (Duffy and Défago 1999), existence in soil and efficacy (Gu and Mazzola 2001). Since *P. indica* is an axenically grown endophyte; therefore, the media and growth requirements are quite different than the typical fungus culture media. Tripathi et al. (2015) analyzed the four different media (Hill and Käfer medium, PDB medium, CZ medium and ME medium) to find out the suitable media for proper growth of *P. indica* on both solid and liquid culture conditions. The fungus showed significant results under Hill and Käfer Medium in terms of growth and biomass production in solid and liquid media conditions, respectively. The maximum fungal biomass recovery under Hill and Käfer Medium was obtained at 30 °C temperature, 120 rpm agitation speed, and 6.5 pH; moreover, glucose as a carbon source, potassium dihydrogen phosphate as a phosphorus source, potassium chloride as potassium source and peptone as nitrogen source (Kumar et al. 2012). Kumar et al. (2011) compared five different media (YPG medium, PDB medium, ME medium, Hill and Käfer medium, and Gamborg medium) to evaluate and find out the suitable media for culturing *P. indica*, observed the supraoptimal growth of fungus in Hill and Käfer Medium. Though Hill and Käfer medium was found suitable for growing *P. indica* culturing, various other media were also successfully used for culturing. Baishya et al. (2015) cultured *P. indica* on successfully PDB media with increased agitation speed (200 rpm) with nearly similar temperature and pH conditions (30 °C temperature and 6.5 pH) used by earlier researchers. Bagheri et al. (2014) cultured *P. indica* on liquid Aspergillus Complex Medium (CM) at 18–22 °C temperature; however, *P. indica* was cultured on Aspergillus minimal media (Peškan-Berghöfer et al. 2004; Kumar et al. 2009;

Yadav et al. 2010; Mohd et al. 2017) and modified Aspergillus medium (Pham et al. 2004). Several reports have shown the culturing of *P. indica* on medium without using numerous chemical nutrients in the media viz., Attri and Varma (2018) used jaggery, Osman et al. (2020) formulated vegetable juice-based media and Anith et al. (2015) utilized coconut water for co-cultivation of the two biological agents.

Johnson et al. (2013) explained the two important factors for co-cultivation of *P. indica* and host under in vitro conditions. The first important factor is the optimal supplementation of nutrients in media. The change in concentration of nutrients (more or less) in media may lead to a shift from mutualism to parasitism (Johnson and Oelmüller 2009). The surplus nutrient may allow one of the partners to dominate and the dearth of important nutrients creates a negative impact on plant growth in the later stages of development. Therefore, the co-cultivation shows remarkable results in minimal media compared to the highly supplemented media (Sherameti et al. 2005, 2008a, b). The second critical factor that affects the co-cultivation is the pH of the growing culture. The optimum pH (6.5–7.0) is required to grow both the symbionts in harmony. Under in vivo conditions, inoculum size is an important factor.

4.6.1 Media Composition

A large variety of synthetic and complex media are employed for the activation and culturing of *P. indica*. As mentioned earlier in this chapter that *P. indica* can be cultivated on various media but the most commonly used media are Hill and Käfer medium (Kaefer 1977), modified aspergillus medium (Varma et al. 2001a, b), and PDA medium (Martin 1950).

4.6.2 Maintenance and Culturing of *P. indica* on Solid Media

The frozen ampoule of *P. indica* can be revived by thawing as per the procedure recommended by ATCC (American Type Culture Collection). Place the ampoule in a water bath at 25°C to 30°C for 5 min without shaking and wipe it out with 70% ethanol. Transfer the 50 µL culture to the culture media aseptically and incubate at 30 °C (Varma et al. 1999). The stock culture of *P. indica* was maintained for 10 days at 30 °C on Hill and Käfer medium slants (Prasad et al. 2005) prepared with 15 g/L agar. The fungus was cultured on solidified media in Petri plates at 28± 2 °C for 7 days in an inverted position under dark conditions (Pham et al. 2004).

4.6.3 Cultivation of *P. indica* on Incubator Shaker

The initial small-scale optimization of culture development conditions in shake flasks helps to minimize the effectors to perform the large batch and fed-batch

bioreactor cultures (Hamilton-Miller et al. 1999; Bareither et al. 2013; Kaur and Roy 2015).

The fungus *P. indica*was culturedin shakers at 28 ± 2 °C temperature, 125 rpm agitation speed and 80 μmol/m^2/s illumination for 10 days (Das et al. 2012; Bertolazi et al. 2019).

The maximum biomass recovery of *P. indica* was obtained at low oxygen concentration, more working volumes, and minimum agitation speed (Varma et al. 2001a, b) in the rotary shaker incubator. Kumar et al. (2011) recovered the highest spore yield within 8 days at 200–300 rpm and 20% working volume. The actual sporulation was started after 6 days when the entire carbon source in the culture media was depleted.

4.6.4 Cultivation of *P. indica* in Fermenter

The verification of laboratory procedures and their modification is required to scale up the *P. indica* production. The microbial biomass yield can be enhanced without creating the microbial stress in the mass multiplication processes by employing a suitable statistical experimental design in a bioreactor (Sukumaran et al. 2005; Strube et al. 2012). The quality and quantity of microbial biomass in bioreactor depends upon several physical and physiological parameters (Amanullah et al. 2010; Bareither et al. 2013) viz., temperature, pH, cell viability, substrate concentration, and oxygen/carbon dioxide pressures (Hamilton-Miller et al. 1999).

The culture condition required for the bulk production of *P. indica* was standardized in a 14-L bioreactor by using modified Hill and Käfer medium (Table 5.1) to obtain increased fungal biomass and spore production (Varma et al. 2012a, b). The highest dry cell weight recovered after the period of 42 h was 7.36 g/L. The rate of biomass production and fungal growth per day was reported 0.79 and 1.15, respectively. The sporulation was started after 48 h and finally produced 9.25 10^7 spores/mL after 60 h. The spore starts to appear early in the bioreactor compared to the shake flask method due to proper distribution of the fungal inoculums, faster utilization of glucose, and quicker depletion of glucose. The scarcity in carbon source caused a drop-down in pH but in such an acidic condition the fungal growth remains unaffected until pH 4.5 (Varma et al. 2012a, b).

For the production of cell-free products and culture filtrate of the *P. indica,* various techniques were analyzed by Bagde et al. (2011) and Kumar et al. (2012).

Further, Varma et al. (2012a, b) formulated soil extract and jaggery (Product of the *Saccharum officinarum*)-enriched media (Table 5.2) for the mass multiplication of *P. indica.* Though the media prepared from the extract of jaggery was found economically cheaper, the consistency in results was observed only for 14 days; therefore, the Hill and Käfer medium was found superior over this media.

The maximum *P. indica* production was obtained in a 5-liter capacity fermenter at optimized conditions viz., pH 6.7, temperature 30 °C, and oxygen supply rate 0.2 VVM (Vessel Volume/Minute) and 100–200 rpm agitation speed (Bajaj et al. 2014).

Table 5.1 Hill and Käfer medium composition (Hill and Käfer 2001)

Composition	Amount
Glucose	10.0 g/L
Peptone	1.0 g/L
Yeast extract	1.0 g/L
Soybean meal	1.0 g/L
KH_2PO_4	1.83 g/L
$MgSO{\cdot}7H_2O$	0.65 g/L
Macro element stock solution[a]	50.0 mL/L
Microelement stock solution[b]	2.5 mL/L
Vitamin stock solution[c]	1.0 mL/L
$CaCl_2$, 0.1 M	1.0 mL/L
$FeCl_3$, 0.1 M	1.0 mL/L
Macronutrient mix/L stock solution	
$NaNO_3$	12.0 g
KCl	10.4 g
$MgSO_4{\cdot}7H_2O$	10.4 g
KH_2PO_4	30.4 g
Microelement mix/L stock solution	
$ZnSO_4{\cdot}7H_2O$	2.2 g
H_3BO_3	1.1 g
$MnSO_4{\cdot}4H_2O$	0.5 g
$CoCl_2{\cdot}5H_2O$	0.16 g
$CuSO_4{\cdot}5H_2O$	0.16 g
$(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$	0.11 g
Vitamin mix/L stock solution	
Thiamin	0.1 g
Glycin	0.04 g
Nicotinic acid	0.01 g
Pyridoxine	0.01 g

[a] Macro element stock solution
[b] Microelement stock solution
[c] Vitamin stock solution

Table 5.2 Composition of fungal culture media enriched withjaggery

Composition	Amount
Sucrose	60–85 g/L
Glucose	5–15 g/L
Protein	0.4 g/L
Fat	0.05 g/L
Calcium	0.4 g/L
Magnesium	0.045 g/L
Phosphorus	0.045 g/L

4.7 Co-Cultivation Methods

The treatment of *P. indica* was applied to micropropagated plants by different methods in various species.

4.7.1 In Vitro Co-cultivation Method

P. indica—host plant in vitro co-cultivation model was described by Johnson et al. (2013) in *A. thaliana* plants. Prasad et al. (2019) has succeeded with an identical type of co-cultivation experiment between *P. indica* and *A. thaliana*. The plant seeds and *P. indica* were cultured separately and later grown in the same media. This facilitates the interaction of both plant and fungus within the same media which is not possible for the other VA mycorrhiza because of the specific requirement of pH and other growth conditions of each interacting partner. In the experiment fungus, *P. indica* was cultured on the slightly acidic modified Käfer medium supplemented with sugar and protein for 4 weeks. The fungus plug of fully grown in *P. indica* transferred to the PNM medium and allowed to grow for 7 days. Meanwhile the 12-day-old *A. thaliana* seedlings grown on MS medium were also shifted to the same PNM medium in which *P. indica* fungal lawn is grown. Both the fungus and plant were grown in the same culture at 22 °C for 7 days (Johnson et al. 2013). The PNM is considered a suitable medium to grow both fungus and the host plantlet simultaneously (Sherameti et al. 2005, 2008a, b). Tsai et al. (2020) mixed the *P. indica* mycelium (3 g) in the plant culture media and inoculated germinated rice seeds in the same media. The *P. indica* co-cultured rice seedlings were transferred to the soil for further analysis. Dolatabad et al. (2017) co-cultured *P. indica* and 30- day-old micropropagated peppermint plants in the MS medium. After confirmation of co-culture of both the fungus and the host plant, they were shifted to in vivo conditions. The seedlings of tobacco and arabidopsis grown on MMN1 culture medium were inoculated with *P. indica* by placing a 5 mm fungal disc near the vicinity of the seedling roots (Sherameti et al. 2005). The *P. indica* endophyte showed significant growth enhancement in peanut (*Arachis hypogaea*) upon in vitro co-cultivation (Fig. 5.1).

4.7.2 Seed, Seedling, or Propagule Treatment

The seeds of rice (*Oryza sativa*) were treated with *P. indica* by mixing the fungal mycelium with vermiculite carrier, and this mixture was applied to the seeds (Saddique et al. 2018). After 10 days, the co-cultured seedlings were shifted to Yoshida nutrition media (Yoshida et al. 1976) for hardening. The *P. indica* fungal biomass was applied to turmeric (*Curcuma longa*) propagative buds by using raw talcum powder as substrate; moreover, juice of *S. officinarum* was used as an adhesive substance.

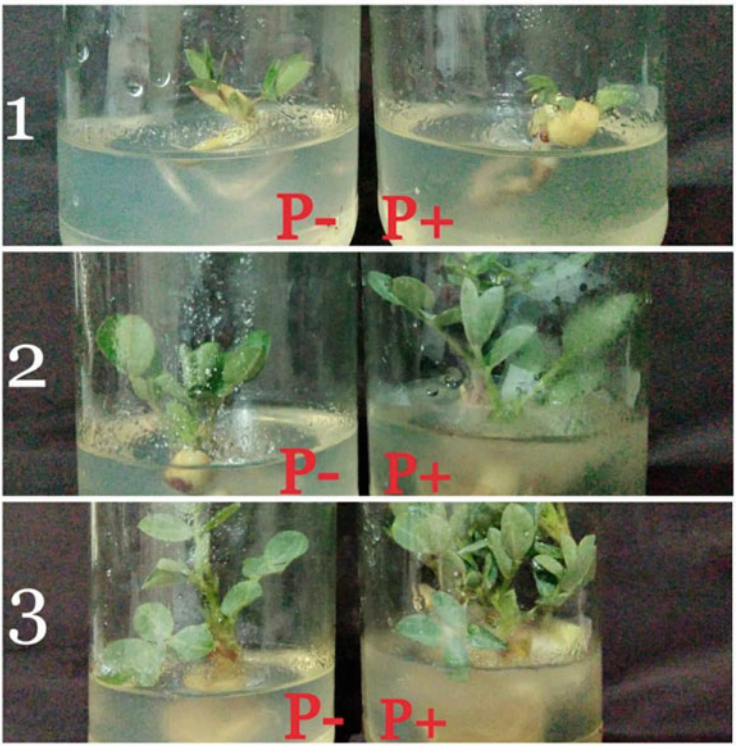

Fig. 5.1 Effect of in vitro *P. indica*—groundnut seedling co-cultivation on growth of the plant, where 1 refers to 12-day-old *A. hypogaea* plants, 2 refers to plants after 25 days, and 3 refers to plants after 45 days. (Credit: Shilpa Hanmantrao Tarte)

The inoculation of *P. indica* with the corn and white clover plant root was done by keeping the fungal disc below the soil (1 cm) at the time of seedling planting (Baghaie and Daliri 2020). A similar method was used in (sweet wormwood) *Artemisia annua* and *P. indica* co-cultivation (Rahman et al. 2020).

4.7.3 Root Treatment

Bagheri et al. (2014) inoculated *P. indica* to the growing roots of 4-day-old germinating seeds by direct immersing plant roots in *P. indica* homogenate for 12 h. The homogenate was prepared by maintaining the spore concentration at 5×10^5 spores/mL. The inoculation was confirmed by staining (Vierheilig et al. 1998). This method was employed in rice by dipping the radical of plantlets in 10^6 spores/mL for 2 min and further transferred to water agar plate for 1 day and finally cultured in hydroponic conditions. In this method, the pattern of colonization was found uniform in the roots of the host plant (Mohd et al. 2017). Bertolazi et al. (2019) inoculated *P. indica* in rice roots through irrigation. The *P. indica* mycelium pellet was obtained

by centrifugation at 4000 x g for 7 min and the crushed pellet was suspended in distilled water prior to irrigation. Dabral et al. (2019) directly poured the *P. indica* spore suspension (5×10^5 spores/mL) in the punctures made near the vicinity of roots to make it available in the rhizospheric zone of the rice seedlings.

4.7.4 Substrate and Soil Treatment

Waller et al. (2005) inoculated 2 g of *P. indica* mycelium in 300 g of the substrate prior to sowing of barley seeds. After sowing 4 g of *P. indica*, mycelium was mixed in soil and plants were grown in the growth chamber for the hardening purpose. The *P. indica* was inoculated on a hard substratum made from sterile sand and soil in a 3: 1 ratio in a pot culture experiment of maize seedlings (Krishnaveni et al. 2014). Kumar et al. (2009) also performed the co-culture of *P. indica* and maize by inoculating *P. indica* directly in sterile soil by mixing 10 g wet fungal mycelium and 100 mL Hoagland's solution in 1000 g of sterile soil. The sandwich-like model was utilized by Attri and Varma (2018), in which live inoculums of *P. indica* were sandwiched between two layers of the sterile substratum. The sterilized bagasse: perlite (50:50) was used as carrier to inoculate *P. indica* (Amini et al. 2020). One percent of *P. indica* mycelium was mixed in the sterile planting material (w/v) before planting the chilli (*Capsicum annuum*) seedlings in pro-tray cavities (Nandana and Anith 2020). The 200 µl of 1% *P. indica* suspension grown in ASP liquid medium was applied in the soil in the vicinity to the arabidopsis plant roots (Pan et al. 2017; Jiang et al. 2020).

4.8 Effect of P. indica *on the Host*

The plant naturally employs various mechanisms at the cellular level to get relief from the biotic and abiotic stresses (Bray 1997). The microbial symbiosis and osmotic potential regulation (Ruiz-Lozano 2003) help plants to diminish the detrimental effects generated in the plant due to biotic and abiotic stresses (Sylvia et al. 1993). The plant itself can overcome adversities by expanding the surface area of the root (Subramanian et al. 2006). The symbiotic endophytic fungus retains the moisture-producing glomalin soil aggregations (Ruiz-Lozano 2003).

4.8.1 Biotic Stress

P. indica found a promising endophyte, which develops resistance in several host plants against fungal pathogens. The *P. indica* develop resistance against the foliar pathogens by producing ROS and antioxidants in cereals (Waller et al. 2005; Serfling et al. 2007; Kumar et al. 2009). The *P. indica*-colonized fungal infected

host plant mimics the well-characterized pathways associated with systemic resistance developed by rhizobacteria (Van Loon et al. 1998).

The pH boosted by *P. indica* in barley plants infected with (*Blumeria graminis*) f. sp. hordei, suggesting a triggered systemic reaction in the host plant (Felle et al. 2009). The *P. indica* enhanced antioxidant activity, glutathione reductase activity, and glutathione pool, in the *B. graminis* f. sp. hordei infected barley plants (Waller et al. 2005). The glutathione-associated antioxidative capacity assists the partners in the mutual association by protecting against abiotic stress (Kranner et al. 2005). The *P. indica*-treated and *Golovinomyces orontii*-infected plants initiate and retaliate defense reaction in arabidopsis leaves (Waller et al. 2005; Stein et al. 2008). The disease resistance ameliorated in *P. indica* colonized barley plant against the pathogenic fungus *Fusarium culmorum* (Waller et al. 2005) and *F. graminearum* (Deshmukh and Kogel 2007). The detailed mechanism behind the systemic resistance developed by *P. indica* against fungal infections was studied in *A. thaliana*. The active jasmonate pathway was found associated with the primed systemic responses against fungus *Golovinomyces orontii* in the host plant, upon colonization with *P. indica* (Stein et al. 2008). The molecular analysis of barley roots inoculated with the *P. indica* at 1, 3, and 7 dai, indicated the differential regulation pattern in 1300 genes from about 40,000 probe sets, imbibed on a microarray (Schäfer et al. 2009). The gene related to resistance against pathogens were expressed earlier and stronger in *P. indica*-treated barley plants compared to control plants (Molitor et al. 2011). The pathogenic infection of *Verticillium dahliae* can induce and accumulate the phytohormone into the arabidopsis leaves; this was found restricted by *P. indica* root colonization to generate a systemic response (Sun et al. 2014).

4.8.2 Abiotic Stress

The endophyte *P. indica* has been reported to help the host plant in overcoming abiotic and biotic stress, lag the withering of plants, and lift the secondary metabolites and antioxidant production in the host plant (Varma et al. 1999; Waller et al. 2005). The root colonization in in vitro grown Arabidopsis plant encouraged the expression of several drought stress regulating genes (Sherameti et al. 2008a, b). The hampered photosynthetic efficiency due to drought conditions was found revived by *P. indica* colonization in Chinese cabbage (*Brassica rapa* subsp. pekinensis) (Sun et al. 2010). The salt stress effects produced in *Triticum aestivum* were mitigated by *P. indica* root colonization (Zarea et al. 2012), identical plant stress reliving mechanism reported in strawberry (Husaini et al. 2012). Several organic components related to stress-induction are primed in the plant during the stress conditions. The proline level increases in the plants exposed to abiotic stress (El Moukhtari et al. 2020). Co-culture of plants with *P. indica* has been shown to increase the amount of proline as compared to control plants. The increase in proline content assists in *P. indica* inoculated host plant in building up the tolerance against the osmotic stress (Zarea et al. 2012). The reactive oxygen species (ROS) and the stress-associated antioxidants are the major players in the alleviation of stress (Foyer and Shigeoka

2011). The defense mechanism of the host plant under abiotic stress depends upon the activation and agglomeration of aforementioned components (Hamilton and Bauerle 2012). In the stress tolerance conferred by *P. indica,* the increase in levels of ascorbate from dehydroascorbate resulted in the enhanced glutathione in barley (Waller et al. 2005). The gene expression profile of the *P. indica*-treated barley plants under stress revealed the involvement of ROS activity (Baltruschat et al. 2008). The amount of malondialdehyde in the Chinese cabbage was found reduced due to unsaturated lipid breakdown by ROS (Sun et al. 2010). Gahlot et al. (2015) found that *P. indica* up-regulated six salt stress tolerance inducing genes out of 36 screened genes. These genes are associated with several important cellular mechanisms in the host plant cell. *P. indica*-colonized rice plants executed better performance under salt stress conditions corresponding to control plants (Jogawat et al. 2013). The *P. indica* treatment secured rice, from arsenic toxicity. *P. indica* aided plants to overcome the arsenic stress by arresting the availability of free arsenic in the plant rhizosphere, conversion of arsenic into insoluble form and regulating antioxidative activities (Mohd et al. 2017). *P. indica* has the potential to reduce the H_2O_2 accumulation (Schafer et al. 2007) and increasing CAT, GR, and SOD activities in the host plant (Mohd et al. 2017). Similar significant benefits were conferred by *P. indica* against cadmium (Dabral et al. 2019). *P. indica* sustained the chlorophyll in rice foliar (Abadi and Sepehri 2016), ultimately affected the quantum yield of PSII (Fv/Fm) significantly (Vahabi et al. 2016) in the host plant. During abiotic stress, the levels of Fv/Fm were enhanced by the endophyte in the host plant was reported in *A. thaliana* (Sherameti et al. 2008a, b; Bakshi et al. 2017). *P. indica* not only eases the host plant to tolerate the salt stress but also improves its growth parameters (Bagheri et al. 2013). In response to salt stress, the increased Na^+/K^+ ratio, auxin, CAT, SOD, and the elevated expression of *LeNHX1* gene was observed in *P. indica*-colonized tomato plant roots compared to the control (Abdelaziz et al. 2019). *P. indica* not only imparts the stress endurance ability against biotic, abiotic, and metal toxicity but also shows a significant growth in host plant viz., barley (Ghaffari et al. 2019), barley (Ghabooli et al. 2013), maize (Hosseini et al. 2018), melon (*Cucumis melo* L.) (Hassani et al. 2019), tomato (Ghorbani et al. 2019), rice (Mohd et al. 2017), cenna (*Cassia angustifolia*) (Nanda and Agrawal 2018), tobacco (Hui et al. 2015), wheat (*Triticum aestivum*), and sunflower (*Helianthus annuus*) (Shahabivand et al. 2012, 2017).

4.8.3 Growth Parameters

In regular mycorrhizal symbiosis association, the host plant gets benefited by increased leaf area, photosynthetic efficiency, carbon assimilation, and chlorophyll content (Yano-Melo et al. 1999; Ceccarelli et al. 2010). Similar beneficial effects were reported in *P. indica*-colonized plants which resulted in better growth, enhanced development, and increased biomass production (Waller et al. 2008; Jurkiewicz et al. 2010). In *P. indica*-black pepper (*Piper nigrum*), colonization the growth parameters viz., the number of leaves, leaf area, chlorophyll content, dry

weights, total fresh weight of berries, and spike set (Anith et al. 2018). The early flowering and increased biomass were reported in *C. forskohlii* due to *P. indica* colonization (Das et al. 2012), similar results were observed in the case of barley plants in the pot cultures experiment (Achatz et al. 2010). *P. indica* effectively encouraged early germination, increased the viability, quality, and quantity of seeds in various crops viz., improved seed germination (Blechert et al. 1999), enhanced seed production in *A. thaliana* (Shahollari et al. 2007), increased seed yield in *H. Annuus* (Bagde et al. 2010), and enhanced seed viability (Harrach et al. 2013).

4.8.4 Yield Parameters

The yield parameters get positively influenced by *P. indica* due to several factors viz., vigorous foliar growth, ample number of inflorescences or flowers, higher seed weight (Rai et al. 2001; Peškan-Berghöfer et al. 2004; Barazani et al. 2005; Dolatabadi et al. 2011a), increased number of ears (Waller et al. 2005), and a higher number of pods per plant (Ray and Valsalakumar 2010). The increased number of fruits per plant was reported due to the co-cultivation of *P. indica* with tomato plants (Fakhro et al. 2009). The yield parameters of proso millet (*Panicum miliaceum* L.) (grain yield, panicles/plant, number of grains/panicle and 1000-grain weight) were found enhanced by *P. indica* co-culture (Ahmadvand and Hajinia 2018).

4.8.5 Quality

The *P. indica* could express various plant quality influencing compounds viz., antifungal compound (spilanthol), medicinal compound (podophyllotoxin), essential oils, glycosidic compound (saponin) in paracress (*Spilanthes calva*) (Rai et al. 2004), flax *(Linum album)* (Baldi et al. 2008), sweet fennel (*Foeniculum vulgare*) (Dolatabadi et al. 2011a, b), *Chlorophytum* sp. (Gosal et al. 2010), respectively. The oil quality of the host seeds was influenced by *P. indica* in peppermint (*Mentha piperita*), and moldavian dragonhead (*Dracocephalum moldavica* L.) (Dolatabad et al. 2017; Amini et al. 2020). A high content of erucic acid and glucosinolates in oil is considered unsuitable for human consumption; *P. indica* reduced the amount of erucic acid and glucosinolates in rapeseed (*Brassica napus* L.) (Zhen-Zhu Su et al. 2017). Total oleoresin and piperine content in berries of *P. nigrum* (L.) was enhanced by *P. indica* (Anith et al. 2018).

4.9 *Possible Reasons for Beneficial Impact of* P. indica *on Host Plant*

P. indica colonization impacts the host plant in various ways. The maximum significant changes in the host plant could be due to the earlier expression of developmentally regulated genes (Waller et al. 2008), influence on plant hormone expression (Lee et al. 2011) and efficient mineral absorption (Nautiyal et al. 2010).

4.9.1 Mineral Absorption

The mutualistic soil microorganisms can influence the plant growth by providing the nutrients in the most absorbable form to the host plant. Both macro- and micronutrients were made available from the rhizosphere to host plant by *P. indica* (Franken 2012). The endophyte *P. indica* increased the absorption of phosphorous and nitrogen from the soil (Varma et al. 2001a, b; Shahollari et al. 2005; Kumar et al. 2011). *P. indica* assists in the transportation of the phosphate through the phosphate transporter (PiPT) (Yadav et al. 2010). *P. indica* could successfully create a significant impact on N, P, and K uptake in chickpea (*Cicer arietinum*) and black lentil (*Vigna mungo*) plants (Nautiyal et al. 2010; Kumar et al. 2012). The endophyte efficiently provided Fe and Cu in the sugar cane plants (Gosal et al. 2011), P in arabidopsis (Shahollari et al. 2005), and N in the tobacco plant (Sherameti et al. 2005). The starch hydrolysis activity detected in the *P. indica*-colonized roots indicates the possibility of the involvement of the endophyte in creating an adjunct carbohydrate sink in the plant (Sherameti et al. 2005). The starch-degrading enzyme can deplete the carbohydrate level in the host plant, but the endophyte compensates it by enhancing CO_2 assimilation rates in the barley plants (Achatz et al. 2010). Surprisingly, the *P. indica* inoculated root rhizosphere could degrade traces of diesel oil in soil (Baghaie and Daliri 2020). The recent investigation revealed that the freezing tolerance can be provided by the *P. indica* in arabidopsis; moreover, it can induce the expression of CBF-dependent pathway-related genes. *P. indica* increases the absorption of several minerals viz., N, Ca, Mg, P, K, S, B, Fe, and Zn and also significantly affect the agronomic parameters of B. napus (L.) (Zhen-Zhu Su et al. 2017). The absorption of sulfur was found increased by *P. indica*, ultimately ameliorated the overall growth in maize plants in low-sulfate environments (Narayan et al. 2021).

4.9.2 Plant Growth Hormones

Plant hormones may hold the key to understanding *P. indica* diverse host range. (Shahollari et al. 2005; Schäfer et al. 2009; Kumar et al. 2011; Lee et al. 2011). The production of phytohormone auxin by *P. indica* in brinjal (*Solanum melongena* L.) was confirmed by UV visible spectroscopy, Fourier transform infrared spectroscopy,

and mass spectroscopy. The enhanced level of auxin affected the host plant significantly (Swetha and Padmavathi 2016). Similar results were reported by Hua et al. (2017) by using high-throughput gas-chromatography-based mass spectrometry in *P. indica*-colonized Chinese cabbage roots. The involvement of Auxin in *P. indica* cultured host plant growth has been observed (Sirrenberg et al. 2007; Vadassery et al. 2008; Lee et al. 2011). However, the amount of auxin was varied depending upon the different plant species colonized by *P. indica.* The auxin production remains unaffected in *P. indica*-colonized arabidopsis (Vadassery et al. 2008), but auxin regulatory genes were found up-regulated in *P. indica*-colonized Chinese cabbage roots (Dong et al. 2013). In the co-culture experiment of *P. indica* and *A. thaliana* the production of IAA was increased which resulted in profuse root branching in the host plant (Sirrenberg et al. 2007). The auxin-enhanced root branching of *P. indica*-colonized soybean (*Glycine max*) resulted in enhanced absorption ability of the plant (Shen et al. 2006). The auxin produced through *P. indica* colonization developed bushy root hair in Chinese cabbage, which ultimately increased the absorption of water and minerals (Lee et al. 2011).

Vadassery et al. (2008) found that *P. indica*-colonized host plant produces relatively more amount of cytokinins. *P. indica* altered the expression of cytokinin regulatory genes during colonization in several host plants has been reported (Vadassery et al. 2008; Johnson 2014; Zhang et al. 2018). The *P. indica* co-cultured rice plants developed tolerance against biotic stress by utilizing GA as a signal component (Cosme et al. 2016). *P. indica* established the cell root colonization through GA signaling (Jacobs et al. 2011). Kim et al. (2017) studied the effect of *P. indica* colonization in Arabidopsis under long day conditions. The results showed the early flowering which was corroborated by increased transcript levels of the flowering associated genes. Thus, the *P. indica* encouraged early flowering in arabidopsis by promoting the GA expression (Pan et al. 2017).

ABA is a classical stress hormone and imparts resistance against host plantsfor salt and drought stress. The ABA is required for the symbiotic association between microbe and the host plant (Lievens et al. 2017). In arabidopsis, the expression of ABA was found to increase much before the physical interaction with the *P. indica* during the initial recognition phase (Vahabi et al. 2015). This ABA-dependent stress response was triggered by some mobile signaling molecules secreted by the *P. indica* viz., cellotriose and small secreted proteins (Akum et al. 2015). Such signaling molecules help in the establishment of symbiosis even under stress conditions. The prime role of ethylene is to regulate flowering, ripening, and senescence. Ethylene is also involved in plant adaptation under abiotic and biotic stress (Kende 2003; Abeles et al. 2012). In *P. indica* co-cultivation with the host plant, the expression of ACC synthase was up-regulated (Khatabi et al. 2012a, b; Ansari et al. 2013). The methionine synthase that provides substrates for ethylene synthesis in the host plant were enhanced by *P. indica* (Peškan-Berghöfer et al. 2004). During *P. indica* colonization, ethylene and jasmonic acid worked synergistically in the host (Verma et al. 1998). Ethylene activates innate immune reactions against harmful pathogenic microbes, but surprisingly responds mildly to the useful root-colonizing microbes. Ethylene tries to hinder the secondary colonization of the *P. indica,* but it

recognizes the beneficial symbiotic association its response become mild towards fungus. Thus ethylene helps to maintain the useful and harmful colonization traits by signaling components in *P. indica* colonization (Camehl et al. 2010).

Taken together, plants hormones may participate in the successful colonization of *P. indica* in host plant roots; moreover, plant hormones are indirectly involved in producing beneficial effects in the host plant.

5 Conclusions and Future Prospects

The survival of substantial numbers of tissue cultured plants mostly depends upon their adaptation ability to the changing environment. The pre-sensitized micropropagated plants by using fungus *P. indica* can increase the survival frequency. The axenically cultivable, broad host range and potential to impart several significant benefits to the host plant by the *P. indica* provides a better alternative to conventional bio-hardening agents. The *P. indica* successfully exploited in tissue cultured hardening processes of a few medicinal, important horticultural, and endangered plant species. In the future, there is scope to employ *P. indica* in various important plant species; moreover, the complex mechanism of interaction, commercial production, the effect of fungal derivatives, development of cheap alternative media for fugal cultureand genetic fidelity of the host crop after *P. indica* interaction with host plant is needed to be further studied.

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Chapter 6
Application of Biostimulants in Establishing and Acclimatizing In Vitro-Raised Plants

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Abstract In the past few decades, biostimulants have emerged as new and promising products able to increase crop yields and quality through efficient nutrient uptake, assimilation and translocation, water use, and the promotion of crop tolerance towards environmental factors. Although there is a large body of knowledge on the application of biostimulants in greenhouse and field trials, information on their use in plant tissue culture/micropropagation remains limited. In addition, micropropagation is highly dependent on the use of plant growth regulators, which are often costly and may cause the development of aberrant and undesirable physiological and epigenetic disorders. To mitigate these challenges, biostimulants can be applied as a suitable additive as they often contain plant growth-promoting constituents including various phytohormones such as cytokinins, auxins, and

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gibberellins that are generally applied in micropropagation. The application of biostimulants may holds much promise for the production of tissue cultured plants that are of economic value in generating higher plant yields and value-added plant-based products that are of superior quality whilst maintaining their physiological, biochemical, and genetic integrity for commercial exploitation at a larger scale. In this chapter, we provide a detailed assessment of the potential role of biostimulants in in vitro propagated and ex vitro acclimatized plants of economic value. The mechanism of action of the biostimulants during plant growth and development is discussed.

Keywords Phytohormones · Biochemicals · Gene expression · Micropropagation · Plant endophytes · Secondary metabolites · Shoot proliferation

1 Introduction

Plant tissue culture/micropropagation/in vitro propagation remains a powerful tool and technique enabling rapid crop improvements via biotechnological approaches (Kumlay and Ercisli 2015). It can be utilized to generate healthy, vigorous, and virus-free plantlets, independently of the climate, season, or geographical constraints (Gulzar et al. 2020). Production of certain crop species, such as strawberries (Hernández-Soberano et al. 2020), require an in vitro seed germination step, allowing for the identification of plants with desirable agronomic characteristics. Undifferentiated callus cultures may be used for transformation purposes or mutation breeding. Important plant metabolites and extracts may also be generated via bioreactors (Gulzar et al. 2020; Moyo et al. 2011). Additionally, micropropagation can contribute to the sustainable cultivation of over-exploited or rare species (Carmo et al. 2020; Moyo et al. 2011). Despite the numerous benefits offered by micropropagation, whether for agricultural, horticultural, or conservation purposes, the technique is undeniably costly, particularly with respect to the use of plant growth regulators (PGRs) and may be time-consuming to optimize (Gulzar et al. 2020). This is particularly related to the choice of nutrient medium, gelling agent, type and concentration of PGRs, light intensity and photoperiod requirements, and choice of explant (Gulzar et al. 2020). These optimized protocols may not be transferrable between species, cultivars, or even explant types and thus generally need to be established for every new sample that is to be cultured. Even so, not all genotypes will be easily cultured, and some plant species appear to be almost completely recalcitrant to micropropagation. Furthermore, the use of relatively high, non-physiological levels of PGRs is often associated with somaclonal variations (Bairu et al. 2011), which generally results in aberrant phenotypes and physiological, genetic, and epigenetic abnormalities.

Biostimulants have been shown to have considerable benefits in enhancing agricultural yields and productivity, enhancing aspects such as nutrient uptake,

nutrient translocation and assimilation, water use efficiency and enhanced tolerance to a variety of both biotic and abiotic factors (Kaur et al. 2021; Yakhin et al. 2017). There are several advantages that can benefit commercial plant tissue culture of horticultural crops in relation to the application of biostimulants. Generally, biostimulants are relatively low-cost options for enhancing plant growth and are used at extremely low concentrations (Carmo et al. 2021; Kaur et al. 2021). Often, biostimulants contain a variety of PGRs (Yakhin et al. 2017), consequently, they may provide a suitable option to help mitigate some of the challenges and costs associated with micropropagation. These either reduce or entirely supplant the use of PGRs and improve the quality of the plant material being cultured during the in vitro propagation stage and their acclimatization, whilst providing the plants with a head-start for further cultivation.

2 Overview of Biostimulants

Biostimulants are generally defined as substances that are applied at low concentrations that do not provide the plant with nutrients or direct protection against external stress, but rather stimulates the plant's endogenous metabolism to modify its physiological processes (du Jardin 2015, 2012). The earliest mention of this concept, known as a 'biogenic stimulant', was discussed in 1993 (Yakhin et al. 2017). Although it is generally accepted that the term 'biostimulant' was originally coined and defined in 1997 (http://grounds-mag.com, reviewed by du Jardin 2015) and only appeared in a peer-reviewed paper describing the alleviation effects of biostimulants on perennial ryegrass (*Lolium perenne* L.) in 2007 (Kauffman et al. 2007), the term 'plant biostimulant' was, however, used twice before this, in a paper on *Zea mays* (Ohlrogge 1977), and *Solanum lycopersicum* (Castro et al. 1988).

There is still no consensus on the definition of a biostimulant, but the United States Food and Drug Administration (US FDA) published the following: '[A] substance or micro-organism that, when applied to seeds, plants, or the rhizosphere, stimulates natural processes to enhance or benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, or crop quality and yield'. Since this definition could still include general fertilizers and crop protectors, the European Biostimulants Industry Council (EBIC) added two further caveats to the definition (www.biostimulants.eu/):

1. *Regardless of nutrient presence, plant biostimulants operate through different mechanisms than fertilizers.*
2. *They do not act directly against pests or disease, but rather affect the plant's vigour, therefore differing from crop protection products.*

One of the recent definition of a biostimulant is the following:

'A material which contains substance(s) and/or microorganisms whose function when applied to plants or the rhizosphere is to stimulate natural processes to benefit

nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and/or crop quality, independently of its nutrient content'. (Ricci et al. 2019).

Based on the review by Yakhin et al. (2017), the challenges in defining biostimulants were highlighted and the following definition was proposed: 'A formulated product of biological origin that improves plant productivity as a consequence of the novel or emergent properties of the complex of constituents, and not as a sole consequence of the presence of known essential plant nutrients, plant growth regulators, or plant protective compounds'. However, this definition excludes synthetic biostimulants. They have also been referred to as bioeffectors (Van Oosten et al. 2017), metabolic enhancers, phytostimulators (Yakhin et al. 2017), or elicitors (Mire et al. 2016). An elicitor is a biostimulant with a biotic origin (Vargas-Hernandez et al. 2017; du Jardin 2015). The term biostimulants will be used in this chapter, regardless of its origin or mechanism of action.

Biostimulants have a broad effect on plants, including stimulating plant growth by increasing plant metabolism, enhancing photosynthesis, inducing germination, and increasing the absorption of nutrients from the soil (Kaur et al. 2021; Yakhin et al. 2017). In addition, alleviation of abiotic stress in the presence of biostimulants is frequently cited (Blaszczak et al. 2016; Bulgari et al. 2019; Guinan et al. 2013; Sharma et al. 2014; Shukla et al. 2018; Trevisan et al. 2019; Van Oosten et al. 2017; Yamauchi 2018).

Biostimulants can be categorized into several groups based on their source. These groupings include humic and fulvic acids, beneficial bacteria and fungi, protein hydrolysates, seaweed and other botanical extracts, biopolymers such as chitosan, and inorganic compounds (du Jardin 2012; Gupta et al. 2021). Although these diverse biostimulants exert different aspects of plant growth, a vast overlap of effects has been reported. These include increased tolerance against abiotic and biotic stress (Trevisan et al. 2019) and increased uptake of nutrients (Desoky et al. 2018; Halpern et al. 2015). Nutrient use efficiency in terms of nutrient mobilization, uptake from the soil, transport, storage, and assimilation can also be improved by biostimulants (du Jardin 2015). Furthermore, biostimulants lead to crops of higher quality and yield and exhibit phytohormone-like activity (Colla et al. 2014). The stimulatory effect is usually holistic, improving several aspects of plant growth and health simultaneously. Biostimulants and their effects on plants is still a relatively novel field of research, and as such, is generally poorly understood (Gupta et al. 2021; Yakhin et al. 2017). Due to their holistic effects on the plant, it is difficult and often expensive to analyze the effects of biostimulant treatments. As a result of this lack of fundamental research into their active ingredients and modes of action or mechanisms of action (Yakhin et al. 2017), it is also challenging to classify products that may act as biostimulants (Fleming et al. 2019). However, the number of publications focusing on biostimulants is growing exponentially (Fig. 6.1), with scientific and commercial interest on the rise over the last decade (Rouphael and Colla 2018). From 2010 till present, close to 400 scientific documents have discussed the topic of biostimulants, particularly in the field of agriculture and related biological sciences. For this chapter, biostimulants will be characterized into five groups, namely plant growth-promoting rhizobacteria (PGPR), arbuscular mycorrhizal fungi (AMF),

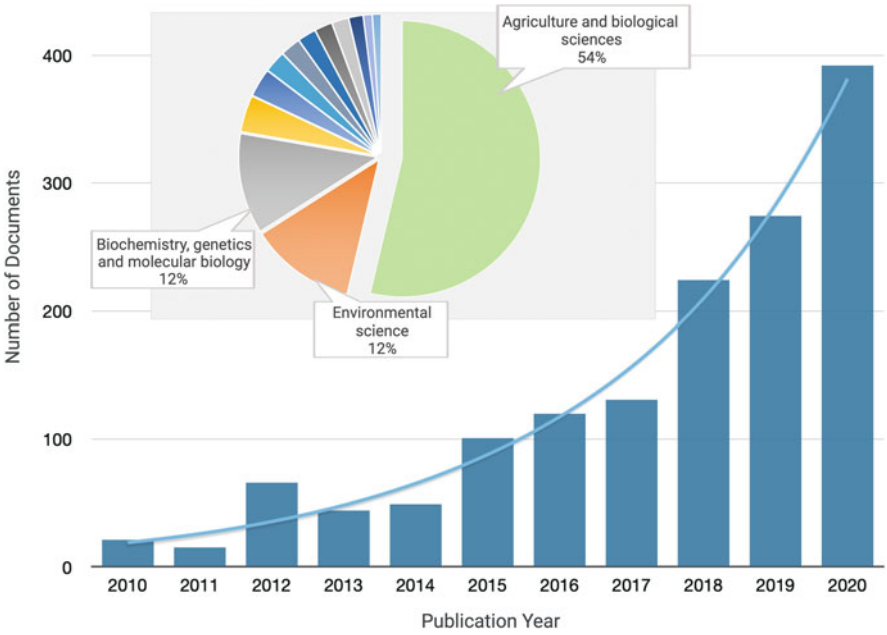

Fig. 6.1 Publication trend analysis demonstrating increasing scientific interest and research outputs in the area of biostimulants. Data were retrieved from Scopus (https://www.scopus.com; accessed 29 June 2021) using the keyword 'biostimulants' for the bar graph. The pie chart indicates major research fields that are represented in published documents

chitosan, compost/humic substances, and plant/seaweed-derived biostimulants. Although biostimulant research is still in its infancy, there have been a number of cases where biostimulants have been utilized to enhance the efficacy of micropropagation protocols for a variety of agriculturally and horticulturally important plants. In this chapter, we will be addressing the ways in which these various forms of biostimulants have been applied in micropropagation/plant cell tissue culture protocols, as well as discussing the possible underlying mechanisms by which these biostimulants influence plant growth, both during the in vitro growth stage and ex vitro acclimatization.

3 Effects of Biostimulants on Micropropagation Stages

The potential benefits of biostimulants have been explored at different stages (I–IV) of plant growth and development, for example, (I) establishment of aseptic cultures, (II) proliferation of axillary shoots, (III) in vitro rooting, (IV) hardening and acclimatization. During these various stages, responses of cultured explants and regenerating tissues are species-specific (Fig. 6.2). The application of biostimulants

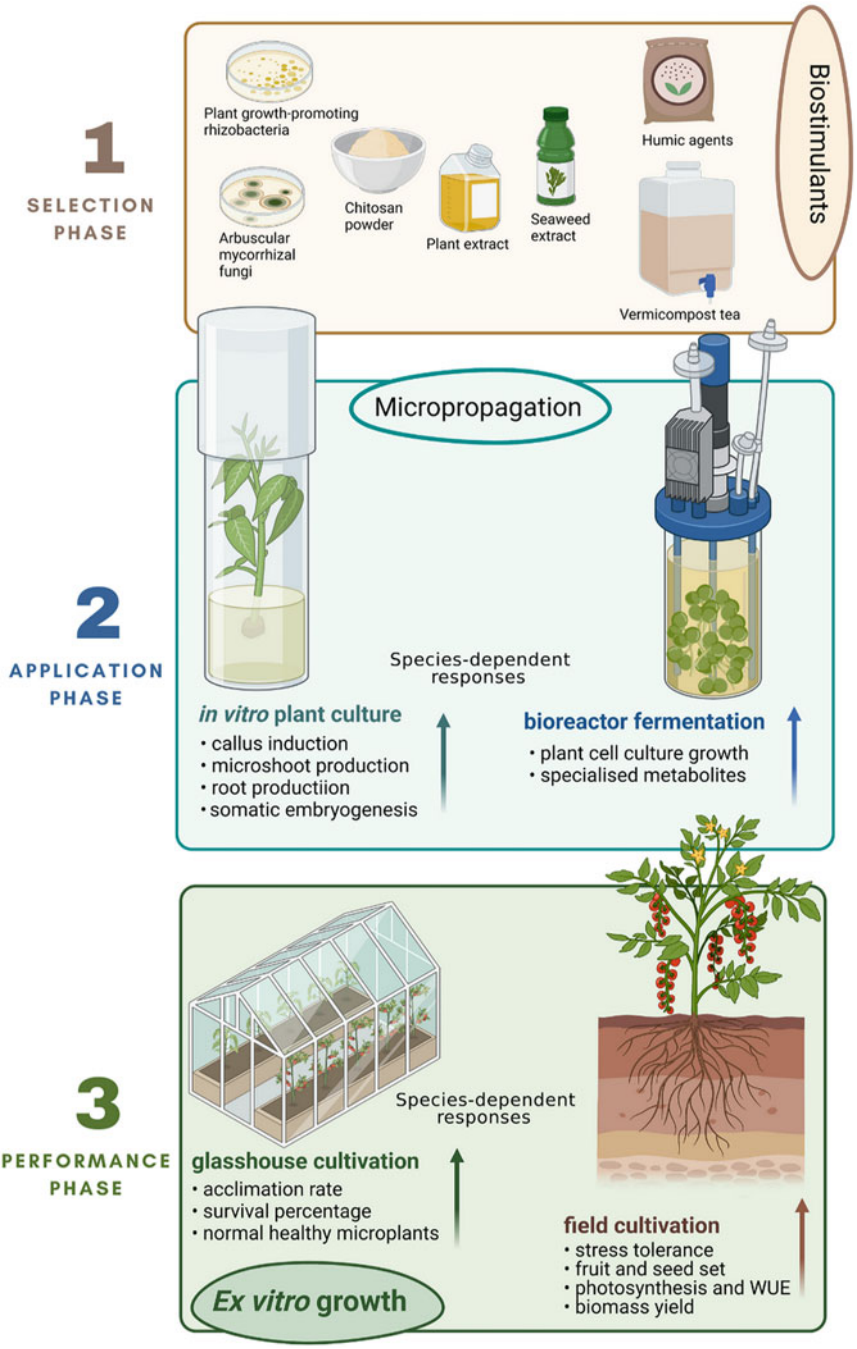

Fig. 6.2 Application of biostimulants in plant micropropagation and associated benefits. (1) Selection phase—examples of commonly used biostimulants; (2) Application phase—Biostimulant (s) application to solid and/or liquid cultures in vitro encourages prolific culture establishment and alternative routes for accessing specialized metabolites of industrial value; (3) Performance

during micropropagation requires three different phases. As there are several types of biostimulants that can be applied in micropropagation, during the selection phase, after choosing a biostimulant, the mode of application, and concentration of a biostimulant may require optimization and both solid and liquid culture systems offer the opportunity to test the effectiveness of a biostimulant on plant growth and development during micropropagation to encourage plantlet regeneration in vitro (Fig. 6.2).

Beyond the beneficial impact on plant physiology, biostimulants are able to influence plant biochemicals, central (primary) and specialized (secondary) metabolites, whilst regulating plant epigenomic, proteomic, and transcriptomic metabolism (Drobek et al. 2019; Ganugi et al. 2021; González-Morales et al. 2021). Microbial originated volatile organic compounds (VOCs) modulate plant physiological and hormonal pathways to increase biomass and yield production in plants by producing and releasing phytohormones including gibberellins, auxins, cytokinins (CKs), jasmonic acid, and ethylene (López-Bucio et al. 2015; Mohanram and Kumar 2019; Sharifi and Ryu 2018).

4 Micropropagation Stage I

Biostimulant activity during micropropagation stages is modulated by different factors such as plant species/genotype, stages of growth, and applied PGRs. Application of diverse biostimulants with/without PGRs exerts diverse morphological changes in plants at different micropropagation stages (Table 6.1). Traditionally, micropropagation involves the use of PGRs in order to enhance growth and development, but at times direct organogenesis is not successfully achieved and callus cells are formed. These types of cells are induced in an auxin-rich medium. However, biostimulants have also been used to enhance callus formation in some plants. For instance, PGPR (Avercom, Avercom nova-2, Violar, and Phytovit) or vermicompost tea in combination with PGRs (auxins and CKs) stimulated callus initiation and formation in common wheat and sainfoin (Beyaz and Türkay 2019; Tsygankova et al. 2017). In common wheat, the application of PGPR was more beneficial during callus formation relative to PGRs (auxins and CKs) as they resulted in low callus regeneration efficiency. An innovative nanotechnology (zinc oxide nanoparticles) system together with humic acid was used by Al-Mayahi (2021) to increase callus induction and callus-producing buds in date palm. Interestingly, infecting callus with an endophytic strain of *Methylobacterium extorquens* prevented browning in *Pinus sylvestris* cultures, yielding green and healthy callus cells (Pirttilä et al. 2008). Even though callus is not often desirable for the production

Fig. 6.2 (continued) phase—growth productivity during ex vitro acclimation leads to plants that are suited for field cultivation and can cope with environmental stresses

Table 6.1 Examples of biostimulant utilization at different stages involved in the micropropagation of different plants

Micropropagation stages	Biostimulant category	Biostimulant type/treatment	PGR	Plant species (common name)	Reported outcome	References
Callus induction (stage I)	PGPR	Avercom, Avercom nova-2, Violar, Phytovit	2,4-D, BAP, IAA	*Triticum aestivum* L. (common wheat)	Avercom, Avercom nova-2, Violar and Phytovit increased callus cell formation in *T. aestivum* when combined with 2,4-D	Tsygankova et al. (2017)
	Compost	Vermicompost tea	BA, NAA	*Onobrychis viciifolia* Scop. (Sainfoin)	Application of 20% vermicompost tea in combination with PGRs resulted in callus initiation	Beyaz and Türkay (2019)
	Humic substances	Humic acid (HA) with/without zinc oxide nanoparticles (ZnO-NPs)		*Phoenix dactylifera* L. (date palm)	Supplementing media with HA and ZnO-NPs resulted in high levels of callus induction and callus-producing buds	Al-Mayahi (2021)
	Seaweed	*Gracilaria edulis*, *Padina boergesenii*		*Eleusine coracana* Gaertn. (finger millet)	Seaweed treatments enhanced embryogenic callus induction in finger millet	Satish et al. (2016)
Shoot proliferation (stage II)	PGPR	*Arthrobacter agilis*, *Bacillus methylotrophicus*, volatile compounds from bacteria		*Fragaria* x *ananassa* Duch (strawberry)	Exposing in vitro propagated strawberry plants to *A. agilis* significantly improved shoot fresh weight, with volatile compounds of *A. agilis* mainly stimulating shoot fresh weight	Hernández-Soberano et al. (2020)
	PGPR	*Pseudomonas putida* strains (SJ04, SJ25, SJ48), *P. fluorescens* CS417r strain		*Mentha piperita* L. (peppermint)	Direct inoculation of *M. piperita* explants with native (Argentina) PGPR strains improved shoot ramification number, leaf number, shoot	Santoro et al. (2015)

					fresh weight, and the number of trichomes	
	AMF	*Cladosporium sphaerospermum*		*Nicotiana tabacum* L. (tobacco)	Tobacco plants exposed to *C. sphaerospermum* had increased stem length, shoot biomass, leaf length, and leaf biomass	Li et al. (2019)
	Chitosan	*N*-acetylated chitosan		*Ipomoea purpurea* L. (purple morning glory)	Chitosan oligomer mixture stimulated shoot induction, shoot length, and leaf number in *I. purpurea*	Acemi et al. (2018)
	Chitosan	Shrimp, fungal, Oligomer chitosan		*Dendrobium phalaenopsis* (orchid)	Application of chitosan in *D. phalaenopsis* stimulated plantlet regeneration (5–7 plantlets) within 12 weeks	Nge et al. (2006)
	Chitosan	Chitosan	BAP	*Coleus aromaticus* Lour. (Mexican mint)	Combination of chitosan and BAP induced shoot multiplication in *C. aromaticus*	Govindaraju and Indra Arulselvi (2018)
	Chitosan	Chitosan		*Stevia rebaudiana* Bertoni (Candyleaf)	*Stevia rebaudiana* growth parameters including shoot length, shoot number, and dry weight were stimulated with the application of chitosan	Rasouli et al. (2021)
	Seaweed	*Hypnea Pseudomusciformis* extract, *Gracilaria* spp. (GrE) extract		*Comanthera mucugensis* (Giul.) L.R. Parra and Giul. (Empre-viva de Mucugê)	*Gracilaria* spp. extract increased bud formation in *C. mucugensis* explants, whereas *H. pseudomusciformis* extracts improved aerial bud growth	Carmo et al. (2020)

(continued)

Table 6.1 (continued)

Micropropagation stages	Biostimulant category	Biostimulant type/treatment	PGR	Plant species (common name)	Reported outcome	References
	Seaweed	*Turbinaria decurrens*	BAP, IBA	*Solanum surattense* Burm F. (yellow-fruit nightshade)	*Turbinaria decurrens* extract combined with BAP increased shoot number in *S. surattense*	Gurusarava et al. (2017)
	Seaweed	*Fucus spiralis, Cystoseira myriophylloides*, *Laminaria digitata*		*Nicotiana benthamiana* Domin (Benth or Benthi)	Seaweed treatments enhanced explant regeneration and shoot elongation	Esserti et al. (2017)
	Seaweed	*G. salicornia, Padina gymnospora, P. boergesenii, Gelidiella acerosa*		*Solanum melongena* (L.) cv Pusa purple long (Brinjal)	Seaweed extracts enhanced the elongation of shoots in brinjal	Satish et al. (2015)
	Seaweed	*Caulerpa cylindracea*, *Asparagopsis taxiformis*		*Prunus armeniaca* (L.) cv 'Canino' (apricot)	Seaweed-treated apricots had improved plantlet regeneration and produced multiple shoots	Alburquerque et al. (2019)
	Seaweed	*G. salicornia, Kappaphycus alvarezii*		*Bacopa monnieri* (L.) Pennell (water hyssop)	Seaweed extract concentrations improved shoot induction in *B. monnieri*	Rency et al. (2017)
	Seaweed	*G. edulis*, *Sargassum wightii*		*Lycopersicon esculentum* L. (tomato)	*Gracilaria edulis* and *S. wightii* enhanced shoot proliferation in tomatoes	Vinoth et al. (2019)
	Humic substances	Humic fractions		*Actinidia deliciosa* Liang and Ferguson (kiwifruit)	Shoot weight was enhanced by the application of high molecular weight humic fraction	Marino et al. (2008)
Embryogenesis (stage II)	AMF	*Laccaria bicolor*, *L. laccata*, *Pisolithus tinctorius*		*Abies cephalonica* Loudon (Greek fir)	Treatment of plants with ectomycorrhizal fungi reduced embryogenic cell line proliferation. Dual culture during the embryogenic cell	Krajňáková et al. (2012)

					proliferation favoured embryo formation and maturation of *A. cephalonica*	
	Seaweed	*Caulerpa scalpelliformis, G. corticata*	BAP	*Lycopersicon esculentum* L. (tomato)	Seaweed extracts were beneficial in embryo development and the conversion to plantlets	Vinoth et al. (2014)
	Seaweed	*Ulva lactuca* and silver nanoparticles using *U. lactuca* extract (ULAgNPs)	BAP, KIN, NAA, ABA	*Gloriosa superba* L. (flame lily)	The combined effect of *U. lactuca* extract, ULAgNPs and PGRs influenced somatic embryo formation, maturation, and conversion into plantlets	Mahendran et al. (2018)
	Seaweed	*Gracilaria edulis*, *Padina boergesenii*		*Eleusine coracana* Gaertn. (finger millet)	Treatment enhanced the maturation of embryos and their conversion to plantlets	Satish et al. (2016)
In vitro rooting efficiency (stage III)	PGPR	Metabolites secreted by *Pantoea agglomerans*	IBA	*Pyrus communis* L. cv Dar Gazi (European pear)	Root formation in *P. communis* plants treated with metabolites secreted by *P. agglomerans* emerged within 7 days, and the treatment stimulated root elongation. Combining exo-metabolites with IBA improved root number and root percentage in *P. communis* plants	Luziatelli et al. (2020)
	PGPR	*Pseudomonas fluorescens* VUPF5, *Bacillus subtilis* VRU1		*Pistacia* spp. (UCB1 pistachio)	*Pseudomonas fluorescens* VUPF5 and *B. subtilis* VRU1 nanocapsules improved root length in UCB1 pistachio plants. Likewise, nanoformulation of VUPF5	Pour et al. (2019)

(continued)

Table 6.1 (continued)

Micropropagation stages	Biostimulant category	Biostimulant type/treatment	PGR	Plant species (common name)	Reported outcome	References
					metabolite strain stimulated root length in plants	
	AMF	*Rhizophagus irregularis*, *Claroideoglomus etunicatum*, *C. etunicatum*, *Funneliformis mosseae*		*Tectona grandis* Linn.f. A (teak)	Successful colonization of AMF was achieved in *T. grandis* roots. *Tectona grandis* plantlets inoculated with *R. irregularis* had increased root length colonization percentage	Chaiyasen et al. (2017)
	AMF	*Rhizoglomus irregulare*		*Linum usitatissimum* L. (flax)	Inoculation of plants in *R. irregulare* caused a reduction in available phosphorus, which lead to changes in plant responses and root system modification	Kokkoris and Hart (2019)
	Endophytic fungi	*Colletotrichum gloeosporioides*		*Centella asiatica*	Co-cultivation of *C. gloeosporioides* with C. *asiatica* improved root length and root number of co-cultivated plants	Gupta and Chaturvedi (2018)
	Seaweed	*Chlorella sorokiniana*	BAP, IBA	*Schomburgkia crispa* Lindley (Schomburgkia)	*Chlorella sorokiniana* extract combined with IBA had a stimulatory effect on *S. crispa* rooting capacity	Pereira et al. (2018)
	Seaweed	*Fucus spiralis, Cystoseira myriophylloides*, *Laminaria digitata*		*Nicotiana benthamiana* Domin (Benth or Benthi)	Seaweed treatments enhanced plant rooting	Esserti et al. (2017)

	Seaweed	*Fucus spiralis, Cystoseira myriophylloides*, *Laminaria digitata*	BAP, IBA	*Vitis vinifera* var. Doukkali (grapevine)	Seaweed treatments enhanced rooting in grapevine	Esserti et al. (2017)
	Seaweed	*G. edulis*, *Sargassum wightii*		*Lycopersicon esculentum* L. (tomato)	Seaweed biostimulants enhanced the rooting in tomatoes	Vinoth et al. (2019)
	Seaweed extract	*Turbinaria decurrens*	BAP, IBA	*Solanum surattense* Burm F. (yellow-fruit nightshade)	The combined effect of the seaweed extract with IBA improved the rooting frequency of *S. surattense* plants	Gurusarava et al. (2017)
	Plant extract	Citrus-based plant biostimulant (BC204)		*Arabidopsis thaliana* (Arabidopsis)	The citrus-based extract BC204 improved growth parameters in *A. thaliana* including root length as well as fresh and dry weight of harvested roots after exposure to salt stress	Loubser and Hills (2020)
	Humic substance	Humic acid		*Rhododendron* section *Tsutsusi* (azaleas)	Rooting (%), root number, and root length increased in plants treated with humic acid	Elmongy et al. (2018)
	Humic substance	Landfill leachate and leonardite humic substances		*Alnus glutinosa* L. Gaertn (Alder) and *Betula pendula* Roth (birch)	Humic substances and their humic acid fractions promoted root growth in plants through the modulation of ABCB transporter transcript levels (*ABCB1* and *ABCB19*)	Tahiri et al. (2016)
	PGPR	*Arthrobacter agilis* and *Bacillus methylotrophicus* and volatile compounds produced by bacteria		*Fragaria* x *ananassa* Duch (strawberry)	Inoculating *Fragaria* x *ananassa* plants with *A. agilis* UMCV2 or *B. methylotrophicus* separately did not show any significant	Hernández-Soberano et al. (2020)

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Table 6.1 (continued)

Micropropagation stages	Biostimulant category	Biostimulant type/treatment	PGR	Plant species (common name)	Reported outcome	References
					changes in plant yield after plants were transferred to the greenhouse. Combined inoculation of bacterial strains in plants improved *Fragaria* x *ananassa* yields, producing fruit with high pH and soluble solids	
Acclimatization (stage IV)	AMF	*Cladosporium sphaerospermum*		*Nicotiana tabacum* L. (tobacco)	After tobacco transplantation in greenhouse conditions, plants exposed to the fungus retained higher rates of growth	Li et al. (2019)
	Chitosan	*N*-acetylated chitosan (chitosan oligomer mixture with a degree of polymerization)		*Ipomoea purpurea* L. (purple morning glory)	During acclimatization stages, 86% of the plants survived with no recorded morphology variation	Acemi et al. (2018)
	Chitosan	ChitoPlant, ChiPro GmbH Bremen		*Solanum tuberosum* L. cv. Désirée (potato)	Transplantation of in vitro propagated plants to the greenhouse increased the number of mini-tubers and yields. Chitosan application *to* in vitro- and greenhouse-grown plants enhanced seed quality of mini-tubers	Kowalski et al. (2006)
	Seaweed extract[a]	Kelpak		*Acacia mearnsii* De wild. (black wattle)	The applied Kelpak treatment contributed to a 90% survival rate in black wattle	(Beck et al. (1998)

	Seaweed[a]	Kelpak		*Anthurium parvispathum* Hemsl.	Acclimatization of *A. parvispathum* was improved with seaweed treatment	Atta-Alla et al. (1998)
	Seaweed[a]	Kelpak		*Dierama luteoalbidum* I.Verd.	All acclimatized plantlets formed corms after 6 months following the application of Kelpak	Madubanya et al. (2006)
	Seaweed[a]	Kelpak		*Eulophia cucullata* (Afzel. Ex Sw.) Steud. (foxglove orchid)	Application of Kelpak every second week resulted in a 70% survival rate	McAlister and van Staden (1998)
	Seaweed[a]	Kelpak		*Eulophia streptopetala* Lindl. (orchid)	*Eulophia streptopetala* had a 90% survival rate after Kelpak application	McAlister and van Staden (1998)
	Seaweed[a]	Kelpak		*Eulophia petersii* (Rchb. f.) Rchb.f. (orchid)	Kelpak resulted in a 60% survival rate during the acclimatization of *E. petersii*	McAlister and van Staden (1998)
	Seaweed[a]	Kelpak		*Hosta* spp. 'gold drop'	Kelpak treatments had a 100% survival rate similar to the control treatment	Ördögh et al. (2019)
	Seaweed[a]	Kelpak		*Impatiens flanaganae* Hemsl. (Mrs Flanagan's impatiens)	Kelpak solution enabled successful acclimatization of regenerated plantlets	Nikolova et al. (1996)
	Seaweed[a]	Kelpak		*Kniphofia pauciflora* baker (dainty poker)	Kelpak improved *K. pauciflora* rooting and plant establishment during acclimatization	Lindsey et al. (1998)
	Seaweed[a]	Kelpak		*Scilla krausii* baker (wild squill)	*Scilla krausii* increased rooting and plantlet establishment during plant acclimatization	Lindsey et al. (1998)

(continued)

Table 6.1 (continued)

Micropropagation stages	Biostimulant category	Biostimulant type/treatment	PGR	Plant species (common name)	Reported outcome	References
	Seaweed[a]	Kelpak		*Pinus patula* Schiede ex Schltdl. & Cham. (Patula pine)	Acclimatization in pine plantlets improved with the application of Kelpak resulting in higher conversion frequencies (about 40.3%)	Jones and Van Staden (1997)
	Seaweed[a]	Kelpak		*Salvia africana-lutea* L. (Golden sage)	Sage plantlets acclimatization rate reached 88% (within a month) with Kelpak application	Makunga and van Staden (2008)
	Seaweed[a]	Kelpak		*Solanum tuberosum* cv. 'BPI' (potato)	During the transplantation process, leaf/soil drench application of Kelpak delayed rooting, decreased root fresh and dry weights and increased the shoot: root ratio	Kowalski et al. (1999)
	Seaweed[a]	Kelpak		*Thapsia garganica* L. (deadly carrots)	Kelpak enhanced rooting and plantlet establishment during acclimatization	Makunga et al. (2006, 2003)

Abscisic acid (ABA); Arbuscular Mycorrhizal Fungi (AMF); 6-Benzylaminopurine or benzyl adenine (BAP or BA); Indole-3-butyric acid (IBA); Indole-3-acetic acid (IAA); Kinetin (KIN); 1-Naphthaleneacetic acid (NAA); Plant Growth-Promoting Rhizobacteria (PGRP); Zinc oxide nanoparticles (ZnO-NPs)

[a] (Stage IV): Kelpak applications listed were mainly applied as treatment during the acclimatization stage for the in vitro regenerants

of plants that are true-to-type using an indirect organogenesis route, callus is more readily exploitable for large-scale production using industrial fermenter technologies. The production of meristemoidal callus and/or somatic embryogenic callus also offers opportunities for long-term cryopreservation of genotypes and germplasm. This is often the chosen route for maintaining superior genotypes and/chemotypes in industrial settings as it saves on manual labour which requires routine subculture in commercial production pipelines.

5 Micropropagation Stage II

Proliferation helps to increase the formation of axillary shoots typically cultured on medium supplemented with relatively high CK concentration (Gaba 2005). The production process of this stage has high-cost implications and some of the costs are attributed to the use of PGRs. Therefore, evaluating the possible use of biostimulants in the proliferation stages could be of immense value due to their low cost and proven ability to enhance crop productivity at low concentrations. This is of particular interest to those that operate commercial plant tissue culture laboratories with the intention of generating horticultural crops at an industrial scale. Microbial inoculants (PGPR and AMF) and bacterial volatile compounds (BVCs) promoted shoot proliferation and shoot biomass in three valuable horticultural crop species, namely strawberry, peppermint, and tobacco (Hernández-Soberano et al. 2020; Li et al. 2019; Santoro et al. 2015). The beneficial effect of such microbes might be due to a number of factors such as microbe cultivation and conditions, BVCs employed and dosage control, plant growth stage, and duration. Chitosan application with/without PGRs also stimulated shoot proliferation in several plant species (Acemi et al. 2018; Govindaraju and Indra Arulselvi 2018; Nge et al. 2006; Rasouli et al. 2021). Due to the biostimulants biocompatibility, biodegradability, and bioactivity, application of chitosan in agriculture and horticulture has been shown to regulate various physiological processes leading to growth efficiency in commercial plants.

Somatic embryogenesis is a desirable method for plant clonal propagation because of its biological (growth and development process beginning with a somatic embryo) and practical (development of root and shoot meristems) advantage. The method allows for preservation of embryogenic cell lines for prolonged periods in cryo-storage. Although not a common practice, efficient protocols have been developed to assess the influence of biostimulants in plant species (Greek fir, tomato, flame lily, finger millet) regeneration (Krajňáková et al. 2012; Mahendran et al. 2018; Satish et al. 2016; Vinoth et al. 2014). Additives such as polyamines and malt extracts have also been used to achieve high yields of somatic embryos and plant regenerants in economically valuable species such as banana, citrus, ginseng, and coconut amongst others (Hussain et al. 2016; Natarajan et al. 2020; Rakesh et al. 2021). Advantages associated with somatic embryogenesis are evident, yet few studies have investigated the effect of biostimulants on the proliferation of

embryogenic cell masses and the subsequent after-effects related to maturation of embryogenic cultures. This provides a strong impetus for more research directed towards understanding the role of biostimulants when using somatic embryogenesis and organogenesis systems for plant regeneration.

6 Micropropagation Stage III and IV

Bacterial volatile compounds are considered as a 'chemical language' used by bacteria to communicate with plant partners in turn able to promote aerial (enhance photosynthesis) and root architectural organization (Sharifi and Ryu 2018). Rhizobacterial volatile compounds increased rooting frequency of European pear and pistachio (Luziatelli et al. 2020; Pour et al. 2019) as well as shoot fresh weight of strawberries (Hernández-Soberano et al. 2020). The role of biostimulants in the promotion of in vitro rooting efficiency has been reported (Elmongy et al. 2018; Loubser and Hills 2020; Pereira et al. 2018), a crucial step before transplantation of micro-cuttings into greenhouse and field conditions. The success of protocol development in micropropagated plantlets is highly dependent on the transfer and re-establishment of plants in greenhouse conditions (Trigiano and Gray 2005). In the observed studies, a limited number of in vitro propagated regenerants were transferred and successfully acclimatized in the greenhouse, for example, strawberries (Hernández-Soberano et al. 2020), tobacco (Li et al. 2019), and purple morning glory (Acemi et al. 2018) and potato (Kowalski et al. 2006). However, in a number of in vitro propagated plantlets biostimulants were mainly applied during acclimatization period to minimize transplant shock and improve plant survival in greenhouse conditions. During the transplantation stage, the application of biostimulants contributed to improved growth, yields, and seed quality of potato mini-tubers.

To our knowledge, the adoption of biostimulants, with the exception of seaweed-based extracts, is not currently at a broad scale for commercial plant tissue culture environments even though there is increasing scientific evidence emanating from research laboratories of the usefulness of biostimulants in micropropagation protocols. For large-scale commercial production of plants, there are now industrially formulated biostimulant products that are a convenient solution for the cultivation of horticultural crops using micropropagation (Parađiković et al. 2019). However, commercial plant tissue culture operations may show some reluctance in using biostimulant technology if an additional cost to production will offset a return on the investment as setting up a plant tissue culture facility and producing plants using micropropagation is capital intensive, especially in developing countries (FAO/IAEA-TECDOC 2004). For more reliable use of biostimulants in commercial in vitro plant propagation, more information is needed in terms of the dosages that can assist with increasing seed, plant vigour, and production yields (Parađiković et al. 2019). Nonetheless, a more widespread use of various biostimulants in commercial laboratories that sell tissue cultured plant products is likely to follow

as more scientific research shows evidence that biostimulants increase proliferation rates of micropropagated materials that may be better able to cope with environmental stresses after transplantation across a wide range of economically important plant species. It is imminent that commercial plant tissue culturists will soon realize the potential of including a wide range of biostimulants in their operations and micropropagation protocols if cost reductions are associated with using this type of technology to deliver plants of high quality and integrity that meet market demands, especially if the utilization of biostimulants during micropropagation reduces the frequency of subcultures, shortening the culture time in vitro to ex vitro plantation.

7 Effects of Biostimulants on Physiological, Biochemical, Phytochemical, and Gene Expression in Micropropagated Plants

The importance of photosynthetic efficiency, antioxidant enzymes, central and specialized metabolite production in in vitro propagated plants cannot be overemphasized. On this basis, a few studies have explored the effects of biostimulants on biochemical, gene expression, and phytochemicals in in vitro regenerants (Table 6.2). Biochemical composition in date palm, kiwi, and azaleas varied in response to the application of humic substances in the growth media (Al-Mayahi 2021; Elmongy et al. 2018; Marino et al. 2008, respectively). Significant variations were evident in the quantity of the micro- and macro-elements, total soluble proteins, chlorophyll content, antioxidant enzymes, and endogenous phytohormones. Furthermore, changes in the biochemical profile facilitated by the applied biostimulants, triggered the expression of genes involved in photosynthesis, phytohormone homeostasis and defence responses, sucrose and glycine betaine metabolism, and antioxidative activities in spinach (Fan et al. 2013) and peppers (Li et al. 2019). Such changes are of significance in the context of producing plants of higher nutritional value that can be easily adapted to growing under variable climatic stress conditions.

Elicitation using both biotic and abiotic factors to elevate the accumulation of specialized metabolites has a long history in biotechnological strategies that aim to exploit plant cells as biofactories for chemical and pharmaceutical industries and many examples are provided in the review of Ramirez-Estrada et al. (2016). As an example, the application of PGPR and chitosan stimulated the production of major specialized metabolites including steviol glycosides, stevioside, rebaudioside A, and monoterpenes quantified from essential oils (Rasouli et al. 2021; Santoro et al. 2015). A range of polyphenolics were much higher in concentration in chitosan-treated callus cultures, and chitosan not only increased catechins but also resulted in elevated biomass in callus cultures of *Fagonia indica* (Khan et al. 2019). Moreover, chitosan and seaweed application in cell suspension cultures of *Taxus baccata* and *Withania somnifera* stimulated the production of taxol (Paclitaxel), a novel

Table 6.2 Examples of the effects of biostimulant utilization on the biochemical, phytochemical, and gene expression of different micropropagated plants

Biostimulant category	Biostimulant type/ treatment	Plant species (common name)	Reported outcome	References
PGPR	*Pseudomonas putida* strains (SJ04, SJ25, SJ48) and *P. fluorescens* WCS417r strain	*Mentha piperita* L. (peppermint)	Total essential oil and major monoterpenes present in *M. piperita* were enhanceed by some of the tested bacterial strains	Santoro et al. (2015)
AMF	*Cladosporium sphaerospermum*	*Nicotiana tabacum* L. (tobacco)	Plant exposure to the fungus resulted in the expression of genes involved in cell expansion, cell cycle, photosynthesis, phytohormone homeostasis, and defence responses	Li et al. (2019)
Chitosan	Chitosan	*Coleus aromaticus* Lour. (Mexican mint)	In vitro treated plants accumulated high levels of alkaloids, flavonoids, saponins, terpenoids, total phenolic content, and tannins. Increased *PAL* gene expression was relative to in vivo propagated control plants	Govindaraju and Indra Arulselvi (2018)
Chitosan	Chitosan	*Stevia rebaudiana* Bertoni (Candyleaf)	Chitosan stimulated the production of steviol glycosides, stevioside, and rebaudioside A which are major phytochemicals in *S. rebaudiana*	Rasouli et al. (2021)
Chitosan	Chitosan and amino acid complex (liquid form, INAGROSA company, Spain)	*Taxus baccata* L. (English yew)	Taxol production increased in cell suspension cultures of *T. baccata* with the application of chitosan and amino acid complex	Najafabadi et al. (2020)
Chitosan	Chitosan	*Fagonia indica* Burm.f. (White spine)	Chitosan application increased phenolic and flavonoid content in *F. indica*. The enhanced production of phytochemicals correlated with antioxidant enzyme activity of plants	Khan et al. (2019)

(continued)

Table 6.2 (continued)

Biostimulant category	Biostimulant type/ treatment	Plant species (common name)	Reported outcome	References
Chitosan	Chitosan	*Psammosilene tunicoides* W. C. Wu et C. Y. Wu	Hairy roots of *P. tunicoides* contained high levels of saponin content. Differential expression of genes encoding antioxidant enzymes (*SOD*, *POD,* and *GR*), stress-responsive transcription factors (*WRKYs* and *NACs*) and terpenoid biosynthetic enzymes (*DXS*, *GPPS,* and *SE*) increased in chitosan exposed plants contributing to saponin metabolism	Qiu et al. (2021)
Seaweed	*Ascophyllum nodosum*	*Spinacia oleracea* L. (spinach)	*Ascophyllum nodosum* extract increased total soluble protein content, phenolics, flavonoids, chlorophyll, and antioxidant activity. The extract induced transcripts and upregulated genes involved in sucrose and glycine betaine metabolism. Furthermore, *A. nodosum* led to higher cytosolic glutamine synthetase, betaine aldehyde dehydrogenase, choline monooxygenase, and glutathione reductase transcription in plants	Fan et al. (2013)
Seaweed	*Sargassum wightii* and *Gracilaria edulis*	*Withania somnifera* (L.) Dunal (Indian ginseng)	*Gracilaria edulis* extracts significantly increased withanolides production (withanolide A, withanolide B, withaferin A, and withanone) in plants	Sivanandhan et al. (2013)

(continued)

Table 6.2 (continued)

Biostimulant category	Biostimulant type/ treatment	Plant species (common name)	Reported outcome	References
Humic substance	Humic acid	*Rhododendron* section *Tsutsusi* (azalea)	Humic acid increased endogenous phytohormones (IAA, GA, ZR, iPA) accumulation and the upregulation of enzyme activity (POD, SOD, APX, CAT, and PPO) and total soluble protein in azaleas	Elmongy et al. (2018)
Humic substance	Humic acid with or without zinc oxide nanoparticles (ZnO-NPs)	*Phoenix dactylifera* L. (date palm)	Mineral elements such as N, P, K, S, and Zn increased in shoots with the application of humic acid. A similar trend was also observed in antioxidant enzymes (CAT and POD), chlorophyll content, and the endogenous IAA concentration	Al-Mayahi (2021)
Humic substance	Humic fractions (low molecular weight and high molecular weight)	*Actinidia deliciosa* Liang and Ferguson (kiwifruit)	The lower molecular fraction humic substance increased micro- and macro-elements in kiwi plantlets	Marino et al. (2008)

compound, and withanolides in plants. The progress in the use of biostimulants as elicitors in plant cell culture is deemed to accelerate as better resolution of how these functional agents alter specialized metabolism at both the genetic and biochemical levels (Mrid et al. 2021) and those biostimulants that act via jasmonic acid signalling mechanisms *in planta* that elicit defence-related gene expression induce the accumulation of transcripts associated with phenylalanine ammonia lyase (PAL), tryrosine aminotransferase, superoxide dismutase (SOD), catalase (CAT), and peroxidase and subsequent production of these enzymes. Using a transcriptomics approach, Qiu et al. (2021) studied the effects of chitosan in a set of hairy root cultures in *Psammosilene tunicoides* and genes encoding for key regulatory enzymes that act in the terpenoid biosynthesis were regulated via nitric oxide signalling pathways that are dependent on ROS-mediated activation. The main important transcription factors were from the NAC, WRKY, and AP2/ERF classes. There is an increasing body of knowledge associated with complex molecular genetic

networks that control physiological and biochemical mechanisms that assist with plants to respond to various stresses that are controlled by biostimulants in plants and although many studies thus far have utilized whole plants growing in ex vitro environments, many of these mechanisms are displayed by those plants growing in in vitro culture. For a comprehensive review, the work of González-Morales et al. (2021) provides detailed information on how plant extracts such as biostimulants, including those manufactured from seaweeds, are implicated in salinity, drought and heat, and several molecular markers such as heat shock proteins and late embryogenesis abundant (LEA) proteins are expressed. There are thus many opportunities to explore these types of biostimulants as scientific tools to study stress adaptations of plants in vitro.

8 Mechanisms of Action of Biostimulants Under In Vitro Prorogation and Acclimatization Phase

Increasing evidence has shown that biostimulants improve various aspects of in vitro plant growth by their effect on biochemical and physiological processes. However, biostimulants are complex heterogeneous products, which do not regulate plant physiological processes and promote growth by targeting a single specific biochemical site (Omoarelojie et al. 2021). Yakhin et al. (2017) distinguish between 'mode of action' and 'mechanism of action' in explaining the regulatory effects of biostimulants in plant growth and development. According to Yakhin et al. (2017), *mode of action* refers to a distinct effect of a bioactive molecule on a discrete biochemical or regulatory target, whereas the *mechanism of action* describes the integral effect of multiple biochemical events that influence plant growth and development. Given the increasing beneficial role of biostimulants in micropropagated plants (Tables 6.1 and 6.2), diverse mechanisms of action have been postulated relating to each biostimulant type.

Plant growth-promoting rhizobacteria and related microorganisms stimulate plant growth through direct and indirect mechanisms of action. Plant–microbe interactions enhance nutrient acquisition and exert beneficial effects through several mechanisms including nitrogen-fixation and hydrogen cyanide production, which indirectly increases P availability (Backer et al. 2018). The PGPR also excrete phytohormones, for example, auxins, CKs, and gibberellins, for uptake by roots, thereby regulating hormone homeostasis in plants (Gupta et al. 2015). However, molecular mechanisms of PGRP-secreted phytohormones are sparsely understood (Backer et al. 2018; Spaepen et al. 2014).

Humic substances exert plant growth-promoting effects through complex transcriptional networks involving multifaceted mechanisms of action via auxin-dependent and independent pathways (Trevisan et al. 2011). In *Arabidopsis thaliana*, humic substances stimulated lateral root formation in auxin-like activity, which was confirmed by enhanced transcription of *IAA19*, an early auxin-responsive

gene (Nardi et al. 2016). In another study, Mora et al. (2010) attributed shoot growth-promoting effects of humic substances on root H^+-ATPase activity and the distribution of root-shoot nitrate concentration, which mediate a significant increase in shoot concentrations of certain CKs and polyamines. Upregulation of H^+-ATPase activity in cell membranes triggered by the application of humic substances also contributes to root architecture modifications and elicitation of biochemical pathways regulating nutrient uptake (Canellas and Olivares 2014).

Biochemical and molecular responses triggered by bioactive molecules in seaweed extracts are diverse and complex but remain largely unknown (Carmo et al. 2021). Due to the multi-component composition of seaweed extracts, it is difficult to ascertain their exact mechanisms of action. Seaweed biostimulants modulate complex transcriptional networks, which control metabolism-associated genes and plant development-related genes (De Saeger et al. 2020). For example, *A. nodosum* extract (ANE) is known to influence the balance of endogenous phytohormones by modulating their homeostasis. Rayorath et al. (2008) used a DR5:GUS expression system to demonstrate the localization of auxins in *A. thaliana*, which was hypothesized to account for enhanced plant growth. Furthermore, expression of the CK oxidase4 (*CKX4*) gene, which encodes for a CK oxidase that causes CK degradation, was strongly inhibited by ANE, demonstrating modulatory effects of CK homeostasis (De Saeger et al. 2020). Seaweed extracts also regulate nitrogen metabolism notably nitrate transport and assimilation as demonstrated by upregulation of *BnNRT1.1* and *BnNRT2.1* genes encoding nitrate transporters (Billard et al. 2014). Likewise, increased expression levels of *BNSULTR4.1* and *BnSULTr4.2* that generate sulphate transporters were observed, which indicated a significant impact on sulphate metabolism. The multidimensional influence of seaweed extracts has been reported for several development-associated genes involved in carbon assimilation. Increased expression of ribulose-1,5-biphosphate carboxylase/oxygenase and other photosynthesis-related proteins, cell cycle, cell wall organization, and chloroplast division are influenced by the application of seaweed extracts (De Saeger et al. 2020). Difficulties persist in unravelling the intricate molecular mechanisms regulating the growth of seaweed extract-treated plants, partly because of the complexity in separating direct and secondary effects of the observed polygenic responses.

The application of seaweed extracts to different horticultural plant species in tissue culture, summarized in the review of Carmo et al. (2021), has paved the way for the possible uptake of scientific research into commercial plant tissue culture industries. As more scientific information comes to light, the translation of the knowledge associated with the use of seaweed extracts and other plant biostimulants from research laboratory-based activities into commercial practice is thus imminent. It has been proposed that this may lead to the replacement of synthetic plant growth regulators with plant biostimulants in cases where they offer a cost-saving, time-efficient, and manual labour reducing option that leads to micropropagules that resemble wild types but are generated at higher yields.

9 Concluding Remarks

In view of the increasing benefits associated with plant growth and development in the presence of biostimulants, researchers are continuously exploring their potential in the four stages (I - IV) of micropropagation of plants. Application of biostimulants such as chitosan, PGPR, AMF, humic substances, and seaweed extracts (individually or in combination with conventional PGRs) have been effectively employed to enhance the clonal propagation of model plants as well as valuable horticulture and plantation crops. Some of the developed micropropagation protocols utilizing biostimulants offer additional benefits in their ability to enhance important biochemicals in in vitro regenerants and subsequent survival upon ex vitro acclimatization. Based on the limited evidence, the mechanisms of action for many biostimulants currently used under in vitro conditions are partly attributed to the hormonal-like (e.g. CKs, auxins, and polyamines) activity, modulation of complex transcription network(s), and enhancement of nutrient acquisition. Application of biostimulants as broad-spectrum acting growth-promoting agents is still in its infancy and their use in the future will likely expand across an even more diverse range of plant taxa and become more readily adopted in commercial plant tissue culture operations that aim to generate high-quality horticultural-based products at a large scale, as new scientific information that defines their fundamental molecular effects *in planta* comes to light.

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Chapter 7
Nanomaterials and Plant Tissue Culture: Developmental Path and Contradictory Facts

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Abstract One of the most active fields of research in modern materials science is nanotechnology. Nanomaterials (NMs) are able to enter plant cells and interact with intracellular organelles and different metabolites due to their exceptional physico-chemical properties and nanoscales structure. Plant tissue culture is one of the essential practices for ex situ conservation, industrial-scale propagation, genetic modification, synthesis of bioactive compounds, and growth enhancement of plants. In recent years, the use of NMs has been shown to be effective primarily in removing microbial contaminants from explants. NMs' beneficial effects on somatic embryogenesis, callus induction, plant cell dedifferentiation, redifferentiation, somaclonal variation, genetic modification, and secondary metabolite production have also been demonstrated as well. This chapter seeks to provide detailed research to determine the best NMs available for in vitro propagation and to emphasize the beneficial characteristics of NMs in plant tissue culture.

Keywords Nanomaterials · Plant tissue culture · Industrial scale propagation · Genetic modification · Callus induction · Secondary metabolites

1 Introduction

The tissue culture of plants looks to be a viable biotechnological technique for bioactive chemical synthesis that may be used in a broad range of areas, particularly as part of a larger effort to ensure the long-term conservation and sensible use of biodiversity (Karuppusamy 2009). The broad concept of plant tissue and organ culture is the manipulation of organs and cells under the sterile situations, grown in a culture medium under regulated conditions of light, humidity, and temperature (Smetanska 2008). This regulated plant production technique enables for production

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of higher concentrations of the required phytochemicals, while yet retaining the genetic characteristics of the most productive clones (Chaturvedi et al. 2007).

Nanomaterials are natural or manufactured materials with a diameter ranging from 1 to 100 nm, according to ASTM (American Society for Testing and Materials) standards (Astm E2456–06 2012). NMs are made for a variety of purposes and are primarily divided into the following categories: (1) carbon nanotubes (CNTs), graphene, and fullerenes (C60 and C70); (2) metal-based nanomaterials, for example, gold (Au), silver (Ag), and iron (Fe) NMs, (3) metal oxides, for example, zinc oxides (ZnO), titanium oxide (TiO_2), ceric oxide (CeO_2), and metal salts (nano silicates and ceramics); (3) quantum dots like cadmium selenide (CdSe) and cadmium telluride (CdTe); (4) nanosized polymers (include dendrimers and polystyrene).

Based on certain characteristics, NMs display totally new or enhanced properties. Because of their unique and diverse physicochemical characteristics, nanomaterials (NMs) are used in diverse disciplines such as life sciences, chemical engineering, and electronics. In agriculture, NPs find many uses. The number of seeds per pod and the protein content of seeds substantially increased in black-eyed pea with the application of Fe and Mg nano-fertilizers (Delfani et al. 2014). Gao et al. (2007) described iron oxide NPs (Fe_3O_4) possessing natural peroxidase-enzyme-resembling properties. Other inorganic nanomaterials such as cerium (IV) oxide (CeO_2), trimanganese tetraoxide (Mn_3O_4) and fullerene (C_{60}) NPs also showed significant antioxidant potential (Lee et al. 2013; Yao et al. 2018; Liu et al. 2016). Likewise, seed germination and seedling growth of wheat (*Triticum aestivum* L.) seeds were increased with the treatment of chitosan nanoparticles (CS NPs). The results revealed that CS NPs had greater adsorption on the surface of wheat seeds than chitosan (CS). When compared to CS (50 g/mL), CS NPs showed a growth-promoting impact at a lower dose (5 g/mL). In addition, 5 g/mL CS NPs stimulated auxin-related gene expression, expedited indole-3-acetic acid (IAA) production and transport (Li et al. 2019). All these studies indicate the antioxidant potential, secondary metabolite and phytohormone influencing characteristics of NPs which can be utilized for augmenting seed establishment and plant growth promotion. Nanotechnology has recently gained attention in plant tissue culture to build miniature efficient systems to promote seed germination, plant growth, and development (Wang et al. 2016). Besides, nanoparticles (NPs) have been utilized extensively in plant genetic modification, bioactive chemical synthesis, and plant protection (Cunningham et al. 2018). Table 7.1 shows the use of nanoparticles in plant tissue culture. The use of nanomaterials in plant tissue culture systems offers new methodologies that can be utilized in commercial propagation of in vitro plants.

Table 7.1 Application of NPs in plant tissue culture

Plant species	NPs	Concentration	Role	References
Water-wheel plant (*Aldrovanda vesiculosa* L.)	Ag	5/mg/dm^3	Reduced the contaminations	Parzymies (2021)
Algae *(Pseudo-kirchneriella subcapitata)*	TiO_2 and ZnO	<100 mg/L	Nanomaterial induced hermetic concentration response	Agathokleous et al. (2019)
Mouse-ear cress *(Arabidopsis thaliana* (L.) Heynh.)	Carbon nanodots (CNs)	–	Promoted plant biomass and roots length in plant tissue culture	Chen et al. (2020)
Argan *(Argania spinosa* (L.) Skeels)	TiO_2 and SiO_2	5 ppm	Higher production of α-tocopherol (0.283%) in plant tissue cultures	Hegazi et al. (2020)
Silver birch *(Betula pendula* Roth)	Chitosan nano-fiber (CTS-N) and cellulose nano-fiber (Cellul-N)	0.5% Cellul-N 0.5% CT-N	Highest amount of betulin (0.7 mg g^{-1}) Highest amount of betulinic acid (0.98 mg g^{-1})	Vahide et al. (2021)
Madagascar periwinkle (*Catharanthus roseus* L. G. Don)	MWCNT	50, 100, and 150 mg/L	Significant increase in protein content (34%), phenylalanine ammonia lyase activities (36.5%), soluble phenols (by 23%), and alkaloids (by 1.7-fold), upregulations in the transcriptions of the DAT gene	Ghasempour et al. (2019)
Madagascar periwinkle (*C. roseus* (L.) G.Don)	Ag	75 mg/L	The production of vinblastine and vincristine increased	Shahin (2018)
Madagascar periwinkle (*C. roseus* (L.) G.Don	Co	5, 10, 15, and 20 mg/L	Promoted alkaloids production	Fouad and Hafez (2018)
Chrysanthemum	Ag, 70% red LED, 30% blue LED	7.5 ppm	Significantly reduced microbial counts of 8 tested bacteria and 3 fungi	Tung et al. (2018)
Arabian coffee (*Coffea arabica* L.)	ZnO	25 mg/L	Increased the recovery of in vitro grown leaf explants while minimizing contamination	Devasia et al. (2020)
Clove pink (*Dianthus caryophyllus* L.)	Graphene quantum dots [GQD] and fullerenes [C_{60}]	1 mg/L C_{60} and 25 mg/L GQD	Effectively delayed senescence and abscission of plant tissue	Di Zhang et al. (2021)

(continued)

Table 7.1 (continued)

Plant species	NPs	Concentration	Role	References
Foxglove (*Digitalis purpurea* L.) English lavender (*Lavandula officinalis* Chaix)	Ag	–	Sterilization agent in plant tissue cultures	Nartop (2019)
Dracocephalum (*Dracocephalum kotschyi* Boiss.)	SiO_2	–	Rosmarinic acid, xanthomicrol, isokaempferide, and cirsimaritin increased in the in vitro raised hairy root cultures	Nourozi et al. (2019)
Moldavian balm (*D. moldavica* L)	TiO_2	50, 100 ppm	Luteolin 7-O-glucoside (2.1 mg g^{-1} FW), rosmarinic acid (0.32 mg g^{-1} FW), p-cumaric acid (8.9 mg g^{-1} FW), ellagitannin (4.3 mg g^{-1} FW), increased in greenhouse conditions	Kamalizadeh et al. (2019)
Henbanes (*Hyoscyamus reticulatus* L.)	ZnO	100 mg/L	Production of tropane alkaloids (1.2 fold) and total phenolic contents (3.2 fold) were increased in hairy root culture	Asl et al. (2019)
Garden cress (*Lepidium sativum)*	Fe_3O_4	50 mg/L	Total phenolic (4.65 mg g^{-1} DW) and flavonoid (77.34 mg g^{-1} DW) contents were increased in hairy root culture	Mohebodini et al. (2017)
Flax *(Linum usitatissimum* L.)	ZnO, SiO_2, Al_2O_3	5, 10, 20 mg/L	Lower dose of nano elicitors had a positive effect on callus induction and mucilage formation	Kavianifar et al. (2018)
Tomato *(Lycopersicon esculentum* Mill.)	CeO_2	–	Increased antioxidant potential and biomass	Abdel-Razik et al. (2017)
Bitter gourd (*Momordica charantia* L).	Ag	5 mg/L	Increased production of flavanols, hydroxybenzoic and hydroxycinnamic acids in cell suspension cultures	Chung et al. (2018a, 2018b)
Basil (*Ocimum basilicum* L.)	Cu	0.1, 2.5, 5, 7.5, 10, 12.5, and 15 μM	Significantly increased the percentage of somatic embryogenesis/explant from 15 to 84%. Also increased average number of	Ibrahim et al. (2019)

			regenerated plantlets/explant from 4.3 to 18.7 in comparison to the control treatment	
Rice (*Oryza sativa* L)	TiO_2	40 mg/L	Highest frequency of plant regeneration, regenerated shoots induced roots on NB medium without plant growth regulators	Chutipaijit and Sutjaritvorakul (2018)
Rice (*O. sativa* L., cv. Swarna)	Ag	10, 20, 40 ppm	Significantly increased the chlorophyll *a* and carotenoid content, decreased amount of lipid peroxidation and H_2O_2 content	Gupta et al. (2018)
Palm	Ag	5 mg/L	In vitro explant disinfection	El-Sharabasy et al. (2017)
(poplar × aspen hybrid)	TiS_3 Nanoribbons	1.5 and 3 μg/L	Sterilizing and stimulating agent in the initial growth stage and as a rhizogenesis-activating agent in the rooting stage in plant tissue culture	Zakharova et al. (2021)
Raspberry *(Rubus idaeus)*	Graphene	2 mg/L	Root length, specific surface area, number of root tips, and their bifurcation number were about twice that of the control	Xiao-Fei et al. (2019)
Rice (*O. sativa* cv. IR64)	Ag	10 mg/L	Alleviated abscisic acid and ethylene levels in the plant tissue culture	Manickavasagam et al. (2019)
Rice (*O. sativa*) seedlings	SiO_2, TiO_2, and ZnO	0.5 mg/g 2 mg/g	Fresh weight, dry weight, and plant height of rice seedlings were increased	Yin and Liu (2020)
Sugarcane (*Saccharum officinarum* L.)	Ag	50 mg/L	Increased total phenolic content	Bello-Bello et al. (2017)
Jojoba (*Simmondsia chinensis* (link) C.K. Schneid.)	SWCNTs	0.002 g/L	Phenolics (23.17 mg GAE per g DW), flavonoids (20.66 mg QE per g DW), and tannins (6.35 mg TE per g DW) contents were increased	Gaafar et al. (2018)
Candy leaf *(Stevia rebaudiana* (Bertoni) Bertoni)	CuO	10 mg/L	Increased total phenolic (5.06 lg/mg of DW) and flavonoid (2.23 lg/mg of DW) content in plant tissue cultures	Javed et al. (2018)

(continued)

Table 7.1 (continued)

Plant species	NPs	Concentration	Role	References
S. rebaudiana (Bertoni) Bertoni	Cu–Au bimetallic	–	Total phenolic (54%) and flavonoid (20%) contents were increased	Ghazal et al. (2018)
S. rebaudiana (Bertoni) Bertoni	Zno	1 mg/L	In micropropagated shoots, there was a substantial increase in steviol glycosides (nearly doubled compared to the control)	Javed et al. (2017)
Strawberry (*Fragaria* × *ananassa* Duch.)	Fe	–	Iron nanoparticles along with SA can be a useful method for providing higher quantity in the in vitro culture	Akbar Mozafari et al. (2018)
East Indian balmony (*Swertia chirata* Buch.-ham. Ex wall.)	Ag	–	Improved shoot regeneration of the plant	Saha and Gupta (2018)
Vanilla (*Vanilla abundiflora* J. J.Sm.)	Ag	50 mg/L	Stimulated growth stimulation and reduced bacterial contamination	Spinoso-Castillo et al. (2017)
European wine grape *(Vitis vinifera* L.)	Fe	0.8 ppm	Decreased sodium content and increased potassium content under in vitro salinity-stress conditions	Mozafari et al. (2018)

2 NMS Entry and Their Interactions With Plant Cells

Transporter protein complexes (located on plasma membranes of plant cells) or root exudates mediate NM's entrance into the plant (Yadav et al. 2014). Damages and wounds in aerial and hypogeal regions of plants may potentially serve as feasible pathways for NP internalization (Al-Salim et al. 2011). When NMs are introduced to plant roots, they can be absorbed via both apoplastic and symplastic routes (Rico et al. 2011). The plant cell wall pore size is smaller than 20 nm in diameter; hence, nanoparticles of bigger sizes would have a restriction to penetrate epidermal cells. Their further motions may be affected by capillary forces and osmotic pressure (Deng et al. 2014). Endocytic uptake occurs when a particular receptor interacts with a ligand. Some NMs such as carbon nanotubes can breach the cell membrane and go into the cytoplasm depending on their shape. Through plasmodesmata channels (20–50 nm in diameter), these NMs-endosome/protein complexes can travel to adjacent cells (Wild and Jones 2009; Deng et al. 2014). NMs can enter plant cells via connecting them to particular proteins, ion channels, or via endocytosis. In cell suspension culture, the integration of NMs from the apoplast to the vacuole happened by forming vesicles of the plasma membrane and is mostly dependent on fluid-phase endocytosis (Etxeberria et al. 2009). *Citrus reticulata* plants treated with fluorescent dye labeled iron (III) oxide (Fe_2O_3)NPs in a hydroponic environment revealed that only citrus roots could absorb Fe_2O_3 NPs, but there was no translocation from roots to shoots (Li et al. 2017). In another study, Valletta et al. (2014) found that NPs derived from poly lactic-co-glycolic acid (PLGA) penetrate the leaf cell wall of *Vitis vinifera* L. via stomata openings, absorbed by the roots, and transferred to the shoot via vascular tissues. Transmission electron microscopic (TEM) examination of grown cells revealed that NPs smaller than 50 nm could enter cells whereas larger ones stayed adhered to the cell wall. All these reports indicated that NMs can access plant cells by binding to specific transport proteins, ion channels, or endocytosis, as well as establishing complexes with certain transmembrane proteins or root exudates, and that the precise mechanisms of NMs accumulation in plants varies depending upon the nature and size of NMs and the exposed tissues of different plant species.

3 Nanoparticles for Explant Surface Disinfection

In plant tissue culture, microbial contamination is a major concern. Contamination comes from the explants as well as the laboratory surroundings. A crucial step forward before the in vitro culture is the surface disinfection of explants, and this has a significant impact on culture initiation. Several sterilization agents are used in surface sterilization of explants, such as bromine water (BW), ethanol (EtOH), hydrogen peroxide (H_2O_2), sodium hypochlorite (NaOC1), and mercury chloride ($HgCl_2$). Disinfectant concentration and exposure time influence explant quality. In

addition, various antibiotics and chemicals like silver nitrate ($AgNO_3$) are included into the culture media, which kill or limit the development of endophytic bacteria. Similarly, other metal and metal oxide nanoparticles have been shown to be effective in the elimination of certain bacteria. A broad variety of NPs, such as silver (Ag), iron oxide (Fe_3O_4), aluminum oxide (Al_2O_3), gold (Au), copper oxide (CuO), manganese oxide (MnO), silicon dioxide (SiO_2), silicon (Si), and zinc oxide (ZnO) have been reported with significant antibacterial activity against different microbes. According to Gouran et al. (2014), treatment of Ag NPs had high impact on controlling bacterial contamination but had a modest impact on the control of fungal contamination in plant tissue culture. Murashige and Skoog medium supplemented with Ag and TiO_2 NPs reduced microbial growth substantially (Safavi et al. 2011). Similarly, *Arabidopsis* seeds treated with 100 mg/L Ag NPs (for 1 or 5 min) were shown to be quite effective for decontaminating (100 percent) seeds with no negative effects on seedling survival (Mahna et al. 2013). Shoot buds from potato, tobacco, and barley grown on MS medium enriched with TiO_2 and ZnO NPs (100 mg/L), resulted in contamination-free cultures (Safavi et al. 2011; Mandeh et al. 2012). In a recent study, application of turmeric and benzoin-based fumigation of explants resulted in contamination-free plant tissue culture. This surface sterilization effect is predicted to be produced by the carbon nanomaterial loaded with the inherent antibacterial properties of the natural smog parent material (Sivanesan et al. 2021). NPs' efficacy in eliminating microbiological contamination relies on their size, dosage, dispersion, and type in plant tissue cultures. To determine the optimal dosage with no or minimum plant toxicity, the effects of various types and quantities of NMs on the explants derived from various plant species should be examined. Synergistic impacts in combination with other sterilizers can also increase the efficiency of NPs.

4 Nanomaterials for the Formation of Callus and Organogenesis (Roots and Shoot Growth)

NMs have been found to have beneficial impact on callus induction, shoot regeneration, and plant development. For example, nanoSelenium (Se) (265–530 μM) considerably increased organogenesis and root system development (40%), but selenate had no such effect at any concentration (Domokos-Szabolcsy et al. 2012). Tissues from *Citrus reticulata* nucellus were inoculated on MS media supplemented with 30 μg/mL solution of Zn and Cu NPs. Important findings were achieved in response to the use of Zn NPs to germination parameters, where Zn NPs enhanced the production of antioxidative and non-enzymatic enzymes under in vitro conditions (Hussain et al. 2017). Ag NPs supplemented MS media was efficient in preventing leaf drop and improved in vitro regeneration in *Citrus australasica* (Mahmoud et al. 2020). *C. volkameriana* seedlings cultivated on MS culture media supplemented with uncoated Fe_2O_3 NPs had the highest rates of iron

absorption and the lowest degree of chlorosis. Furthermore, as compared to the control, vegetative growth parameters like fresh and dry weights and chlorophyll content also increased considerably (Saeedi et al. 2016). Treatment of Fe_2O_3 NPs (20 mg/L) increased chlorophyll content by 126.4% in *C. reticulata* plants grown in hydroponic conditions, whereas MDA levels in citrus leaves enhanced by 37.8, 107.2, and 61.5%, respectively, under Fe_2O_3 NPs (20–100 mg/L) exposure (Li et al. 2017).

In *Gerbera ambigua* (Cass.) Sch. Bip., 10 and 30 ppm Ag NPs decreased the efficacy of adventitious roots, whereas 10 ppm Au NPs promoted rhizogenesis (Tymoszuk and Miler 2019). Stem explant of *Tecomella undulata* grown in MS medium supplemented with Ag NPs at 10 mg/L, BAP at 2.5 mg/L, and IAA at 0.1 mg/L showed an increase in shoot induction %, number of shoots, and callus development (Aghdaei et al. 2012; Sarmast et al. 2015). Treatment of Basmati rice with CuO NPs at a concentration of 10 mg/L improved callus induction frequency, with Super Basmati having the highest callus induction frequency (94%) $\geq$ Basmati 385 (90%) $\geq$ Basmati 370 (86%) and Basmati 2000 (86%) (Anwaar et al. 2016). Humic acid (HA) and ZnO NPs combination produced best results in root regeneration and number of roots per shoot (83.34%, 4.4 roots per shoot, respectively) in date palm tissue culture. 2.5 mg L^{-1} HA + 75 mg L^{-1} ZnO NPs led to the maximum content of endogenous IAA (3.644 µg kg^{-1}) (Al-Mayahi 2021). *Silybum marianum* L. callus cultures grown on MS medium with ZnO NPs (0.15 mg/L) generated the greatest fresh callus weight (2294 mg/L FW). Furthermore, metabolite profiling of callus cultures revealed that ZnO NPs significantly enhanced silymarin production and activated the antioxidant system (Shehzad et al. 2021). Likewise, the treatment of Au NPs (10 µg/mL) enhanced the active use of antioxidant enzymes and reduced the production of microRNAs in *A. thaliana* (miR 398 and miR 408) in MS media (Kumar et al. 2013). A low concentration of MgO NPs (1 g/mL) enhanced chlorophyll content in leaves, however, higher concentrations (2 and 4 g/mL) considerably reduced it in *Ananas comos*us (L.) Merr. cultured on MS media (Owusu Adjei et al. 2021).

Similarly, 25–50 mg/mL and 100 µg/mL multi-walled carbon nanotube treatment (MWCNT) treatment in in vitro leaf cultures of *Satureja aintabensis* P.H.Davis (medicinal plant) on B5 medium, stimulated callus formation and markedly improved antioxidant activity suggesting the use of MWCNT as a novel elicitor for antioxidants and secondary metabolites (Ghorbanpour and Hadian 2015). In various physiological processes, NPs play an important role, including redox reaction, respiration, and chlorophyll production. For example, Fe NPs enhanced callogenesis, shoot elongation in chickpea plantlets (Irum et al. 2020). Au NPs enhanced *Gloriosa superba* L. pollen grain germination and mitotic cell division in *Allium cepa* L. root tip cells suggesting the potential of these NPs in pollen germination media and plant tissue culture (Balalakshmi et al. 2017).

Thus, these studies confirm that NPs added into plant culture medium promote callus proliferation, shooting and rooting by alteration of antioxidant enzyme activity, gene expression, ethylene production inhibition, and ROS (reactive oxygen species) production. To acquire a thorough knowledge of the underlying processes

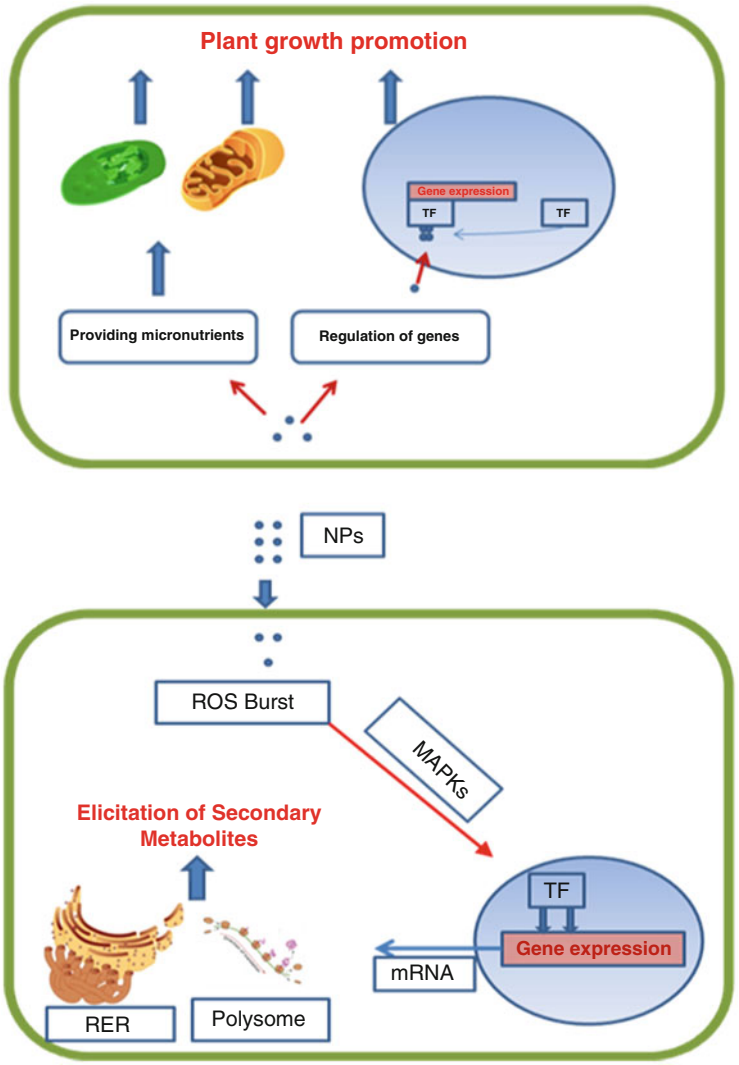

Fig. 7.1 Nanoparticles affect plant metabolism by supplying micronutrients, regulating gene expression, and causing oxidative bursts that acts as important second messengers, upregulating/phosphorylating mitogen-activated protein kinase (MAPK) cascades which control the transcriptional levels of plant secondary metabolites biosynthesis gene regulators. *mRNA* messenger ribonucleic acid, *ROS* reactive oxygen species, *RER rough endoplasmic reticulum*, *TF* transcription factor

behind the function of NPs in plant systems, it is necessary to evaluate the different concentrations, combinations, and sources of NPs. Figure 7.1 shows a proposed process of plant and nanomaterial interactions, as well as their role in plant

development and secondary metabolites production (as adapted from Rastogi et al. 2017, Anjum et al. 2019).

5 Nanomaterial for Improvement of Secondary Metabolites

Plants produce a wide range of secondary metabolites, including phenolics, terpenoids, and alkaloids, recognized for their role as vital interaction mediators for both biotic and abiotic factors. Biotic and/or abiotic elicitors might stimulate the physiology, biochemical, and defense systems of target plants. The use of signaling molecules as elicitors has resulted in a cost-effective method for generating pharmaceutically active chemicals in plants. However, there has been little investigation done on the use of NMs as possible elicitors for the development of industrially usable products. The production of reactive oxygen species (ROS) after exposure to NPs is the fundamental foundation for their ability to elicit plant secondary metabolites. ROS are recognized to be the signaling molecules for the regulation of plant defense, under stress conditions, which can stimulate the synthesis of secondary metabolites (Chung et al. 2018a, 2018b).

Plant growth regulators (PGR) are important for secondary metabolites production. PGRs have been used in combination with various NPs to see how they affect the elicitation of secondary metabolites in different plant species. Hatami and Ghorbanpour (2014) studied the influence of various concentrations of Ag NPs (diameter 5–35 nm) (20, 40, and 80 mg/L) and thidiazuron (TDZ; 0, 50, 75, and 100 μM) used individually as well as their combinations on the essential oil bioaccumulation in geranium (*Pelargonium graveolens* L'Hér.). Ag NPs and TDZ used together resulted in the greatest concentration of the essential oils showing maximum values for key essential oil components viz., citronellol and geraniol. In *Prunella vulgaris* L., the effects of different ratios of Ag and Au NPs alone or in combination with naphthalene acetic acid (NAA) revealed significant effect on callus growth and secondary metabolite synthesis. The combination of Ag and Au NPs (1:3) with NAA resulted in greatest accumulation of phenolics (9.57 mg/g DW) and flavonoids (6.71 mg/g DW) (Fazal et al. 2016).

Copper and Zn play a key role in plant metabolism. There has been a lot of research published on the effects of Cu and Zn deficiency, but less reports are available on the effects of CuO NPs and ZnO NPs and their role as elicitors in plant tissue culture. Oloumi et al. (2015) demonstrated that *Glycyrrhiza glabra* L. (Licorice) seeds cultured on 0.8% agar containing Hoagland solution supplemented with nanosize ZnO and CuO particles resulted in enhanced production of glycyrrhizin (natural sweetener), phenolics and anthocyanins (in comparison to bulk size particles of ZnO and CuO). Similarly, *Thymus kotschyanus* and *T. daenesis* callus cultures maintained on MS medium on treatment with 150 mg L^{-1} ZnO NPs resulted in highest content of carvacrol (0.68 mg/L) and thymol (22.8 mg/L) (Mosavat et al. 2019). In addition, Ag NP and ZnO NPs treatment of *Brassica nigra* showed an increase in phenolics (up to 0.15 μg

GAE/mg) and flavonoids (up to 0.22 μg QE/mg) on Murashige and Skoog (MS) medium (Zafar et al. 2016). In a study by Asgari-Targhi et al. (2021) on nanocomposites, it was found that CS-Zn NPs improved organogenesis as well as substantially influenced plant metabolites production in micropropagation system. Nanocomposites application in tissue culture resulted in increased concentration of chlorophylls, carotenoids, proline (two fold), proteins, and markedly enhanced activity of antioxidant enzymes like catalase, peroxidase, and phenyl ammonia lyase. Besides, CS-Zn NPs treatment also augmented the accumulation of soluble phenols (40%) and alkaloids (60%) indicating a significantly potent elicitation effect on plant secondary metabolism. Similarly, treatment with 100 g/mL MWCNTs considerably increased the amount of two major phenolic acids (caffeic acid and rosmarinic acid) in *Satureja khuzestanica* cultures (Ghorbanpour and Hadian 2015). NPs applied to plant growth media under in vitro conditions may function as both an elicitor and a food source. Poborilova et al. (2015) found that addition of 10–100 mg/mL Al_2O_3 NPs to suspension cultures of tobacco cells enhanced the phenolic content significantly. Suspension culture of a wild medicinal herb *Dracocephlum polychaetum* Bornm. treated with 30 mT Static Magnetic Field (SMF) and Fe_2O_3 magnetic nanoparticles (100 ppm) increased total phenolics, flavonoids, and anthocyanin content, as well as the activities of polyphenol oxidase and phenylalanine ammonia lyase (Taghizadeh et al. 2019). All these reports described above corroborates the use of NPs in plant cell, tissue, and organ cultures as successful and prospective bioactive chemical elicitors. However, more research is needed to assess the ability of additional NPs to stimulate secondary metabolite synthesis in plant tissue.

6 Nanomaterials Influence Somaclonal Variation and Genetic Transformation

Genetic and epigenetic alterations between clonal regenerants and the matching donor plant are designated as somaclonal variations. Tissue culture is a dependable technique for plant propagation and assessing somaclonal variations, which are essential for progeny modification and improvement. This increases a taxon's or population's evolutionary capacity to adapt to changing environmental circumstances. The regenerated plants obtained from somaclones may differ from the mother plant genetically or phenotypically (Ngezahayo et al. 2007; Bairu et al. 2011; Mgbeze and Iserhienrhien 2014). In plant tissue culture, somaclonal variation offers both advantages and drawbacks. *Linum usitatissimum* calli and regenerants produced by Au and Ag NPs treatment demonstrated somaclonal variations (Kokina et al. 2017). Similarly, flax cultivated in culture medium supplemented with AuNPs and Au/SiO2 NPs showed higher tetraploid (23%) and methylation levels in all samples (Kokina et al. 2017). Calli cultivated with Au/SiO2 NPs showed even higher increase in tetraploid cell numbers resulting in somaclonal variants.

Genetic transformation enables the transfer of a foreign gene of interest into the plant cell to introduce the desired characteristic into a plant. For the delivery of the extraneous gene to plant cells, tissues, and organs, electroporation and particulate bombardment (Direct) and agro-modern (Indirect) transformation techniques are utilized. Transferring the genes to protoplasts has been frequently utilized as the electroporation technique. However, it is not that simple to isolate and regenerate protoplasts. In exchange for providing DNA to the tobacco protoplasts with endocytosis, Torney et al. (2007) demonstrated that Si NPs supplied with a gold-capped Mesoporous silicate NPs, gene of interest and chemical inducer can be used as a biolistic gun for targeted genetic transformation (Torney et al. 2007). In cotton, plant exogenous DNA was inserted into pollen grains by using magnetic nanoparticles under magnetic field and transgenic plants have been successfully produced by using these genetically transformed pollens (Zhao et al. 2017). Calcium phosphate (CaP) NPs were used to transport the GUS (β-glucuronidase) gene-containing pCambia 1301 (vector backbone) into *Brassica juncea*, wherein CaP NPs showed the highest transformation efficiency (80.7%), followed by *Agrobacterium tumefaciens* (54.4%) (Naqvi et al. 2012).

7 Concerns About Toxicity and Safety

Nanomaterials have distinct features relative to their bigger counterparts due to the enormous surface area to volume ratio and quantum scale impacts; one such unique attribute is an additional toxicological trait that NMs may have, in comparison to their bulk counterparts. A nano-boom causes a lot of good changes, but at the same time created an ongoing pollution that is too tiny to detect or manage. Certainly, it is far simpler to fight a recognized danger than an invisible one. Nanomaterial phytotoxicity is thought to be influenced by their chemical composition, dosage, size, stability, type, as well as the medium of culture composition, method of application and plant and explant type. Sometimes, nanomaterials applied to the culture media can have unfavorable impacts on explant survival, cell viability, organogenesis, shoot growth, seed germination, and seedling development. The effect also varies from plant to plant. For example, NanoTiO_2 improved spinach development by boosting the activity of Rubisco activase enzymes, and lowering chloroplast oxidative damage induced by ultraviolet radiation (Gao et al. 2008). However, in case of *Chlorella vulgaris*, nano-TiO2 is cytotoxic to the cells (Clément et al. 2013). In case of *Cucurbita pepo* (zucchini), treatment with multi-walled carbon nanotubes (1000 mg/L) showed no effect on germination; however, it reduced biomass to 60% compared to the control under hydroponic conditions (Stampoulis et al. 2009). Similarly, exposure of 1000 mg/L and 2000 mg/L MWNTs drastically decreased the root and shoot lengths of lettuce, red spinach, and cucumber after 15 days of hydroponic cultivation (Begum et al. 2012).

Agents for capping play an important part in the toxicity findings in nanotoxicological investigations. NMs have been coated with a variety of compounds,

which can affect their behavior in the environment as well as their toxicity to living creatures (López-Moreno et al. 2018). For example, in *Lemna gibba*, modified CuO NPs with an organic polymer shell poly (styrene-co-butyl acrylate) caused significant toxic effects as compared to bare CuO NPs (Perreault et al. 2014). Besides capping agents, dosage of NPs is also very significant factor governing its advantages or disadvantages in a plant system. Hence, dose-dependent research is needed to find the optimally safe NP dosages, with beneficial effects on plant development, with lower environmental and plant-negative effects. The manufacture, storage, and distribution of these NPs, as well as their application and possible abuse, and disposal, should all be considered. Because each NM is distinct, NPs bioaccumulation, penetration, and translocation in plants should be examined in-depth, specifically for each of the NP employed.

8 Conclusions and Future Prospects: A Headlight for Future Targets

While research on the implications of NMs in tissue culture is still in its early stages, this area deserves more attention due to its significant benefits. The nanotechnological approaches implemented in tissue culture of commercially important horticulture and plantation crops are oriented to the task of developing new techniques to generate precision horticulture in order to efficiently meet micronutrient requirements with the goal of improving plant disease resistance, plant growth, and yield. Nanotechnology is an interdisciplinary science and provides unlimited coverage in many fields. While a broad range of NPs are known to have significant antibiotic action but only a few NPs, such as ZnO, TiO_2, Ag, and Zn NPs, have been mainly employed in plant tissue culture to reduce microbial contamination in crops like rubber (Moradpour et al. 2016), foxglove (Nartop 2019), palm (El-Sharabasy et al. 2017) etc. Exploration of more and more new-age environment friendly materials are required for NPs synthesis aiming its application in plant tissue cultures. In terms of plant biotechnology, NMs have plenty to offer. Besides, decontamination of explants, differentiation of callus, genetic transformation, induction of somaclonal variations, and secondary metabolite release are the major applications of NPs in plant tissue culture. A variety of commercially significant plant species such as *Aloe vera* (Raei et al. 2014), *Dracocephalum moldavica L.* (Kamalizadeh et al. 2019), vanilla (Spinoso-Castillo et al. 2017), strawberry (Akbar Mozafari et al. 2018), and tomato (Abdel-Razik et al. 2017) have been treated with NPs approach for improving plant establishment and augmenting secondary metabolite synthesis. The interaction of NPs with in vitro plants must be thoroughly investigated. There can be no genuine progress without assessing this interaction and hence deciphering this mechanism of action of this interaction is essential for successful and sustainable use of the NPs. Apart from this, another crucial information gap to be filled is the potential environmental and ecological implications of NMs. This is the biggest

issue that nanotechnology sector is facing. Green synthesis of NPs can be a bigger boon solving this problem to a great extent. Furthermore, to achieve a more realistic knowledge of the possible impacts of NMs important biotic and abiotic characteristics of the ecosystems should be involved and field research is required. Despite all of these unknown factors lurking in the background, nanotechnology still emerges as a prominent, promising field with great promise for commercial plant tissue culture. The more targeted studies are needed to explain and simplify the procedure to exploit just the beneficial features without exposure to unfavorable effects.

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Chapter 8
Somatic Embryogenesis in Cashew (*Anacardium Occidentale* L.)

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Abstract Somatic embryogenesis has seen many advances. However, many aspects are not fully understood especially on cashew (*Anacardium occidentale*) despite several studies conducted for the technique improvement. Regenerate in vitro viable embryos through asexual cells (haploid or diploid) is the target of somatic embryogenesis. This process leads to the production of bipolar structures with root/shoot axis well defined. The in vitro culture of cashew is brought out with emphasis on the critical factors that influence the explants response and plantlet regeneration. The recalcitrant nature of cashew has been attributed to abnormal development observed in the calli derived from its explants in some cases and to the limited success recorded up to here in tissue culture of the plant. This review highlights advances, challenges, and future prospects in somatic embryogenesis research of cashew.

Keywords Cashew · Somatic embryogenesis · True-to-type plants · Explant Browning · Growth Regulators

Abbreviations

ABA	Abscisic acid
BA	6-Benzyl adenine
BAP	6-Benzylaminopurine
B5	Gamborg media
Ca	Calcium

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CE	Corn extract
CW	Coconut water
DSE	Directly somatic embryos
2,4-D	2,4-Dichlorophenoxyacetic acid
GA_3	Gibberellic acid
GRS	Growth regulators substances
H_2O_2	Hydrogen peroxide
IAA	Indole-3-acetic acid
ISE	Indirect somatic embryos
LS	Linsmaier and Skoog, 1975
M	Molar
μM	Micromolar
MS	Murashige and Skoog, 1962
N6	Chu et al., 1975
NAA	α-Naphthaleneacetic acid
ORG	Organogenesis
O_2	Oxygen
PAE	Papaya extract
%	Percentage
PGRs	Plant growth regulators
pH	Potential of hydrogen
PE	Potato extract
PVP	Polyvinylpyrrolidone
PPO	Polyphenol oxidase
POD	Peroxidase
PAL	Phenylalanine ammonia lyase
SH	Schenk and Hildebrandt, 1972
SE	Somatic embryogenesis
TE	Taro extract

1 Introduction

Somatic embryogenesis is the greatest expression of cell totipotence in plant cells. In short, totipotence is the ability of a plant cell to undergo a chain of complex coordinated metabolic and morphological steps to generate a complete and normal plant or sporophyte without the participation of sexual techniques. The in vitro technique by which any somatic cell develops into a zygotic structure that finally produces a plant theoretically is somatic embryogenesis (Rao 1996; Jiménez 2005). Somatic embryos have a single cellular origin like zygotic embryos (Rao 1996). Several authors have come to this conclusion, especially in *Agave tequilana* (Blue agave), with the unicellular origin of somatic embryos that have been reported (Gutiérrez-Mora et al. 2004; Portillo et al. 2007). The somatic embryogenesis

process consists in general of two main steps, the induction and the expression of embryos obtained (Rodríguez-Garay et al. 2000; Gutiérrez-Mora et al. 2004; Jiménez 2005). Theoretically, any part of the plant can be used to initiate somatic embryogenesis with somatic cells. However, significant differences in competence were found in practice. Generally, the cells coming from young tissues including immature zygotic embryos that are more competent for somatic embryogenesis (Gutiérrez-Mora et al. 2012). Somatic embryos induction requires simple manipulation of in vitro culture conditions. The growth regulators substances (GRS) such as auxins, cytokinins, abscisic acid, and gibberellins are one of the principal components in the culture medium. Moreover, auxins are the most important elements in the induction of the process (Rao 1996; Dodeman et al. 1997; Jiménez 2005; Fehér 2006; Jiménez and Thomas 2006). Also, it is important to notify that endogenous hormone play important roles in somatic embryogenesis. The signal for the cell polarization and the asymmetric division given by auxins as it may happen in the zygotic counterparts is a necessity to somatic cells (Gutiérrez-Mora et al. 2004; Pagnussat et al. 2009). Regarding, the initial steps of the development of a somatic embryo, the induction is usually initiated by the action of selected auxins [the most used auxin for most species is 2,4-Dichlorophenoxiacetic acid (2,4-D)] (Nomura and Komamine 1986; Jiménez 2005). In vitro somatic embryogenesis is practiced in many tissue culture laboratories with many species, genotypes, and explants, but the biological background of the process is still largely unknown.

The cashew tree is highly valued for its edible nuts and cashewnut shell liquid. It has attracted interest from conventional plant breeders and biotechnology programs with the goal of improving productivity. It is the most widely cultivated tree fruit in the world. Its culture has spread to several countries and has become a staple of cooking, especially in India. In 2019, world production is 3,960,680 tonnes (FAOSTAT 2019). The high demand for cashew kernels in the confectionery industry has led to increased cultivation of the crop. It is an achene rich in proteins, vitamins, trace elements, and monounsaturated fatty acids that regulate cholesterol levels. In addition, cashew fruits have high vitamin C levels with approximately 200 mg/100 g of juice, four times higher than that of orange juice (Trevisan et al. 2006). However, current propagation methods have become a limiting factor in supplying adequate planting material (Gogate and Nadgauda 2003). Planting material production request the use of technology such as in vitro tissue or cells culture in order to produce a large quantity of clone in a short time with low cost. In addition, micropropagation techniques essentially take two ways: organogenesis (micropropagation) and somatic embryogenesis (Margara 1989; Saadi 1991). The successful micropropagation method in woody plants is somatic embryogenesis. This method makes it possible to produce whole plants in a very short time without going through the constraints usually experienced by micro-cuttings (caulogenesis and rhizogenesis steps) (Daikh and Demarly 1987; Roguet 1989).

The aim of this review is to summarize the vast amount of information published so far on somatic embryogenesis in cashew (*Anacardium occidentale* L.) that can be utilized for the commercial cultivation of in vitro plants.

2 General Characteristics of Somatic Embryogenesis (SE)

Shoot organogenesis (ORG) or somatic embryogenesis (SE) are the two alternative morphogenetic pathways that in vitro culture techniques are taking to enable plant regeneration (Villalobos and Engelmann 1995). Both SE and ORG may be induced at the same time under the same tissue culture conditions (Fiore et al. 1997; Pasternak et al. 1999; Castillo et al. 2000). So, the difference between somatic embryogenesis and organogenesis can sometimes be difficult, and even a detailed comparative histological analysis of the morphogenic process can only insinuate an embryo-like origin of developing structures (Bakos et al. 2000). However, they can be separated in space and time (Ma and Xu 2002; Mithila et al. 2003; Singh et al. 2003; Vikrant and Rashid 2003) with the use of appropriate medium composition, principally type or concentration of plant growth regulators (PGRs). Somatic embryos are distinguished by bipolar structure presenting shoot and root meristems, with a closed tracheal system separated from the maternal tissue and, frequently single-cell origin and production of specific proteins. Although genetic components determine the potential of species/genotypes to form somatic embryos, the expression of embryogenic competency at the cellular level is defined by developmental and physiological cues. Somatic embryos can be developed indirectly, through callus tissue (ISE) or directly from explant tissue (DSE). Somatic embryos developing via DSE are formed from competent explant cells which, contrary to ISE, are able to undergo embryogenesis without dedifferentiation, i.e., callus formation. It is believed that both processes are extremes of one continuous developmental pathway (Carman 1990). The distinction between DSE and ISE can be difficult (Emons 1994), and both methods have been observed to occur simultaneously in the same tissue culture conditions (Turgut et al. 1998). Somatic embryogenesis can be induced directly or through callus in the culture of somatic embryos. This last process is called secondary somatic embryogenesis in contrast to primary somatic embryogenesis induced from explant cells. It has been found that secondary somatic embryogenesis has much higher efficiency compared to primary somatic embryogenesis for many plant species (Raemakers et al. 1995; Akula et al. 2000; Vasic et al. 2001). Some cultures are able to maintain their competence for secondary embryogenesis for many years and thus provide useful material for various studies, as demonstrated for *Vitis rupestris* (Sand Grape) (Martinelli et al. 2001). The most frequent mode of embryogenesis is the indirect type of regeneration.

In cultures of *Anacardium* explants, indirect somatic embryo formation was also the most frequently observed mode, since, in the majority of the protocols, callus development preceded somatic embryo formation.

3 Factors Crucial for SE Induction

Physiological state of an explant-donor plant, genotype, age, the external environment which includes composition of media and physical culture conditions (temperature, light), type of plant and developmental stage of an explant are some factors that determine the in vitro development of cells and tissues. Somatic cells from an explant, callus, or suspension cells produce embryogenic cells which turn into somatic embryos. Just a few somatic cells are responsive to embryogenesis induction factors and capable of enduring somatic embryogenesis. Such cells, which represent an intermediate state between somatic and embryogenic cells, are called competent. Competent cells exhibit sensitivity to physical and chemical stimuli that initiate the embryogenic pathway of their development, while embryogenic cells are already established during embryogenesis. Embryogenic cells undergo embryogenesis without stimulation by external stimuli (De Jong et al. 1993). A prerequisite for the successful establishment of somatic embryogenesis is a proper choice of plant material. The experiments establish in the special conditions required successful embryonic induction. This is without knowing why a given genotype/explant has embryogenic potential and how and why competence or commitment is reached or what is the real initiator of the development of the embryo.

4 Effects of Media Composition on Growth and Development of Cashew

The components of plant tissue culture media can be classified into inorganic salts, organic compounds, complex natural preparations, and inert support materials (Huang and Murashige 1977). The nutrient media determines the success of plant cell and organ cultures. The recalcitrance of certain species can rise above by manipulating other media components (Birhman et al. 1994). The study of Samson et al. (2006) has shown that two- or four-fold dilution of the MS salts increased the development of coffee embryogenic callus rate by 2.6 and 5.7, respectively, in comparison to full strength MS salts. This clearly shows that modifications of the medium, mainly manipulating the doses of inorganic salts and vitamins, can have a significant effect on somatic embryogenesis possibly by altering the osmotic potential of the medium. The ions of different types are the active factor in the medium rather than the compounds. One type of ion can be provided by more than one compound. Levels developed by Murashige and Skoog (1962) for tobacco tissue culture (Nassar 2004) "MS medium" (Murashige and Skoog 1962) is the most widely used plant culture medium (Vuylsteke 1989) where inorganic salts levels used in most plant tissue culture media are based. In cashew culture, formulations media were multiple with modifications of MS media (MS/2; MS/4) (Browning et al. 1987). Other popular media include B5 (Gamborg et al. 1968), SH (Schenk and Hildebrant 1972), N6 (Chu et al. 1975), and LS (Linsmaier and Skoog 1975) media

(Hussein 2012; Saad and Elshahed 2012). The proximal part of the cotyledons produced somatic embryos when they were initially cultured on SH medium with NAA and BA (Sy et al. 1991). Hegde et al. (1994) observed that pieces of cotyledons produced somatic embryos on LS medium with Ca pantothenate.

5 Carbon Source

Sugars are an essential energy source in all tissue culture media and create the appropriate osmotic conditions for cell growth in vitro. The type and concentration of sugar added in the media influence somatic embryogenesis. The carbohydrate of choice as a carbon source usually is sucrose probably because it is the most common carbohydrate in plant phloem (Murashige and Skoog 1962; Thorpe 1980; Lemos and Baker 1998; Fuentes et al. 2000; Ahmad et al. 2007). Sucrose has been used most often to induce somatic embryos in different plant species including cultures of *Asparagus officinalis* (asparagus) (Levi and Sink 1990). It has been showed that carbohydrates added to the culture medium may play several roles, including the histo-differentiation of somatic embryos through the direct regulation of gene expression (Lipavská et al. 2000).

In the cashew case, sucrose is an important element in the induction of somatic embryos and has been most frequently employed for their induction. Kembo and Hornung (1999), Gogte and Nadgauda (2000), and Martin (2003) induced embryogenic cultures by using 3% of sucrose while Cardoza and D'Souza 2002 used 2% of sucrose. Gogte and Nadgauda (2000) induced somatic embryos by using 4% of sucrose whereas Ananthakrishnan et al. (1999) and Shirly and Thimmappaiah 2005 used 6% of sucrose to obtain somatic embryos.

6 Light Conditions

One of the most important environmental signals is light, and its numerous effects on plant growth and development are so much known. Despite the fact that in every protocol on somatic embryogenesis induction light requirements were described, systematic research on the light effect on in vitro response of cultured explants was limited. Morphogenic response and its efficiency can be influenced by the spectrum, intensity, and duration of the light supplied for in vitro cultures. To minimize the production of inhibitory compounds from tissues in the culture medium, it has been suggested that cultures should be maintained in reduced light intensity or in darkness (Evans et al. 1983). 49% and 44% of the surveyed protocols for SE induction showed the necessity of mostly photoperiod or darkness requirement, respectively (Gaj 2004). Several works in cotyledon culture like *Helianthus annuus* (common sunflower) (Fiore et al. 1997), *Malus domestica* (apple) (Paul et al. 1994), leaf culture of *Camellia reticulata* (Camellia) and *Camellia japonica* (Camellia)

(San-Jose and Vieitez 1993) have revealed the beneficial effect of darkness on somatic embryogenesis induction.

Generally, *Anacardium occidentale* experiments were done in dark because of the richness of phenolic compounds (Aliyu 2005). Studies in the nucellar culture of cashew revealed a gainful effect of darkness on somatic embryogenesis induction (Shirly and Thimmappaiah (2005)).

7 Explant Type

The most important factor which determines the embryogenic capacity of the culture seems to be the type of explant and its well-defined developmental stage. Efforts to select a responsive explant were focused on finding plant tissues containing competent cells, i.e., capable of undergoing somatic embryogenesis following stimulation by applied external factors (Gaj 2004).

The explants are a source of competent cells, and, on the other hand, determination of physical and chemical factors which switch on their embryogenic pathway of development.

Different types of explants used to induce somatic embryogenesis showed variable responses involving the effect of several factors such as tissue or organ type, harvest time, age, light effect and genotype as found by Fiore et al. 1997 in the cotyledon culture of *Helianthus annuus*. Limited embryogenic capacity at a short developmental stage has been frequently observed in zygotic embryos used for SE induction. In the case of zygotic embryos used for somatic embryogenesis induction, embryogenic capacity restricted to a short-lasting stage of development was often noticed. The greater embryogenic potential is most frequently presented by younger zygotic embryos (Maheswaran and Williams 1984; Eady et al. 1998; Garin et al. 1998). However, between embryogenic ability and the degree of zygotic embryo maturity, a reverse correlation was reported in some plants (Turgut et al. 1998; Choi et al. 1998) including Arabidopsis (Gaj 2001).

Cashewnut was found difficult to propagate in vitro from mature plant tissues (nodal segments or shoot apices) due to its recalcitrant nature, microbial contaminations, and high phenolic exudation (Aliyu 2005). Therefore, efforts have been made to find a suitable alternative explant source. In this effort, nucellus tissues from developing seeds were selected because the embryogenic ability of the nucellus was well established in *Vitis vinifera* (European grape) (Mullins and Srinivasan 1976), *Ribes rubrum* (Red currant) (Zatyko et al. 1975), *Myrciaria cauliflora* (Jaboticaba) (Litz 1984), and *Mangifera indica* (Mango) (Litz et al. 1984). Although the morphogenetic potential of the nucellus has been found apparent in the past several years, there have been only a few successful attempts to exploit this tissue for in vitro studies involving woody plants (Bonneau et al. 1994). The first attempts to induce embryogenic cashew cultures involved seed explants (Jha 1988; Lakshmi Sita 1989; Hegde et al. 1990, 1991; Sy et al. 1991). Somatic embryos were obtained from cotyledon pieces of 6–8-week-old germinated seedlings (Lakshmi Sita 1989),

cotyledons from mature seeds (Sy et al. 1991), and sections of immature cotyledons (Hegde et al. 1994). Jha (1988) reported morphogenesis in callus cultures derived from zygotic embryos and the occurrence of globular protuberances which developed into embryo-like structures while Hegde et al. (1992) observed embryogenesis in cotyledonary segments. However, the obtained embryos could not be germinated. Cardoza and D'Souza (2000) reported induction of direct somatic embryos from the radicular end of zygotic embryos. Secondary embryos developed from the primary embryos. However, conversion of embryos to the whole plant was not achieved. Kembo and Hornung (1999) explored the possibility of developing a simple but efficient method of optimizing cultural conditions necessary for the induction of callus using plumular tissue excised from mature zygotic embryos of cashew as explants and comparing with the response of cotyledonary tissue. Responses extended from swelling of embryos and cotyledons to the proliferation of adventitious roots and white callus-like structures. A number of combinations produced abnormal plants with curled leaves and multiple shoots. Ananthakrishnan et al. (1999) and Gogte and Nadgauda (2000) induced embryogenic cultures from (elite) nucellar cultures. The mature embryos germinated but not convert into complete plantlets. Plant regeneration through direct somatic embryogenesis was established on cashew using seed coat explants (Martin 2003). Induction of somatic embryos on different explants excised from immature zygotic embryos like excised cotyledons and excised hypocotyl with radicle, as well as on intact zygotic embryos as small as 1–2 mm has been obtained.

8 Stress Factors

Nowadays, it is known that somatic cells can acquire embryogenic potential as a result of various external chemical and physical stimuli, generally referred to as stressors. Dudits et al. (1995) claimed that stress is an essential component of embryogenesis where the development of somatic embryos is induced. Its action in the induction of embryogenesis of microspore cultures has been demonstrated by the work of Touraev et al. (1996) and Dunwell (1996). Various factors, such as osmotic pressure, heavy metal chlorides, pH, high or low temperatures, starvation, mechanical injury of explants, or high level of auxin can stimulate the embryogenic competence of somatic cells cultured in vitro. This observation is corroborated by the work of Kiyosue et al. (1993). The importance of the interaction between auxin and stress signaling which results in the acquisition of the embryogenic competence of the somatic cell by a broad cellular reprogramming manifested at different levels (Feher et al. 2003) is one of the hypotheses highlighted on the mechanisms involved in stress-induced embryogenesis. Regardless of the precision of the mechanism, stress treatment triggers the expression of factors that affect cell cycle regulation, gene expression and thus induce somatic embryogenesis. As several experimental observations show, the differentiated fate of plant cells, depending on positional information and developmental signals, can be easily modified under in vitro

conditions. Exposure of injured cells or tissues to suboptimal nutrients or hormones supply (e.g. under in vitro culture conditions) constitutes a radical change in the cellular environment, which generates significant stress effects. The response to stressful conditions depends on two main parameters: the stress level and the physiological state of the cells. If the stress level exceeds cellular tolerance, the cells die. In contrast, lower stress levels improve metabolism and induce coping mechanisms (Lichtenthaler 1998). Adaptations include reprogramming of gene expression, as well as changes in the physiology and metabolism of cells. Stress alters source/sink regulation by activating sink-specific enzyme genes alongside the defense against stress (Roitsch 1999).

9 Endogenous Hormones

Endogenous hormone levels can be considered as major factors in determining the specificity of cellular responses to rather general stress stimuli. In recent years, a large number of experimental observations have focused on the central roles of endogenous levels of indoleacetic acid (IAA) and abscisic acid (ABA) during the early stages of embryogenesis (Feher et al. 2003). The different levels of endogenous phytohormones in various explant tissues could be a factor influencing the requirements of exogenous growth regulators. The effect of genotype on somatic embryogenic competence has been clearly demonstrated. The presence of varying levels of endogenous phytohormones, in particular cytokinins, in different genotypes could influence their response to somatic embryogenesis. Wenck et al. (1988) observed that orchard grass genotypes in which embryogenesis was difficult to induce contained significantly higher levels of endogenous cytokinins than embryogenic genotypes.

Plants are sessile organisms that have endogenous signals to cope with biotic and abiotic challenges (Gilroy and Trewavas 2001). Phytohormones are chemical signals that produce low concentrations and travel around the plant, triggering various responses in tissues and cells. The level of endogenous phytohormones is considered to be one of the crucial factors influencing the embryogenic potential of explants. The quantity and quality of endogenous hormones (auxins, cytokinins, and ABA) were found to be different in the petioles of *Actinidia deliciosa* (kiwifruit) (Centeno et al. 1996), in the zygotic embryos of *Corylus avellana* (Hazel) (Centeno et al. 1997), *Triticum aestivum* (Wheat) (Hess and Carman 1998), and the cotyledons of *Panax ginseng* (Chinese Ginseng) (Choi et al. 1997) show different embryogenic potential. In *Arabidopsis*, an elevated level of auxins has been reported in cotyledons primordia (Ni et al. 2001), which correlates with the embryogenic competence displayed by cotyledonary parts of zygotic embryos (Luo and Koop 1997; Gaj 2001).

10 Role of Plant Growth Regulators (Auxins and Cytokinins) on Embryogenesis

Plant growth regulators play a critical role in determining the development and developmental pathway of the plant cells in tissue culture. This may be due to the accumulation of specific biochemical compounds. Addition of one or more growth regulators to the medium resulted in the maintenance of specific and balanced inorganic and organic compounds in the growing tissue. This leads to the development of cells or tissues into shoots or roots, or even death. Dahot (2007) underlined this well in these works. The high efficiency of 2,4-D for the induction of the embryogenic response found in different in vitro systems and plant species indicates a specific and unique character of this plant growth regulator. It is an auxin herbicide, synthetic growth regulator which appears to act not only as an exogenous auxin analog, but also as an effective stress agent. Several works including that of Feher et al. (2003) prove how 2,4-D causes various changes in the physiology and gene expression of cells, implying its possible role as a stress factor triggering the model of embryogenic development in plant cells in culture. The stress-like action of plant growth regulators should also be taken into account when very high concentrations of exogenous auxins for induction of somatic embryogenesis were required to induce somatic embryos in certain plant systems. For example, 452 M of 2, 4-D was effective in the culture of *Serenoa repens* (saw palmetto) (Gallo-Meagher and Gerrn 2002) and more than 200 M of NAA in *Pisum sativum* (Pea) (Özcan et al. 1993).

Studies on cashew have revealed the importance of PGR_s. According to Lakshmi Sita (1989), cotyledon explants on medium containing Naphthalene Acetic Acid (NAA), 2,4-D, and Benzyl Adenine (BA) developed embryogenic cultures. Then, somatic embryos formed in the presence of NAA, BA, or kinetin. Sy et al. (1991) showed that the proximal part of the cotyledons produced somatic embryos when they were initially cultured on Schenk and Hildebrandt (SH) (1972) medium with NAA and BA, in the presence of casein hydrolysate and adenine sulfate. Hegde et al. (1994) noticed that pieces of cotyledons formed somatic embryos on LS medium with pantothenate of Ca, IAA, and BA under a photoperiod of 20 h. It was also observed that leafy shoots and roots develop on the medium supplemented with activated charcoal. Despite this, there was in general poor organization of shoot meristems.

Somatic embryogenesis potentially offers many possibilities for large-scale plant propagation. Biotechnological applications such as genetic modification of trees to select desired stress tolerance traits and gene transfer are uses of somatic embryogenesis. Recently, the use of nucellus tissues from developing seeds for the induction of somatic embryogenesis has been investigated by Ananthakrishnan et al. (1999) and Cardoza and D'Souza (2000). Ananthakrishnan et al. (1999) observed callus induction from nucellar explants excised from 1-month-old developing cashew fruits on MS medium containing 6.78 μM 2,4-D. The differentiation of the calli into somatic embryos was noticed when calli were transferred to a liquid MS medium

supplemented with 4.52 μM 2,4-D. Different stages of development of the somatic embryos were recorded but there was no further development of the torpedo stage in this liquid medium containing 2,4-D. The conversion of somatic embryos to whole plants has not been achieved. In 2000, Gogte and Nadgauda have mentioned early cotyledonary somatic embryos with a combination of 2,4-D and GA_3 from cashew nucellar tissue. But these embryos did not fully develop. Studies using nucellar calluses in the presence of picloram were conducted by Cardoza and D'Souza (2000) who revealed the development of globular somatic embryos. The maturation of these globular somatic embryos was carried out in the presence of picloram and putrescine and germination was obtained in basal MS medium. Moreover, somatic embryogenesis has also been reported using the immature zygotic embryo as an explant (Cardoza and D'Souza 2000; Gogate and Nadgauda 2003). In both cases, somatic embryos were formed directly from the tip of the radicle or the end of the radicle of the immature zygotic embryo. Therefore, the presence of picloram (2.07 μM) (Cardoza and D'Souza 2000) or 5 μM 2,4-D + 5 μM BAP + GA3 (3 μM) (Gogate and Nadgauda 2003) favored the formation of somatic embryos. A medium with 20 μM of ABA and 3% maltose was used for the maturation. Gogate and Nadgauda (2003) and Cardoza and D'Souza (2000) reported the germination of somatic embryos on a medium lacking growth regulator.

11 Effect of Other Biochemical Factors on Somatic Embryogenesis

Some researchers: Ichihashi and Islam (1999), Islam et al. (2003), and Rahman et al. (2004) have pointed out that the addition of complex organic extracts, such as coconut water, the extract of taro, potato extract, corn extract, and papaya extract are essential for somatic embryogenesis in some species. In addition, it has also been noted that these organic extracts were either non-mutagenic or less mutagenic compared to conventional growth regulators. As such, Lam et al. (1991) asserted that their incorporation into culture media can minimize somaclonal variations. However, because it was not possible to determine which particular constituent of the extract promotes somatic embryogenesis and ensure the consistency of the actual extract each time it was prepared, it can also be said that organic extracts were undefined components.

12 In Vitro Recalcitrance, Phenolic Exudation, and Tissue Browning

Several studies including that of Krishna and Singh (2007) on woody species showed that some of the major problems in the culture of plant tissues of woody species include in vitro recalcitrance of plant tissues and associated phenolic exudation on browning of the media and explants. These factors are closely related, and their interactions are not fully understood. Recalcitrance occurs when plant cells, tissues, or organs do not respond to in vitro culture manipulations that would otherwise induce somatic embryogenesis or organogenesis. This is a major obstacle in plant tissue culture applications (Benson 2000). In particular, *Anacardiaceae* species are well known to be recalcitrant to in vitro treatments. Several authors (Boggetti et al. (2001); Ananthakrishnan et al. (2002)) reported the in vitro recalcitrance of *Anacardium occidentale*. Benson (2000) demonstrated that physiology of the donor plant, in vitro manipulations, and in vitro plant stress physiology are a number of factors that influence or trigger recalcitrant responses. Thorough knowledge of the life cycle of the donor plant, such as the phases of reproduction, rejuvenation, and dormancy, is therefore necessary to minimize the effects of recalcitrant plant tissues as shown by the work of McCown (2000). Careful handling of the in vitro environment can help overcome the problem of recalcitrant plant tissue. According to Benson (2000), this can be achieved by having an optimal balance of auxins and exogenous cytokinins in the growth medium. Further, he suggested applying compounds that are not strictly plant growth regulators, such as polyamines and antioxidants, as a means of alleviating in vitro recalcitrance. Kumar et al. (1998) have shown that in vitro stress physiology can induce an accumulation of ethylene in culture vessels, which ultimately reduces cell proliferation. Many plants are rich in phenolic compounds. Therefore, after tissue damage, these compounds are oxidized by polyphenol oxidases and the tissue turns brown. Evans et al. (1983) demonstrated that oxidation products are known not only to darken tissue but also inhibit the activity of various proteins which may have an inhibitory effect on somatic embryogenesis. According to Gannoun et al. (1995), Raghuvanshi and Srivastava (1995), Thimmappaiah et al. (2002a), Tabiyeh et al. (2006), and Krishna et al. (2008), browning of excised explants and the resulting discoloration of culture media remains one of the main challenges in plant tissue culture systems of *Anacardiaceae* species. The browning encountered in plant tissue culture was caused by the oxidation of phenolic compounds which are released into the medium during the excision of explants. Raghuvanshi and Srivastava (1995) reported that exudation of phenolic compounds from the wound site of the explant impairs the regenerative capacity of plant cells in vitro. Krishna et al. (2008) showed that when phenolic compounds are released from vacuoles due to excision of explants, they are released into the cytoplasm where the oxidative enzyme system is activated, resulting in tissue browning. As the work of Robards et al. (1999) has shown, one of the key enzymes involved in the oxidation process is polyphenol oxidase (PPO), a copper-containing oxidase responsible for the catalysis of O_2 dependent oxidation of

catechols to quinones. The optimum pH of 5.8 used in plant tissue culture is conducive to PPO activity, the optimum pH range of which is 5.0–7.0. Other enzymes in the wound-induced browning process include peroxidase (POD) and phenylalanine ammonia lyase (PAL). Tabiyeh et al. (2006) demonstrated that the activity of PAL, a key enzyme in the phenylpropanoid pathway, triggers the synthesis of phenylpropanoid compounds responsible for browning. In addition, PPO has been shown in research works of Robards et al. (1999) and Krishna et al. (2008) to synergistically stimulate the activity of POD through the generation of its substrate (H_2O_2) during the oxidation of phenolic compounds. To mitigate the negative effects of tissue browning in the micropropagation of *Anacardiaceae s*pecies, various pretreatments targeting inhibition of key oxidative enzymes have been used. Gannoun et al. (1995), Thimmappaiah et al. (2002a), Onay et al. (2004), Tabiyeh et al. (2006), and Krishna et al. (2008) had shown that several antioxidant compounds, used alone or in combination, such as ascorbic acid, citric acid, and salicylic acid have provided effective levels of browning control in in vitro culture of *Anacardiaceae* species. Additionally, adsorbent materials such as polyvinylpyrrolidone (PVP) and activated charcoal have been used to control browning effects in vitro. Success in controlling browning using PVP has been reported in the in vitro culture of *Pistacia vera* (pistachio) (Gannoun et al. 1995), *Mangifera indica* (Raghuvanshi and Srivastava 1995), and *Anacardium occidentale* (Ananthakrishnan et al. 2002). Likewise, in several cashew studies Das et al. (1996, 1999), Ananthakrishnan et al. (1999), Gogte and Nadgauda (2000), and Thimmappaiah et al. (2002b, c) the control of browning in vitro has been controlled or minimized by the application of activated charcoal. Activated carbon was often used in plant tissue culture to enhance cell growth and development (Pan and Van 1998). Its influence on growth and development can be attributed mainly to the adsorption of inhibitory substances in the culture medium (Fridborg et al. 1978; Horner et al. 1977; Theander and Nelson 1988; Weatherhead et al. 1978, 1979), drastically reducing phenolic oxidation or accumulating brown exudates (Carlberg et al. 1983; Liu 1993; Teixeria et al. 1994), changing the pH of the medium to a level optimal for morphogenesis (Owen et al. 1991) and establishing a darkened environment in the medium and thus simulating soil conditions (Dumas and Monteuuis 1995). Other anti-browning techniques described by Krishna et al. (2008), such as etiolating of mother plants and frequent subcultures in fresh medium, have had limited success in the in vitro culture of *Anacardiaceae* species. The exudation of phenolic compounds and the subsequent browning of the explants were reduced to some extent by frequently (initially every 2 days for the first week, followed by weekly subcultures during the induction phase) subculturing the explants, by using activated charcoal (0.3%) and PVP (0.5%) in the culture media, and incubation in the dark. Explant necrosis has also been attributed to the effect of strong disinfection. The use of explants from seedlings germinated in vitro or young shoots treated with a fungicide has been shown to improve the success rate considerably.

13 Conversion of Somatic Embryos into Plants

Further development and completion of maturation are induced by abscisic acid applications and the imposition of a drying period as well as the initiation of embryo development is stimulated by the removal of PGRs. Joy IV et al. (1991) showed that both treatments are necessary for the production of morphologically and physiologically mature embryos. Under a dissecting microscope, embryos appear as yellow nodules surrounded by translucent embryogenic tissue. After 2 weeks, the size of the embryos increases and they become larger in relation to the tissue mass, due to the increase in mitotic activity. A well-defined protoderm and organized root and shoot tips are visible at this time. Then, the apical poles of the embryos take the shape of a dome due to the formation of functional shoot apical meristem. At the root pole, the root cap begins to form. It is only after 3 weeks of culture that the apical meristems are completely different. At this stage, the embryos have developed a visible procambium and the cotyledons (4–6) that begin to emerge from the apical pole. The axis of the embryo lengthens, and the embryos acquire a creamy yellow coloration indicating the deposition of storage products, namely starch and later proteins. Cotyledonary embryos are produced after 4 weeks of maturation. They have reached a length of about 2–3 mm and are characterized by the presence of fully expanded cotyledons. At this time, embryos have accumulated a large amount of storage products, including proteins and lipids. According to Etienne et al. (1993), slow desiccation improved germination and was more effective in stimulating conversion into plantlets. Slow desiccation resulted in a substantial accumulation of starch and protein reserves necessary for the continued development of immature embryos compared to rapid dehydration. Therefore, desiccation could be used to improve germination as the embryo approaches physiological maturity. In large-seeded species, the sequential events during the formation of a fully mature zygotic embryo are very complex. Wang and Janick (1984) asserted that the lack of complete understanding of these processes underlying maturation, therefore, poses difficulties in reproducing the normal development of somatic embryos in vitro in these species. This may be one of the reasons for the problems encountered in somatic embryo maturation experiments in the current system, as the cashewnut is a large-seeded species whose cotyledons make up the majority of the seed mass. The development of cotyledons and the accumulation of storage products surely play an important role in the physiological maturation of the embryo in vivo. The application of in vitro methods based on somatic embryogenesis for plant regeneration is not only by high efficiency of somatic embryos formation but frequently depends on the capacity of embryos for plant development. Redenbaugh et al. (1986) explained that the process of developmental changes undergone by a somatic embryo, called conversion, involves the formation of primary roots, a shoot meristem with a leaf primordium, and the greening of hypocotyls and cotyledons. Despite the high number of somatic embryos produced, it has often been noticed, problems of lack or low frequency of conversion of embryos into plants.

Gogte et al. (2000) observed developmental abnormalities including fused embryos, lack of cotyledon formation, fused cotyledons, multiple cotyledons, and asymmetric cotyledon development in *Anacardium occidentale*. Jha and Das (2004) found that a period of preconditioning or post-maturation was essential for the germination of somatic embryos and germination was obtained after 4 to 5 weeks of preconditioning on MS media containing BA (1.0 mg/l). However, only 8.05 to 23.2% of cases were somatic embryos induced on different explants. Somatic embryogenesis from immature embryos via the callus phase had been attempted in cashew by Jha (1988) but resulted in the development of neomorph-like structures with disproportionate root/shoot ratios. Direct embryogenesis from pieces of immature cotyledons was attempted by Hegde et al. (1994), and embryoids were obtained without an intermediate callus phase. These embryoids were unable to develop further into complete plantlets. The studies by Lazzeri et al. (1987) and Sofiari et al. (1997) revealed a detrimental effect of 2,4-D on the regeneration capacity of somatic embryos. Frequently, ÖZcan et al. (1993) and Rodriguez and Wetzstein (1994, 1998) observed the development of malformed embryos, including multicotyledon or "fan-shaped" embryos, when 2,4-D was included in an induction medium. Choi et al. (1997) and Yasuda et al. (2000) extrapolated that such morphological abnormalities could result from disturbances in the polar transport of endogenous auxins caused by external PGRs. This has been noticed by Fischer et al. (1997) and Hadfi et al. (1998) in the treatment of developing zygotic embryos in vitro with polar auxin transport inhibitors, or exogenously applied auxins, which led to a wide range of morphogenetic alterations, including polyembryos and others strictly resembling malformed somatic embryos.

14 Defiance in Getting Somatic Embryos

Several advances were reached until now about the development of protocols for somatic embryogenesis of *Anacardium occidentale*. Yet, converting somatic cells into somatic embryos is not an easy task, and among the advances, some problems remain. Considering the several aspects involved in the initiation and permanence of the SE process, several variables may be investigated.

The development of cryopreservation protocols is an important step towards promoting the conservation of genetic material for *A. occidentale*. In 2001, Guerra et al. demonstrated that the mechanical strength of the alginate capsule combined with low-temperature storage prevents the germination of encapsulated somatic embryos of *Acca sellowiana (*Pineapple Guava*)*. Likewise, Cangahuala-Inocente et al. (2007) observed a significant increase in the survival of plantlets compared to the direct conversion of encapsulated somatic embryos. Gogte and Nadgauda (2000) showed that the rate of abnormal somatic embryos in cashewnuts is high. Therefore, conversion to temporary immersion systems, adjustment of plant growth regulator concentrations, duration of induction phase, and conditions for somatic embryos are also fundamental issues for future studies towards an efficient protocol

of somatic embryogenesis of *Anacardium occidentale*. Finally, Aguilar et al. (2006) and Aizen and Vazquez 2006 have concluded that global environmental change has and will have several effects on the reproductive biology of plant populations. The capacity of plant populations to reproduce successfully, with consequences for their demography evolution, and long-term persistence will be all directly impacted by climate change, habitat fragmentation, and pollinator decline. Further research on the biology of plant reproduction will therefore be of crucial importance for transaction with these environmental challenges and for maintaining biodiversity, genetic resources, and human well-being. Considering all these findings led us to envisage that in the worst scenario zygotic embryonic and seed development would be limiting. Hence, the development of trustworthy in vitro regenerative methods based on somatic embryogenesis should be a possible alternative to be considered.

15 Conclusion

Somatic embryogenesis, one of the most important tools in plant biotechnology, can be used to clone an individual plant at a relatively low cost. Under specific conditions, a single cell can reprogram itself from its original specialization to an embryogenic state, especially in plant species considered to be recalcitrant in somatic embryogenesis. Establishing detailed culture conditions, including the sequence and periodicity of the media applied, can be effective. So far, many advances have been made in the somatic embryogenesis of *Anacardium occidentale*, encouraging the use of this technology in new perspectives, in addition to genetic improvement and production of clonal forests. Our growing knowledge of somatic embryogenesis and other in vitro micropropagation procedures gives hope for a bright future, thus allowing progress in the conservation and sustainable use of plant genetic resources around the world.

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Chapter 9
Somatic Embryogenesis and Plant Regeneration in Horticultural Crops

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Abstract Globalization of agriculture leads to competitiveness in the production of food crops, especially in horticulture. The rate of multiplication of desirable plants by traditional breeding methods is slow and nature-dependent. Horticultural crops such as fruits and plantation crops are perennial and natural and must pass across prolonged juvenile phases for seed production. Somatic embryogenesis (SE) is one of the powerful biotechnology tools by which a somatic or vegetative plant cell is transformed into an embryo for commercial propagation. Somatic embryogenesis can help in the rapid and mass multiplication of newly released and improved varieties. Obtaining true-to-type plants in cross-pollinated plants is difficult because of their heterozygous nature. The mass multiplication of true-to-type plants with desired traits in in vitro conditions within a short period is possible by this method. It is a complex process and is controlled by several types of external and internal triggers. Plant regeneration through somatic embryogenesis offer merits over organogenesis in exploiting the feasibility of single-cell origin and accomplishing spontaneous production of embryos on large scale. Synthetic seeds produced through the process of somatic embryogenesis can be availed for direct field planting, and the plants have less bearing time compared with plants produced by conventional breeding methods. The bipolar nature of embryos produced by somatic embryogenesis permits the development of plantlets without the need for the separate root development stage unlike organogenesis in the in vitro method of propagation. In this chapter, the latest advances in somatic embryogenesis of major and commercially important crops are described.

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1 Introduction

Somatic embryogenesis (SE) is a biological process that plays an invaluable role in the plant regeneration system in the line of biotechnology. The development of plants with desirable quality traits through traditional breeding methods is time-consuming and arduous approach (Chandra et al. 2004). Breeding strategies for improving horticultural crops, predominantly fruit trees, are limited due to inherent concerns such as long juvenile periods, perennial nature of the trees, and typical flower structure in some crops, self-incompatibility, sterility, and inbreeding depression. Even though many techniques in fruit crops have already been standardized, there is still a need for innovative breeding techniques because of the long juvenile phase that made these techniques troublesome. The ideal alternative is to resort to SE in which sophisticated technology enables in the development of elite cultivars of substantial populations within a brief period.

Somatic embryogenesis is the process in which an individual or group of somatic cells develop an embryo which further develops into a complete plant under suitable conditions. The induction of somatic embryogenesis in the cells is one of the most spectacular achievements in the plant tissue culture (Reinert 1958; Steward et al. 1958). The basis for the plant somatic cell to become embryonic is the totipotency of the cell, which denotes that the nucleus of the somatic cell has all the genetic information required for a somatic cell to advance into an entire plant (Kamle et al. 2011). SE is an asexual process that uses the inherent totipotency of somatic cells to dedifferentiate a non-zygotic cell and then redifferentiate them, resulting in the production of all cells required to regenerate full plants (Sahrawat et al. 2003).

Somatic embryogenesis comes under the applications of the plant, tissue, and organ cultures. The earliest detection of in vitro SE was made in carrot (*Daucus carota*) (Reinert 1958; Steward et al. 1958). Even after many species of several genera have been experimented with and significant information has been compiled to establish the embryogenic potential of somatic plant cells. A wide range of interactions between bio-regulators such as auxins, ethylene, cytokinins, and several other growth regulators are mandatory for the origin of SE and to alter the genetic information of the cells (Jimenez 2005). Different parameters, including the source of the explant, the physiology of the mother plant, the incubation conditions, and the nature and composition of the culture media, can also influence the generation and advancement of somatic embryos (Loyola-Vargas et al. 2008).

SE is the process by which non-zygotic somatic cells produce bipolar structures that resemble zygotic embryos but lack vascular connections to the original tissue. Somatic embryogenesis is a multi-stage regeneration process that starts with the initiation of proembryogenic masses and progresses to the formation of somatic embryos, maturation, desiccation, and plant regeneration (Arnold et al. 2002).

SE in vitro is categorized as indirect or direct, depending on the presence or absence of a callus development phase (Rout et al. 2006). The embryo is formed directly from pre-embryonic determined cells in direct SE, without the formation of an intervening callus. In general, direct somatic embryogenesis is less common than indirect somatic embryogenesis. Some of the cases of direct somatic embryogenesis include the development of embryos from cotyledons and immature embryos of peach as published by Yan and Zhou (2002), the young embryos of cherry (De March et al. 1993), the leaves of apple (Da et al. 1996), the flowers of chrysanthemum (Mandal and Datta 2005), and the leaves of carnation (Yantcheva et al. 1998). Somatic tissue dedifferentiates into an unorganized mass of dividing cells known as calli during indirect somatic embryogenesis, from which somatic embryos develop (Mathew and Philip 2003). Somatic embryogenesis from carrot is a prime example of indirect somatic embryogenesis. Other cases of indirect SE include mango embryos (Rivera-Domínguez et al. 2004), banana male inflorescences (Grapin et al. 1996), grape leaves (Robacker 1993), and garlic bulbs and stems (Féréol et al. 2002).

Somatic embryos go through stages similar to zygotic embryogenesis during their development. It entails the command of three sequential steps: (1) Induction of embryogenic lines from explants; (2) maintenance and multiplication of embryogenic lines; and (3) maturation of somatic embryos and conversion into viable plantlets (Kamle et al. 2011). SE research has a wide range of application potentials, including genetic engineering, germplasm preservation, fast track breeding, synthetic seeds, embryo rescue in distant hybridization, the induced culture of hybrids of somatic cells, and induction of polyploids. Somatic embryo cultures are frequently derived from a single cell, so they are an ideal system for inducing mutations and checking chimeras. The use of SE in horticultural plants has been demonstrated to be useful in crop improvement, protoplast culture, and germplasm preservation. Dedifferentiation and redifferentiation of somatic cells are the first steps in somatic embryogenesis, whereas double fertilization is the first step in zygotic embryogenesis. Though the two types of embryogenesis begin at different stages, the molecular events occurring during somatic and zygotic embryogenesis are remarkably similar from the very beginning of embryo development. LEC1, LEC2, FUS3, and ABI3 are four key transcription factors that express and govern both types of embryogenesis, and their control systems of gene expression may be identical.

The characteristics of plants regenerated via somatic cell division can be affected by the in vitro culture environment, including the kind and concentration of applied plant growth regulators (PGRs), the genetic origin of the explant, and the number of subcultures (Konieczny et al. 2012). All of these variables can influence the genetic and epigenetic variation in the phenotype (Bairu et al. 2011), by a phenomenon known as somaclonal variation (SV) (Larkin and Scowcroft 1981). In the explant, SV might be a pre-existing genetic variant. Somaclonal variations, on the other hand, are now being utilized to develop disease-resistant clones in various crops like a banana (Ghag et al. 2014).

2 Factors Affecting Somatic Embryo Induction

2.1 Explant and Genotype

The choice of the desired explant is an important factor that decides the success of most tissue culture experiments like somatic embryogenesis (Lo Schiavo 1995). Isolation of explant generates stress on the respective cell which induces somatic embryogenesis (Ikeda-Iwai et al. 2003). In horticultural crops, different explants were used in different crops, phloem of the storage root of carrot, embryos of sugar beet (*Beta vulgaris* L.) (Zhang et al. 2008), immature embryo and cotyledons of peach (*Prunus persica* L.) (Yan and Zhou 2002), leaves of apple (*Malus pumila* Mill.) (Kedong et al. 1996), the scale leaves of lily (*Lilium brownii* var. *viridulum*) (Xuanming et al. 1997), the flowers of chrysanthemum group known as florist's daisy (*Dendranthema morifolium*) (Mandal and Datta 2005), leaves in Solanum sps. (Birhman et al. 1994), petioles and stems in Solanum sps. (Reynolds 1986), corms in taro (*Colocasia esculenta* var. esculenta) (Deo et al. 2009), embryos of mango (*Mangifera indica*) (Rivera-Domínguez et al. 2004), the bulb stems of garlic (*Allium sativum* L.) (Féréol et al. 2002), the male inflorescences of banana (*Musa paradisiaca*) (Grapin et al. 1996), and the cotyledons of cucumber (*Cucumis sativus*) (Elmeer and Hennerty 2008). The explants should also be in the proper developmental stage for successful induction of somatic embryogenesis. The variation in genotypes leads to differential response to SE induction as the genetic makeup of explant descendent from the genotypes (Li et al. 2006). The genotype dependence for SE as a result narrows down the genetic diversity. Sometimes, there is variation in in vitro culture during SE is reported in different horticultural crops like walnut (Chen and Su 2006), vegetable soybean (*Glycine max*) (Ping et al. 2005), and leaf sections of *Anthurium andraeanum* (flamingo lily) were better having higher somatic embryogenic potential than other (Weijie et al. 2006).

2.2 The Base Medium

The different constituents of base media play a critical role in the SE of horticultural crops. SE of horticultural crops has different requirements of base media based on variation in explants, genotypes, and species. For SE of horticultural crops, there are mainly very few established base media like MS, B5, DKW, and SH. It was found in mango as compared to MS, the effect of improved B5 was better for the study of SE (DeWald et al. 1989).

2.3 Plant Growth Regulators (PGR)

The effect of PGR is a critical factor inducing a successful SE and plant regeneration. Usually, SE needs a mixture of different concentration of auxin and cytokinin for morphogenesis in in vitro culture. The requirement of PGR is different for different developmental stages of explant. The concentration of PGRs is very critical, accurate concentration should be required neither it act as an inhibitor when beyond its permissible concentration.

2.3.1 Auxin

In the early stage of SE, bipolar auxin transport and distribution influence the embryo axis formation. Among the auxin, 2, 4-D is widely used in SE in horticultural crops like Chinese quince (*Chaenomeles sinensis* Koehne) (Fitch 1993), grape (*Vitis vinifera*) (Robacker 1993), peach (*Prunus persica* L.) (Yan and Zhou 2002), American chestnut (*Castanea dentata*) (Carraway et al. 1993), and mango (*Mangifera indica*) (Rivera-Domínguez et al. 2004). Differential effect of 2,4-D in different crops, for instance, the effect of naphthalene acetic acid (NAA) and indole-3-acetic acid (IAA) at inducing the SE are more than the 2,4-D in citrus (*Citrus reticulate* Banco.) (Yejiang and Zhongxiong 2001), and gladiolus (Stefaniak 1994) and it also inhibits SE on cherry (De March et al. 1993). Some other auxin also used apart from 2, 4-D in different crops like picloram in banana (Wei et al. 2007) and IAA (Indole acetic acid) in *Begonia cathayana* (Castillo and Smith 1997).

2.3.2 Cytokinins

Cytokinin along with auxin plays a vital role in morphogenesis and the ratio of A/C (auxin/cytokinin) is the deciding factor for the organogenesis of root or shoot. Cytokinin having higher cell dividing activity leads to the formation of several bud embryos from the lateral branches of a primary embryo (Eudes et al. 2003). The high ratio of (A/C) would induce the SE of *Begonia cathayana* Hemsl (Castillo and Smith 1997), American chestnut (Carraway et al. 1993), and cyclamen (Takamura et al. 1995) whereas a high ratio of CTK to auxin would promote apple SE.

2.3.3 Gibberellic Acid (GA) and Abscisic Acid (ABA)

Gibberellic acid (GA) and abscisic acid (ABA) are not usually essential for the induction of SE but in some cases, GA has been reported to induce SE for pear, garnetberry, apple, and cherry and also plays an inhibitory role in citrus SE (Yejiang and Zhongxiong 2001). Plants have differential specificity for the ABA

concentration for induction or inhibition of SE. Application of 10^{-4} M ABA promotes somatic embryo development in carrot apical tip explants. ABA has mostly inhibitory effects and inhibition of the SE of banana embryogenic suspension callus is practically reported.

2.3.4 Other Inductive Factors

Different amounts of nitrogen compounds also play a measurable role in the induction of SE. Thidiazuron (TDZ; N-phenyl-N′- [1, 2, 3 thiadiazolyl]-urea) is a substituted phenyl urea having high potential for both auxin and cytokinin activity. It is more effective in organogenesis in several commercial plantation crops. Several amino acids also play a vital role in the induction of SE, for instance, L-proline induces the petiole SE of apple (Kedong et al. 1996). Carbon as an energy source for explant maintains the osmotic potential in culture media. Cotyledon of melon as explants, direct embryogenesis was noticed when the base medium contained lactose, indirect embryogenesis was observed when glucose was used as the carbon source (Yuting 2007). There was an increase of 6–12 folds in citrus SE efficiency combined with galactose and lactose instead of sucrose alone. The natural additions of malt wort (ME), hydrolyzed casein (CH), and coconut wort (CW) as the cultural media deliver more reduction of N than inorganic N as a result of inducing SE. In several cases, polyamines also play a significant role in the induction of SE, for instance, carrot (Takeda et al. 2002) and eggplant (Singh Yadav and Venkat Rajam 1998).

2.4 Histodifferentiation

During organogenesis, close observation should be done for the development pattern from the early developmental stage to the maturation of the zygotic or somatic embryo. Histodifferentiation, a unique developmental process of tissue and organ through which a globular stage somatic embryo develops into the cotyledonary stage embryo. This process is affected by several factors including the genetic constitution of the species (Li and Grabau 1996). In this process, there is a variation in the concentration of different PGR and nutrients which was needed for the development of the embryo. Removal of both auxin and cytokinin from the base medium was required to initiate embryo differentiation whereas reduction in auxin concentration induced somatic embryo formation. Ethylene concentration in induced base media also regulates the histodifferentiation process of the somatic embryo of horticultural crops. Different other external factors like the intensity of light, darkness, carbon source, temperature, aeration, nutrient source, and diseases affected this process.

3 Somatic Embryo Maturation

Maturation is the final stage of somatic embryo development where embryos start to accumulate storage reserves in this process. It is the critical stage in somatic embryo development, which determines the germination and successful regeneration of somatic embryos, has been examined mainly about the role of different plant growth regulators and some of the nutrients on reserve accumulation and utilization (Lakshmanan and Taji 2000). In this step, there is variation present among different genotypes, in terms of how well the embryos develop into a fully mature embryo and how many mature embryos will develop from induced somatic embryos (Egertsdotter 2019). Embryo maturation and early germination are important stages for obtaining multiple plantlets, and it partially depends on the number of induced quality embryos. Nowadays, a large number of compounds or techniques such as abscisic acid (ABA), activated charcoal (AC), gibberellic acid (GA3), polyethylene glycol (PEG), sugars, sugar alcohol, and low-temperature treatment are provided to medium to improve embryo maturation and early embryo germination (Robichaud et al. 2004).

In important ornamental plants like gladiolus, the somatic embryos were cultured on MS medium supplemented with salts, vitamins, inositol, and GA3. The medium supplemented with GA3 helps to improve embryo quality through promoting embryo size and turning the tips of embryos to green, later which germinated into plantlets. Mujib et al. (2016) recorded 62.15% of embryos maturation in the MS medium supplemented with 0.5 mg/l GA3 after 8 weeks of incubation. The MS medium added with ABA was recorded less efficiently and had little influence on embryos (Mujib et al. 2016). Vieitez (1995) also noted a similar response in hybrid chestnut, i.e., ABA added medium shows poor embryo germination. Whereas, in some coniferous plants, ABA enhanced embryo quality and enabled the embryo maturation process (Lelu-Walter et al. 2008). In Arabidopsis also during somatic embryogenesis, ABA requirement is essential (Bai et al. 2013). During seed or embryo maturation, the ratio of ABA:GA3 is known to be interlinked directly with *LEC* gene expression (Braybrook and Harada 2008). *LEC* (*LEC1, LEC2*) gene and other related genes like *FUS3* form a regulatory system and benefit the building of storage reserve during embryo maturation and cotyledon development (Santos-Mendoza et al. 2008).

In fruit crops like avocado (*Persea americana* Mill.), morphological modifications observed during proliferation were correlated with the subsequent capacity of cultures to develop into mature somatic embryos. Márquez-Martín et al. (2012) found that periods in suspension and inoculum density were important factors that influenced the capability of cultures to undergo maturation in avocado. The maximum number of somatic embryos was recorded when 0.4 g fragile embryogenic callus is grown in 40 ml of MS medium supplemented with 0.41 μM picloram in 9 days (Márquez-Martín et al. 2012).

In banana, the impact of water stress on somatic embryo maturation is one of the most critical aspects (Attree et al. 1991). The embryo begins the process of

desiccation tolerance during this stage, as water is gradually lost (Bewley et al. 2013). Embryo is stimulated to collect reserve proteins, carbohydrates, and lipid compounds during this stage (de Moura Vale et al. 2014). Presently available techniques in bananas reduce the availability of water to developing somatic embryos by using either a greater concentration of gelling gum or filter paper or both. The buildup of starch during embryo maturity is indicated by the white-opaque tint approximately 45 days of culture (Chung et al. 2016). Early responsive to dehydration proteins (ERDs) are important in embryo maturation, according to molecular evidence from transcribed expressed sequences. ERD genes are genes that are rapidly activated during drought stress and are implicated in developmental and stress responses mediated by abscisic acid (Maldonado-Borges et al. 2013).

In case of *Ziziphus jujuba* Mill. (Chinese date), the primary somatic embryos were multiplied by repetitive somatic embryogenesis on MS media with various plant growth regulator combinations. Kim et al. (2006) found that the uppermost repetitive somatic embryogenesis frequency (51.3%) was gained with 0.58 pM gibberellic acid (GA3). GA3 was more effective than cytokinin treatment of BA (Benzyl adenine), zeatin, and 2iP (6-r-r-dimethylallyl amino purine).

Valencia-Lozano et al. (2021) studied the maturation of somatic embryos of coffee (*Coffea arabica* var. Typica) by inducing osmatic stress. In this study, he found that maximum somatic embryos converted to plantlets under osmatic stress by using 9 gm/L of gelrite. The maturation medium is prepared by using MS medium supplemented with 0.2 mg/L BAP, 0.1 mg/L kinetin, 1% glucose, 9.0 g/L gelrite (−1.47 MPa), and pH 5.8.

In papaya (*Carica papaya* L. cv. Co7), an efficient somatic embryogenesis protocol was standardized by Anandan et al. (2012). The liquid half-strength MS medium with 10.5 mg/L ABA and 10.0 g/L sucrose generated the maximum number of mature cotyledonary embryos from somatic embryos.

In oil palm (*Elaeis guineensis* Jacq.), embryogenic callus was grown in a medium that included basal media, plus 0.6 μM naphthalene acetic acid, 12.30 μM 2-isopentenyladenine, 0.3 gm/L activated charcoal, and 500 mg/L glutamine, with subcultures at every 4 weeks for effective maturation by Scherwinski-Pereira et al. (2010) and they reported better maturation. Balzon et al. (2013) also reported good maturation in the similar media composition, i.e., MS medium added with 0.54 μM naphthalene acetic acid, and 12.30 μM 2-isopentenyladenine.

An areca nut (*Areca catechu*) embryogenic callus maturation was reported by Radha et al. (2014) by growing the embryogenic callus in half MS hormone-free medium added with sucrose (3%), agar (0.55%), and activated charcoal (0.25%).

In coconut (*Cocos nucifera*), Pérez-Núñez et al. (2006) reported that even after three multiplication cycles, embryogenic calluses were capable of generating somatic embryos. Abscisic acid at a concentration of 2.5–7.5 μM was added to the culture medium for 3–7 weeks stimulated somatic embryogenesis. The addition of ABA increased the number of somatic embryos produced (Fernando and Gamage 2000).

3.1 Direct Somatic Embryogenesis

Harini and Sita (1993) first reported direct regeneration of somatic embryos from immature zygotic embryos of chilli (*Capsicum annuum* L. var. California Wonder). They identified that somatic embryos were induced mainly on the embryonal axis and cotyledons of the immature zygotic embryos (5–6 mm in length). The somatic embryos induced and matured in MS medium supplemented with the optimum concentration of 2, 4-dichlorophenoxyacetic acid (2,4-D; 1–2 mg/L) and sucrose (10%). Khan et al. (2006) developed an efficient protocol of direct somatic embryogenesis (without involving intermediate callus) from stem segments and shoot tips of *Capsicum annuum* L. Somatic embryos induction and maturation achieved in MS medium supplemented with 0.5 μM of thidiazuron (TDZ).

Martin (2003) using seed coat explants, a strategy for plant regeneration via direct somatic embryogenesis was devised for the first time on cashew nut (*Anacardium occidentale* L.). The type and age of the explant, as well as growth regulators added to the medium, had a considerable impact on the frequency of embryogenesis. Seed coatings obtained from immature nuts between 15 and 40 days following pollination performed better in terms of inducing somatic embryogenesis. MS medium added with 13.3 μM benzyl adenine (BA), 271.5 μM adenine sulfate, and 2.7 μM naphthalene acetic acid produced the best somatic embryos. The growth of more than 10 embryos per primary embryo, i.e., secondary somatic embryos, was aided by transferring primary embryos to a fresh embryo induction medium.

4 Germination and Plant Regeneration

Good germination and regeneration are the reflection of physiological maturity, including desiccation after maturity by somatic embryos. The mature somatic embryos in lab conditions are exposed to a period of desiccation to mimic the process that occurs naturally in seeds before germination. Without desiccation, germination can also occur in some species and cell lines, but it is believed that it will improve germination rate and subsequent plant formation (Egertsdotter 2019).

In important ornamental plants like gladiolus, maximum plantlet regeneration was recorded on MS medium containing 0.5 mg/l BAP, and later they were transferred to MS medium supplemented with IBA of 1.0, 2.0 mg/l for more efficient in promoting roots (Mujib et al. 2016).

Anandan et al. (2012) in *Carica papaya* L. (cv. Co7) found the best regeneration that MS media added with BAP (0.4 mg/L) and NAA (0.02 mg/L), whereas kinetin had a negative impact on regeneration. Greening was seen in the cotyledonary stage embryos within 15 days of culture. Within 30–45 days of culturing on the same media composition, the developing shoots with roots had grown into a full plantlet.

Perán-Quesada et al. (2004) reported a low conversion rate, i.e., 0%–11.11% when a transgenic Avocado material is used. Márquez-Martín et al. (2012)

mentioned that germination rate of avocado ranges from 5% to 10%. So, before transformation work attempting in this species, the lines should be selected carefully, or else, plant recovery could be extremely impaired.

In banana, various genotypes recorded various somatic embryo germination percentages. The plant recovery is achieved through the germination of somatic embryos with the emergence of normal roots and shoots on germination media with or without plant growth regulators. Highest germination reported by Jalil et al. (2003) in Musa spp. cv. Dwarf Brazilian (AAB) was 90–96%. Embryo conversion rates range from 13% in the edible (AA) Pisang Mas banana to 13–25% in the Cavendish subgroup's Grand Nain (AAA), 66.7% in the highland African banana (AAA) (Namanya et al. 2004), and 100% in the wild *Musa acuminata* ssp. malaccensis (AA) (Escobedo-GraciaMedrano et al. 2016).

In Chinese date, repetitive somatic embryos which developed beyond the cotyledonary stage were selected for germination. Desiccation of somatic embryos at 25 ± 1 °C for 2 weeks was the best treatment for germination with epicotyl elongation and root development (Kim et al. 2006). In Japanese black pine (*Pinus thunbergii* Parl.) desiccation of the cotyledonary somatic embryos at high relative humidity leads to a marked increment in germination frequency and subsequently improved plant conversion rate. (Maruyama and Hosoi 2016).

In coffee, typical somatic embryo conversion to plantlets is the major bottleneck. Valencia-Lozano et al. (2021) developed a protocol where 95.9% of somatic embryo conversion to plantlet was achieved in an osmotic stress medium. The medium is prepared by using MS medium supplemented with 30 gm/L sucrose, 1 gm/L activated charcoal, 3 gm/L gelrite, and smoke water 0.5%. The regenerated plantlets were subculture in the flasks contain the same media, until plants were ready to grow in ex vitro conditions.

Radha et al. (2014) transferred matured areca nut embryogenic calli into half MS basal medium added with BA (1 mg/L), agar (0.55%), and activated charcoal (0.1%). Following that, the somatic embryos were transferred to a medium added with BA (5 mg/L), sucrose (3%), agar (0.55%), charcoal (0.1%), and IBA for plantlet development (0.5 mg L-1). Liquid medium enriched with BA (10 mg/L), IBA (1 mg/L), NAA (1 mg/L), sugar (3%), and charcoal (0.1%) for continued development of shoots and roots (0.1%).

In coconut, 2.5–7.5 μM of ABA was found to be effective when used for 5 weeks, when compared to those grown on medium with low 2, 4-D, a substantial number of somatic embryos developed on medium containing ABA established normal branches and entire plants (Fernando and Gamage 2000).

Harini and Sita (1993) recorded the germination of embryogenic calli of chilli. The matured somatic embryos in MS medium supplemented with the optimum concentration of 2, 4-D (1–2 mg/L) and sucrose (10%) were germinated in the presence of 1 mg/L gibberellic acid (GA3). The germinated embryos formed plantlets within 15 days. Later, plantlets were transferred to a liquid medium without growth hormones before transfer to ex vitro conditions. Khan et al. (2006) achieved good germination of *Capsicum annuum* in MS medium supplemented with 0.5 μM of thidiazuron (TDZ). The germinated somatic embryos which contain shoots were

transferred to rooting medium, i.e., MS media supplemented with indole-3-butyric acid (0.5 to 2.0 mg/L).

In cashew nut, during the germination process first cotyledon turn dark green, then the root area swollen and elongated. The conversion of somatic embryos into plantlets took more than 30 days and the emergence of the shoot apex also took a long time. Embryo showed a higher frequency of conversion when they were transferred to MS media containing 4.65 μM kinetin (Martin 2003).

In the case of oil palm (*Elaeis guineensis* Jacq.), Scherwinski-Pereira et al. (2010) transferred the matured somatic embryos to modified media containing half-strength macro and micronutrients, as well as half-strength, 2% sucrose, and 1.0 gm/L activated charcoal and gelled with 2.5 gm/L Phytagel to turn them into plants. While Balzon et al. (2013) regenerated the somatic embryos on Murashige and Skoog macro- and micronutrients at half-strength concentrations added with 20 gm/L sucrose, 2.5 gm/L activated charcoal, and 2.5 gm/L phytagel.

5 Clonal Propagation or Mass Propagation or Micropropagation

Plant tissue culture for the mass propagation of planting materials can be carried out in different ways. For commercial mass propagation, establishment of long-term, high-frequency embryogenic cultures capable of producing clonal plant material is necessary (Lakshmanan and Taji 2000). Normally, differentiated somatic cells are involved in clonal propagation, hence the term somatic embryogenesis. The main advantages of somatic embryogenesis are to produce the intact plant with the apical meristem and the primary root, avoiding organogenesis problems, i.e., senescence, rejuvenation, rhizogenesis, and difficulty to transfer into the soil. (Ji et al. 2011). The plants derived from somatic embryos through somatic embryogenesis are quite similar to seed plants because the zygotic (seed) embryos establish a strong root-shoot link, without the plagiotropism that often comes with cutting propagation. In breeding programs, almost endless number of SE plants may be generated from one original seed embryo for practical purposes for the deployment of the elite clones and SE cultures (clones) can be cryostored for extended periods enables for field testing before the selection of production cell lines (Park et al. 2014). In addition, the SE process is suitable for scale-up and automation, resulting in lowering labor costs and boosting up the production process. The in vitro multiplication of early embryos allows for automation and scaling up desirable genotypes by using a liquid culture medium for commercial plant production (Egertsdotter et al. 2019).

Castillo et al. (1998) experiment in *Carica papaya* L. revealed that embryogenic calli cultivated in a liquid phase maturation media had a much higher frequency of SE and regeneration in comparison to parallel treatments on agar-solidified medium. It was also possible to see the development of secondary embryos from primary SEs.

Kosky et al. (2002) described an improved approach for bulk propagation of the banana hybrid cultivar FHIA-18 (AAAB) in a liquid medium and scaled up in a bioreactor using somatic embryogenesis.

Garcia et al. (2016) conducted a small-scale pilot experiment in *Theobroma cacao* L. and came to the following conclusions viz., (1) a high level of plant production is possible using SE, while efficiency is genotype-dependent; as a result, it is necessary to optimize hormone balance and hormone type and explants type. (2) Through secondary SE, it is possible to increase somatic embryogenesis production by tenfold is achievable, and (3) the observed differences in response between genotypes could be due to differences in endogenous and exogenously provided nutrient hormones.

Maciel et al. (2016) optimized numerous steps in the process for large-scale generation of somatic embryos viz., multiplication of embryogenic aggregates with MM medium (Teixeira et al. 2004); regeneration of somatic embryos in liquid medium containing 2.0 mg L^{-1}naphthalene acetic acid (NAA) or in Petri dishes containing 4.0 mg L^{-1} proline; and use of 2.0 mg L^{-1}6-benzyl amino purine (BA) for the production of cotyledonary embryos in RITA temporary immersion type bioreactors (CIRAD, Montpellier, France) were found to be the best conditions for coffee.

In coffee, SE has allowed the multiplication of two cultivated coffee species, *Coffea arabica* and *Coffea canephora,* since the 1990s. In recent years, coffee SE has advanced to the point that is currently one of the most advanced technologies (Etienne et al. 2018). Awada et al. (2020) created a miniaturized and automated screening system to meet high-throughput standards for the first time in plant micropropagation to speed up the optimization of SE methods by multiplying experimental conditions in a short amount of time.

Various ways of automating SE procedures are used to simplify culture protocols, with a preference for liquid over solid cultures, and the use of robots and automation for the harvest of selected mature embryos, followed by automated germination and planting. As of today, there are no automated systems for the large-scale production of somatic embryo plants that are yet in commercial use (Egertsdotter et al. 2019).

Once the plantlets have formed, the next step is to establish plantlet's growth ex vitro by a gradual transition to growth conditions outside the enclosed culture container used for in vitro culture.

6 Hardening and Acclimatization of Somatic Plants

6.1 Substrates and Their Role in Successful Acclimatization

Plantlets obtained through somatic embryogenesis are hardened in stages, progressing from high to low humidity and low to high light intensity. Most of the agar may be removed gently by washing with water if grown on solid media. Plants can be placed in the shade for 3 to 6 days to acclimatize to their new surroundings

with diffused natural light. The plants are then progressively toughened by being moved to suitable substrates like sand, peat, soil, compost, etc.

Hussain et al. (2016) carried out an experiment in which plantlets regenerated via somatic embryogenesis from the nucellus tissues of kinnow mandarin were acclimated by planting in plastic pots filled with various hardening mixes (sand, sand + peat moss (2:1), sand + soil + peat moss (1:1:1), and sand+ soil + compost (1:1:1). The plantlets were then covered with plastic sheets for 15 days and the acclimation period lasted about 12 weeks. The potting combination with sand and peat moss (2:1) had the best survival rate (100%) followed by sand, soil, and peat moss (1:1:1). The use of sand, soil, and compost resulted in a 90% success rate during hardening off (1:1:1).

Kaur et al. (2011) used several mixes of soil, vermicompost, and biofertilizers to optimize potting mixture for hardening of in vitro grown plants of *Tylophora indica*, an essential medicinal plant. Different combinations used were soil: vermicompost (T_0), soil: vermicompost: azotobacter: pseudomonas (T_1), soil: azotobacter: pseudomonas (T_2), soil: azotobacter (T_3), soil: pseudomonas (T_4) and found that among all the combinations tried, soil: vermicompost: azotobacter: pseudomonas (T_1) (1:1:1:1) recorded the highest survival rate of 92% upon transplantation of plants to the field conditions followed by soil: vermicompost (1:1) with the survival rate of 88%.

Waghmare et al. (2017) conducted studies on somatic embryogenesis in strawberry (*Fragaria ananassa* var. Camarosa). The acquired plantlets were transplanted in soil rite-filled containers. At 32 ° C and 90% humidity, the length and size of the plantlets, as well as the size of the leaves, were increased. Soilrite offered the highest plantlet survival efficiency (100 percent). Biswas et al. (2007) did a similar investigation in the same variety of strawberries for hardening plantlets using sterile sand and garden soil (1:1) and reported a survival rate of 90–95. Soilrite may be the best medium for the primary hardening of strawberry.

Pires et al. (2020) investigated somatic embryogenesis from mature *Olea europaea L. cv. 'Galega* Vulgar' embryos. When the plantlets had grown around 10 cm in length, they were moved to honeycomb trays for acclimatization. The substrate utilized was a 1:1:3 (v/v) combination of sand, perlite, and peat. Finally, the plants were moved to 2 L pots with a similar substrate composition and placed in a greenhouse. At the end of this phase, an average rate of 75 percent acclimatized plants was obtained.

Wafaa and Wahdan (2017) performed studies on the influence of substrates acclimatization of strawberries. Rooted shoots of the two cultivars Festival and Marquez were cleaned with distilled water to completely remove the rooting substrate and transferred to plastic pots (15 cm diameter) filled with peat moss, vermiculite, peat moss+ vermiculite (1:1 v/v), and peat moss+ sand (1:1:1 v/v/v). To produce high relative humidity, pots were moved to the greenhouse and covered with clear polyethylene sheets. The highest rooting percentage was obtained when plantlets were cultured alone in peat moss (96%) and were followed by a mixture of peat moss and vermiculite (90%), but it decreased to 83.33 percent when the plantlets were cultured in a mixture of peat moss and vermiculite.

Ghosh et al. (2018) analyzed thidiazuron-induced somatic embryogenesis in tissue cultures of half-high blueberry plants. After 8 weeks of culture, five plantlets (4–5 cm long) with 8–10 leaves developed on all concentrations of TDZ and ZEA were removed from the glass jars, rinsed in sterilized water, and transplanted into plastic pots containing peat: perlite (3: 1 v/v) with a 100% survival rate. Following acclimation, hardened off seedlings were moved into 6-cm^3 plastic pots containing the same peat-perlite mix and cultivated in a greenhouse with natural light.

Young plantlets derived from somatic embryos of *Hibiscus sabdariffa* L., measuring 3–4 cm in diameter with 5–6 roots were successfully hardened being transplanted onto plastic pro-trays with a 2:1 combination of coco peat and coarse sand for primary hardening. The plants in portrays were incubated for 1 month in a plant growth chamber at a temperature of 25 °C and relative humidity of 75%, with periodic misting's of water, until new leaves emerged. For secondary hardening, 1-month-old primary hardened plants were placed into clay pots containing a 1:1:1 mixture of soil, sand, and organic manure (Konar et al. 2018).

Various workers have reported effective acclimatization of rooted shoots in peat-based compost and autoclaved FYM + Sand (1:1) in the case of guava (Prakash and Tiwari 1996). Somatic embryogenesis-derived plantlets of guava (*Psidium guajava* L.) performed well on coco peat substrate as reported by Bajpai et al. (2016).

The fully formed plantlets of culantro (*Eryngium foetidum* L.) were rinsed clear of agar in flowing water and transferred to paper cups with a combination of sterile garden soil and sand (1:1) kept in a mist chamber at a regulated temperature (27 ° C) with relative humidity (80–90%) for hardening. Following that, the acclimatized plants were transferred to clay pots and kept in in vivo circumstances as published by Ignacimuthu et al. (1999).

The regulation of environmental factors during acclimatization of SE-derived plantlets was studied by Bajpai et al. (2016). To promote growth and minimize mortality in plantlets during the acclimatization period, bio-agents such as *Glomus aggregatum*, *T. harzianum*, and *Piriformospora indica* shown to be extremely efficient in increasing the establishment frequency of the plantlets regenerated by SE.

Bio-agents such as *Trichoderma harzianum* in the carrier substrate not only improves plant survival but also increases fibrous root growth and plant vigor. *T. harzianum* fortification in coco peat had the greatest results for improving guava plant survival and growth as reported (Bajpai et al. 2016).

7 Conclusions and Future Prospects

Somatic embryogenesis is a highly touted method for developing protocols for accelerated multiplication of unique and elite genotypes, artificial seed production, and genome editing research. Cells must dedifferentiate, initiate cell division, and reconfigure their physiology, metabolism, and gene expression patterns during somatic embryogenesis, which follows a distinct developmental pathway marked

by some conspicuous processes. Understanding embryogenic induction, the origin of somatic embryos, and whether they are unicellular or multicellular, is crucial for scientific and biotechnological purposes. The success of embryogenic cells could be measured by cell tracking.

Despite extensive research on somatic embryogenesis in model species such as *Daucus carota* (Raghavan 2006) and *Picea abies* (Von Arnold et al. 2005), plant embryogenesis remains a mystery. Regeneration from embryogenic cultures remains a challenge in the majority of woody angiosperms, which are physiologically distinct from the aforementioned model species. This fact emphasizes the importance of researching the key steps of the embryogenic process in woody angiosperms to gain a better understanding comprehension of the mechanisms involved in the regulation of SE.

Plant somatic embryos can serve as a model experimental system for research into plant cell development and differentiation, plant totipotency expression, crop enhancement, and mutant screening. Also, embryonic callus methods might be used to save endangered plant species. The discovery of hormone-inducible genes has provided insight into the hormonal regulation of gene expression throughout embryogenesis. The identification of genes involved in signal transduction pathways like SERKs has sparked interest in the switching of many signal cascades during somatic embryogenesis. Transcription factors like BBM, LEC1, and LEC2 could be exploited to control embryogenic development. Protein markers can also be used to determine embryogenic potential and to distinguish between different stages of plant growth.

The use of SE in the mass propagation of commercial production of horticulture and plantation crops is the production of efficient true-to-type plants from the single mother plant by avoiding the long juvenile phase of conventional propagation. This SE system facilitates the future crop improvement strategy employing genetic transformation method.

In horticulture plants, SE methods have been mentioned in the literature is in the early stages of industrial development and they are based on small laboratory-scale batch sizes and labor-intensive transfers between process steps. The SE propagation needs significant investment and skilled technicians, but recent improvement focused on automation for the selection and handling of the individual plant. Molecular markers are critical for improving the entire SE process and progressing to full commercialization. Furthermore, it will contribute to a better understanding of embryogenesis and totipotency in higher plants, a crucial biological process that is still poorly understood.

A more comprehensive investigation is essential to unlock the full potential of somatic embryogenesis and make it completely accessible for the creation of plants. Finally, farmers and consumers must get persuaded that plants derived from somatic embryo-based technologies are safe, secure, and necessary.

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Chapter 10
Prospect and Commercial Production of Economically Important Plant Mulberry (*Morus Sp.*) Towards the Upliftment of Rural Economy

Pijush Mallick and Mrittika Sengupta

Abstract Mulberry (*Morus sp.*) is one of the economically important plants across many countries having numerous attributes of sustainable development. Mostly, it is cultivated for the sericulture industry, an agro-based cottage domain that supports the upliftment of the rural economy. The leaf foliage of such hardy, woody, perennial tree species of *Morus* is used for silkworm feeding for better and optimum production of commercial raw silk. However, mulberry crop improvement through modern biotechnological approaches over conventional breeding methods plays a significant role in the production of qualitative and quantitative leaf foliage within a short period of time for commercial purposes. Development of biotic and abiotic stress-tolerant mulberry varieties under various environmental conditions, biotechnological tools such as micropropagation of hard to root genotypes, in vitro tissue culture, callus culture, protoplast fusion, marker-assisted selection, and genetic engineering made a successful contribution towards optimum foliage production followed by healthy economy return. Apart from silkworm feed, mulberry is also being exploited by pharmaceutical, cosmetic, food, and beverage industries along with its utilization in environmental safety approach. Considering all the important characteristics of mulberry, it had established as an ideal crop plant in providing sustainable future and economic growth.

Keywords Crop improvement · In vitro propagation · *Morus* species · Sustainable development · Rural economy

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1 Introduction

The kingdom plant plays a crucial role in keeping mother nature in a stable state by minimizing global warming by absorbing carbon dioxide from the atmosphere and releasing oxygen into the environment, which purifies the air and gives animals and other species to live. Plants also help soil health by retaining water in the subsurface of the soil and cooling hot urban areas via the water cycle's evapotranspiration process (Katul and Novick 2009). The eco-biological recycling of essential minerals from plants back to nature allows soil and forest fertility to be maintained (Barot et al. 2007). The beauty of plants is that they may co-exist in the same habitat and with the same resources like; air, water, soil nutrients, and predatory organisms in a place or unit of area (Anten 2005), resulting in the establishment of a sustainable ecosystem.

However, the mulberry plant (*Morus spp*.) (Fig. 10.1) belongs to the family *Moraceae* and is widely grown in India, China, and other Asian and American countries. They are being primarily cultivated for leaf foliage production, which is the only source of food for the silk-producing *Lepidopteran* insect *Bombyx mori*

Fig. 10.1 Introduction to *Morus* species and its body parts; (**a**) fully grown mulberry plant, (**b**) apical leaf, (**c**) matured fruit, and (**d**) stem

L. Conventionally, mulberry is being grown through vegetative means like; stem cuttings, grafting, or budding with several limitations. However, a series of factors, including the plant's genetic makeup, age, physiological parameters, and environmental conditions, can affect the efficacy of these strategies. Recent advancements of biotechnological applications like tissue culture techniques and molecular genetic engineering for mulberry crop improvement show satisfactory results in quantitative and qualitative progress with minimum failures (Fig. 10.3). Other possible ways like micropropagation of hard to root genotypes, isolation of somaclonal variants, screening of germplasm for abiotic stress tolerance, induction of polyploids, production of synthetic seeds, and cryopreservation of genetic resources are being adopted since the twentieth century.

In general, India's economy mainly depends on agriculture and agro-based industries due to the availability of land and human resources. But due to some social constrains and urbanization, the scarcity of land is being a serious issue where the farmers are having low cash returns, and the fact that agriculture is limited to one or two seasons each year, villages have turned to support rural enterprises like sericulture (Rai 2006). Sericulture is an agro-based cottage industry compiled with a series of activities like mulberry plant cultivation, maintenance to feed the silkworms, silkworm rearing to produce silk cocoons, reeling the cocoons to unwind the silk filament, yarn production, weaving, and fabric processing (Ahmed and Rajan 2011). In India, the total area of mulberry cultivated land is around 282,244 ha and mulberry silk is mainly produced in Karnataka, West Bengal, Jammu and Kashmir, Tamil Nadu, and Andhra Pradesh. Although some other states like Maharashtra, Odisha, Kerala, and Madhya Pradesh have made some progress in this domain under their development plans. Significantly, the five major mulberry silk-producing states collectively account for more than 80% of the total area under mulberry cultivation and 97% of raw silk production in the country and thus growth of farmer's realization, sericulture is gaining ground in non-traditional areas too.

In mulberry, the optimum crop production depends on the recommended package and practices from the government ministry and here the Central Silk Board is engaged in it. The introduction of high-yielding mulberry varieties, irrigation expansion, uses of fertilizer, and higher cropping intensity transformed Indian sericulture in the 1990s, resulting in the stands in the global silk market. However, the situation is changing now on a parallel track with environmental and climate change issues. Drastically lowering of ground water levels, expensive fertilizers and, most importantly, soil health is worsening as a result of excessive use of inorganics like fertilizers, insecticides, and so on for high crop output. This is a common phenomenon for all agricultural crops, and the mulberry is no exception. As sericulture is an agro-based sector, where it can be a useful industry including mulberry cultivation for rural development since it provides year-round revenue and employment (Lakshmanan and Gethadevi 2007). It also provides farmers with an additional source of revenue and employment (Lakshmanan 2006). Furthermore, the mulberry plant is explored as a medicinal tree as it improves and enhances the life of human beings by their biologically active ingredients present in body parts like leaf, stem, and root (Venkatesh and Chauhan 2008; Bao et al. 2016). Mulberry is presently

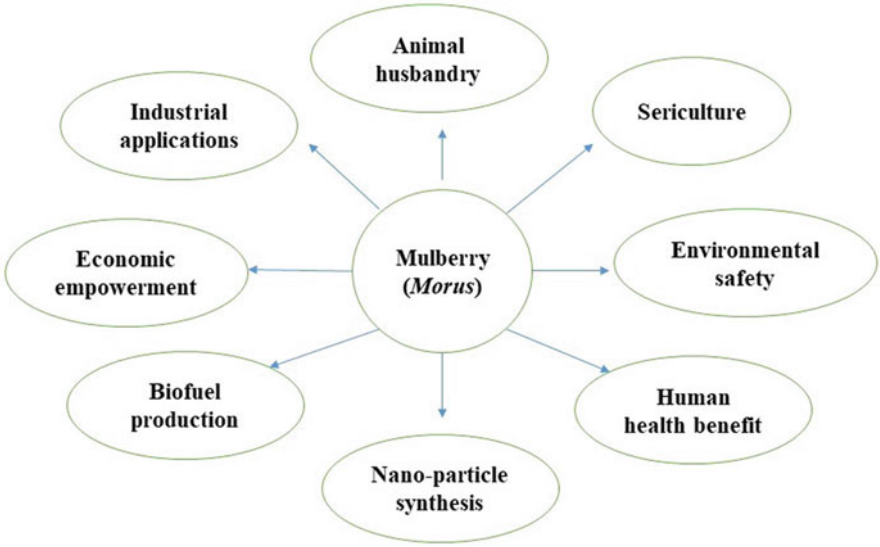

Fig. 10.2 Important domain of *Morus* utilizations

exploited industrially since every part of the mulberry is used in the creation of numerous goods in the pharmaceutical, food, cosmetic, and healthcare industries (Yang et al. 2010; Zhang et al. 2018) (Fig. 10.2).

2 Origin, Diversity, and Importance of Genus *Morus*

Mulberry was originated in the Himalayan foothills and then expanded to Asia, Africa, America, and Europe, among the other places (Yokoyama 1962). It belongs to the genus *Morus* and the family *Moraceae*, and it is classified based on the floral biology and morphological features (Koidzumi 1917; Hotta 1954, and Katsumata 1972), anatomical characteristics such as shape and sizes of cystolith and idioplast, and isoenzyme banding pattern (Metcalf and Chalk 1979). A total of 150 *Morus* species have been identified in the mulberry, but only 68 have been assigned to higher priority, based on their usage in silkworm rearing, medicinal value, and fruit sweetness. Species like, *M. multicaulis*, *M. alba*, and *M. atrpurpuria* are found throughout China's south, north, and west regions (Yong Kang Huo 2000), whereas *M. bombycis* is widely dispersed and widely farmed in Japan's cold regions, and *M. latifolia* is widely distributed and cultivated in Japan's warm regions (Machii et al. 1999). Species *M. macroura* Miq. is abundantly found in northern parts of India (Dhar and Ahsan 1989; Dandin et al. 1993), southern India (Yadav and Pavan Kumar 1996), and in the state of West Bengal (Yadav and Pavan Kumar 1996; Ravindran et al. 1997). *M. alba* L. genotypes are found in Punjab, in the North-

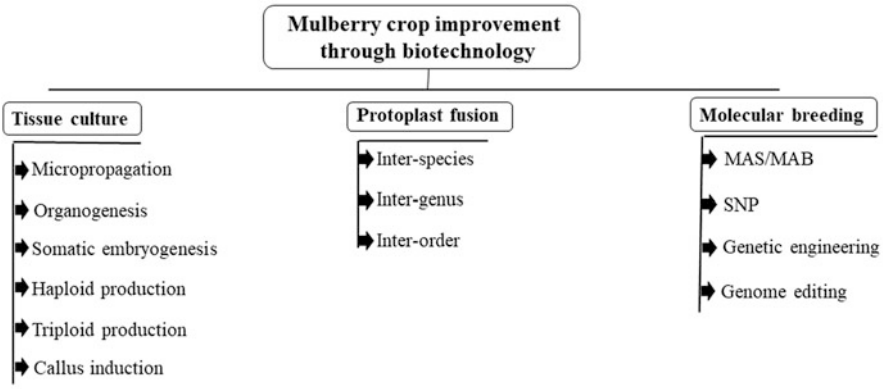

Fig. 10.3 Various approaches of mulberry crop improvement through biotechnology

Western Himalayas, and Western Tibet region (Ravindran et al. 1997). The popular species *M. indica* L. is mostly found in a wide range of habitats, from temperate to subtropical Himalayas, Arunachal Pradesh, Kashmir, and Sikkim, reaching to 2100 m above sea level. *M. serrata* Roxb. is confined largely to the high altitude region of North-Western India (Ravindran et al. 1997), while *M. laevigata* is found across in India in both natural (Andaman and Nicobar Islands) and managed habitats (Tikader and Thangavelu 2003). In India, a Geographic Information System (GIS) was utilized to map the variety diversity of *Morus* species across the plain to the hilly regions (Saraswat et al. 2009). Many accessions (14) belonging to species *M. indica* and *M. alba* were brought from extreme cold regions (Ladakh, Meghalaya, Himachal Pradesh, etc.) in the last few years, and the majority of accessions (75) belonging to *M. indica*, *M. alba*, and *M. laevigata* were brought from extreme dry hot regions (Rajasthan, UP, MP, and Bihar) and kept in CSGRC, Hosur, Karnataka (Ananda et al. 2005, 2011).

However, the common mulberries like *M. alba*, *M. indica*, *M. latifolia*, *M. nigra*, and *M. multicaulis* are cultivated for silkworm rearing, whereas *M. rubra* and *M. nigra* are cultivated for fruits (Yaltirik 1982). *M. laevigata* and *M. serrata* are two wild species utilized for lumber (Tikader and Vijayan 2010). The wood of both species is used to make hockey sticks, tennis rackets, and other items (Yong Kang Huo 2000). *M. alba* leaves are high in protein content and provide a nutritious human diet (Srivastava et al. 2003). Hyperlipemia, constipation, sleeplessness, anti-aging, premonitory, and apoplexy are all treated with syrups and recipes made from *M. alba* fruits (Singh 1997). *Morus* fruits have been discovered to have anti-diabetic and anti-oxidant characteristics, reducing harmful oxidative compounds in the erythrocytes of diabetic rats (Hong et al. 2004). Latex, which is used as a plaster for sores and in the manufacturing of skin ointments, is present in the shoot (Ravindran et al. 1999). The leaf foliage is also fed to cattle, goats, and other animals because it is highly nutritious and palatable to herbivorous animals (Benavides et al. 1994), as well as possessing numerous medical qualities such as anti-oxidants (Yen

et al. 1996) and hypoglycaemial substances (Benavides et al. 1994; Kelkar et al. 1996). Fruit is also grown for human consumption, as well as for the production of jam, jelly, marmalade, frozen desserts, pulp, juice, paste, ice cream, and wine (Koyuncu 2004). Mulberry fruit is a high source of phenolic acids and flavonoids and is used as a traditional treatment for diarrhoea, constipation, hypoglycaemia, and avulsed teeth (Lee et al. 2011; Arfan et al. 2012).

3 Issues of Mulberry Plant Breeding and Foliage Production

In general, plant breeding is an intentional attempt by people to manipulate nature in order to benefit plants' heredity. Such plant modifications are both permanent and heritable types. The desire of humans to improve specific qualities of plants in order to perform new tasks or to enhance existing ones is driving such an attempt to change the status of crop variety. As a result, in a modern scientific society, the terms "plant breeding" and "plant improvement" are frequently interchanged. To produce targeted and directional changes in the nature of plants, the plant breeder employs a variety of breeding tools and approaches. New tools are developed as science and technology evolve, while old ones are honed for use by breeders. Clear breeding objectives are set before beginning a breeding effort, depending on considerations such as producer demands, consumer preferences and needs, and environmental impact. Breeders strive to make the job of crop producers easier and more efficient in a variety of ways.

Mulberry (*Morus*) of the *Moraceae* family is a commercially important tree plant cultivated in India, China, and other Asian countries to feed the larvae of *Bombyx mori* L., a silk-producing *Lepidopteran* insect that converts the mulberry protein morine to sericine and fibroin (Vijayan et al. 2011a, 2012). Mulberry breeding is primarily focused on increasing qualitative leaf foliage productivity, which accounts for more than 38.2% of total sericulture productivity (Banerjee 1998). However, leaf productivity is a multifactorial feature affected by several linked characters such as plant height, number of branches, leaf retention capacity, nodal length, leaf size and weight, and total biomass, enhancing leaf productivity in a difficult way (Doss et al. 2011). The main objectives of mulberry breeding are to develop disease-pest resistant, stress-tolerant quantitative and qualitative genotypes for the sericulture farmers who are directly or indirectly associated with mulberry cultivation. Inbred development is hampered by high heterozygosity and inbreeding depression, and therefore direct breeding hasn't made much progress for mulberry. As a result, heterozygous parents are employed to produce F1 progenies, which are subjected to various genetic evaluation and selection techniques to determine the superior quality of genotypes. Mulberry trees have a high heterozygozity genetic makeup, making it difficult to improve economically important features by conventional breeding and selection but few of them are being popular for the last three decades (Table 10.1).

Table 10.1 Popular high-yielding cultivated mulberry varieties across India (Source: Datta 2000).

Sl. No.	Variety	Cultivated region	Developed by	Origin
1.	Kanva-2	South India irrigated	CSRTI, Mysore	Selection from natural variability
2.	S-36	South India irrigated	CSRTI, Mysore	Developed through EMS treatment of Berhampore local
3.	S-54	South India irrigated	CSRTI, Mysore	Developed through EMS treatment of Berhampore local
4.	Victory-1	South India irrigated	CSRTI, Mysore	Hybrid from S30 × C776
5.	DD	South India irrigated	KSSRDI, Thalaghattapura	Clonal selection
6.	S-13	South India Rainfed	CSRTI, Mysore	Selection from polycross (mixed pollen) progeny
7.	S-34	South India Rainfed	CSRTI, Mysore	Selection from polycross (mixed pollen) progeny
8.	MR-2	South India Rainfed	CSRTI, Mysore	Selection from open pollinated hybrids
9.	S-1	Eastern and NE India irrigated	CSRTI, Berhampore	Introduction from (Mandalaya Mayanmar)
10.	S-7999	Eastern and NE India irrigated	CSRTI, Berhampore	Selection from open pollinated hybrids
11.	S-1635	Eastern and NE India irrigated	CSRTI, Berhampore	Triploid selection
12.	C776	Saline soils	CSRTI, Berhampore	Hybrid from English black and C. multiculis
13.	S-146	N. India and hills of J and K irrigated	CSRTI, Berhampore	Selection from open pollinated hybrids
14.	Tr-10	Hills of eastern India	CSRTI, Berhampore	Triploid developed from "S1"
15.	BC-259	Hills of eastern India	CSRTI, Berhampore	Back crossing of hybrid of Matigare local × Kosen with Koren twice
16.	Goshoerami	Temperate	CSRTI, Pampore	Introduction from Japan
17.	Chak Majra	Subtemperate	RSRS, Jammu	Selection from natural variability
18.	China white	Temperate	CSRTI, Pampore	Clonal selection

Due to the genetic drag and the difficulty of eradicating the bad agronomic traits arise, such type of conventional breeding system prevents favourable traits from being introgressed from wild relatives or species. In such cases, advanced biotechnological tools such as plant tissue culture, transgenesis, allow the introduction and overexpression of desirable genes (Vijayan et al. 2011a), or even RNA interference (RNAi) technology, which allows for the knockout of undesirable genes, are viable

options for improving specific traits without disrupting current trait combinations in mulberry (Vijayan et al. 2011b).

4 Uses of Biotechnological Tools for Mulberry Crop Improvements

The primary goal of mulberry breeding is to create a new variety or genotypes with higher yields, quality, drought, disease, and insect resistance. Biotechnological approaches including plant tissue culture and genetic engineering were established as a useful tool of mulberry crop improvement in the last three decades (Fig. 10.3). Mainly, the plant tissue culture techniques played a significant role in mulberry crop improvement within a very shorter period which ultimately benefitted the sericulture professionals. The use of novel important gene(s) and making transgenic mulberry is successfully established (Table 10.3), but the viability of such transgenic genotypes in the commercial market is poor due to some limitations. Besides that, molecular DNA marker-assisted breeding in mulberry is also well established where single nucleotide polymorphism (SNP) technique and other molecular DNA markers showed optimum positive outcomes (Table 10.2).

Table 10.2 Application of molecular markers in mulberry (Source: Vijayan et al. 2014)

Sl. No.	Marker	Work	Reference
1.	RAPD[a]	Initiated use of molecular markers in the systematic of *Morus*	Xiang et al. (1995)
2.	RAPD	Genetic diversity among mulberry accessions collected from South India	Naik et al. (2013)
3.	AFLP	Elucidated the interrelationships of different *Morus* species	Sharma et al. (2000)
4.	AFLP	Genetic diversity among cultivars of Hunan province	Huang et al. (2009)
5.	ISSR	Genetic relationship between Japanese and Indian mulberry genotypes	Vijayan et al. (2003)
6.	ISSR	Testing the quality of genomic DNA extracted with a new protocol	Anuradha et al. (2013)
7.	SSR	Developed 10 microsatellite primers pairs and tested in 27 mulberry accessions, which could differentiate wild mulberry accessions from cultivated ones	Zhao et al. (2005a)
8.	ITS & *trn*L-F	Phylogenetic relationship among 13 mulberry genotypes belonging to nine species was developed	Zhao et al. (2005b)
9.	SRAP	Genetic relationship among germplasm accessions	Zhao et al. (2009)

[a] *RAPD* random amplified polymorphic DNA; *ISSR* intersimple sequence repeats; *AFLP* amplified fragment length polymorphism; *SSR* simple sequence repeat; *ITS* internal transcribed spacer; *trnL-F*; *SRAP* sequence-related amplified polymorphism; *trnL-F* is a non-coding region of the chloroplast genome used as a marker for DNA barcoding

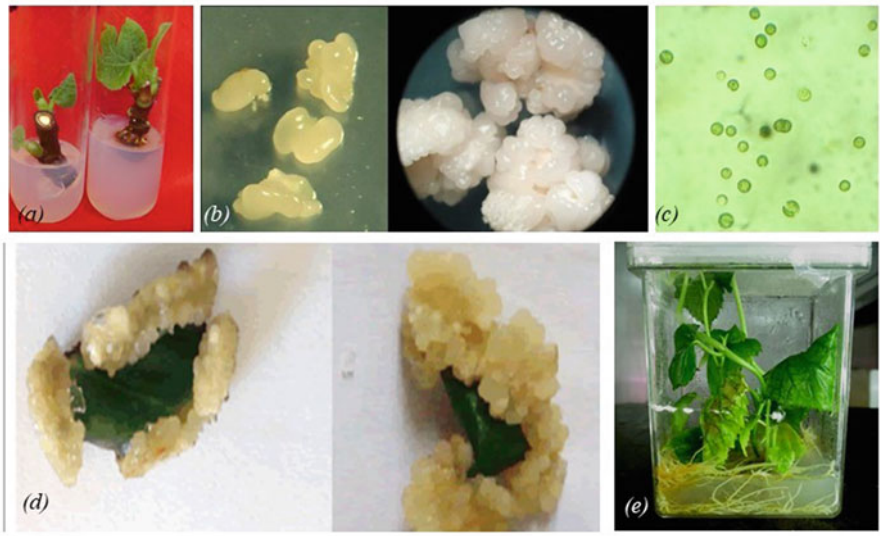

Fig. 10.4 Pictures showing various types of plant tissue culture in mulberry. (**a**) micropropagation of shoot tip, (**b**) freshly isolated somatic embryo and its culture, (**c**) isolated mesophyll protoplast, (**d**) callus induction from leaf disc, and (**e**) in vitro regenerated plantlet through apical shoot culture

4.1 Mulberry Tissue Culture

Mulberry tissue culture technology has evolved and ramified into various fields, such as micropropagation, callus culture, organogenesis, genotypic screening for stress tolerance, polyploid induction, cryopreservation, and transgenesis. Apart from the mentioned, somatic embryogenesis, protoplast technology, haploid culture, synthetic seed production, etc. also found viable in mulberry (Fig. 10.4).

4.2 Micropropagation

Micropropagation is the process of multiplying and/or regenerating plant material in vitro under aseptic and controlled environmental conditions to create thousands or millions of plantlets for field transplantation. Mulberry can be spread conventionally by stem cuttings, grafting, or budding by vegetative means. The success of these methods, however, depends on a series of factors such as mulberry's genetic composition, age, parental physiological conditions, climatic conditions, and cultural practices. Moreover, newly developed mulberry varieties cannot be immediately propagated by stem cuttings since it takes at least 6–7 months for cuttings to be isolated from the parental plant (Kapur et al. 2001). On the other hand, micropropagation permits the plant to propagate under controlled conditions over a short period. In addition, each stem cut creates only one plant by conventional

propagation method by stem cuttings; however, hundreds of plants can be created via micropropagation from one plant composition called "explant". In addition, micropropagation can give rise to seedlings during the entire year, regardless of the seasonal changes. Thus, micropropagation in a relatively short time and limited temporal space is a cost-effective approach for the quick multiplying of mulberry. The generation of non-virus plants in apical meristematic tissues is also facilitated by micropropagation. Micropropagation by restoring complete plants from axillary buds was initiated in *M. alba* by Ohyama (1970), and from nodal explant and shoot apex culture (Sengupta et al. 2016, 2017). Later, several researchers employed various mediums and explants to regenerate plantlets including shoot tips that were discovered suitable for mulberry micropropagation and dormant axillary sprouts.

4.3 Organogenesis in Mulberry

Organogenesis is a complex regeneration process that involves the development of new organs from scratch materials. A variety of criteria including a proper selection of explants, age, compositions of culture growth media, use of specific plant growth regulators, genotype, carbohydrate sources, gelling agents as well as other physical elements (including lighting, temperature, humidity, and other environmental factors) influence on successful organogenesis. Plantlets regeneration may take place directly or indirectly depending on these conditions (Jain and Datta 1992). In direct organogenesis, plantlets develop straight from explants with no intermediate callus formation, while plants develop through the creation of calluses in indirect organogenesis. Callus induction is influenced by a variety of parameters, including the nature of the explants, genotype, medium, and composition. In order to initiate callus production in mulberry trees, several explants have been tested. Cambial regions (Narasimhan et al. 1970), hypocotyls segments (Shajahan et al. 1997), cotyledons (Thomas 2003), stem segments (Vijayan et al. 1998), and young leaves (Chitra and Padmaja 2005) have all been successfully tested, and it has been discovered that the internodal segment from a young shoot is the best explant for callus development (Vijayan et al. 2011b) along with the addition of 2,4-D hormone best suited for it.

4.4 Somatic Embryogenesis

Somatic embryogenesis is an important method for speeding up the genetic improvement of commercial crop species (Stasolla and Yeung 2003). Several research groups had attempted to induce somatic embryogenesis in mulberry, but their success rate is low. *M. alba* was used by Shajahan et al. (1995) to obtain heart-shaped embryos using hypocotyl segments grown on MS medium supplemented with 2,4-D (0.45–4.52 μM) and BAP (2.2 μM). By growing zygotic embryos on MS media supplemented with 0.05 mg/L 2,4 - D + 0.1 mg/L BAP along with 6 percent of

sucrose, primary and secondary somatic embryoids were developed in mulberry by Agarwal (2002) and Agarwal et al. (2004). Somatic embryogenesis has not been successfully established in mulberry that might be applied in the field of crop improvement. This phenomenon is due to the difficulties of hormone directing growth regulations restricted to the production of adventitious branches and roots, like in other agricultural crop plants. To make somatic embryogenesis successful in mulberry and to make them viable in the commercial market, concerted efforts are required.

4.5 Haploid Production

In general, due to the gametophytic nature, only haploid plants have the half number of chromosomes present in their parents. They can be used to generate homozygous lines, which are extremely useful in any type of crop breeding programmes, particularly for tree crops with a long generation cycle and found high heterozygozygosity in the progeny level. This approach has been widely employed in most agriculturally significant plant species since the first successful report on regeneration of haploid plants from pollen grains through the cultivation of datura anthers (Guha and Maheshwari 1964). However, a few reports had been published on tree species. In the case of mulberry, the first haploid production was made though anther culture by Shoukang et al. (1987) and later by Katagiri (1989), Sethi et al. (1992) and Chakraborti et al. (1999), but unfortunately no plants could be regenerated till date. Nonetheless, Thomas et al. (1999) successfully regenerated gynogenic haploids by culturing immature female catkins of mulberry on MS medium supplemented with growth hormones like; BA (2.0 mg/L) and 2,4-D (1.0 mg/L) during the first 3 weeks' culture, and the remaining period with 2,4-D (1.0 mg/L), amino acids glycine (88.8 mg/L), and proline (15 mg/L) for optimum results. However, no further report has been made on haploid production in mulberry though doubled haploidy technique is of much use in mulberry breeding.

4.6 Protoplast Isolation, Culture, and Regeneration of Plantlets

Somatic hybridization via protoplast fusion has opened up a new path for the introduction of important traits into the novel hybrid generation that isn't able to get through traditional breeding (Johnson and Veilleuz 2010). Only a few studies on mulberry plant regeneration from protoplasts and successful viable protoplast culture have been published (Tewary and Sita 1992; Umate et al. 2005; Mallick et al. 2016). The enzyme mixture of 2% cellulase, 1% macerozyme, and 0.5% macerase was found optimum for obtaining viable protoplasts (Mallick et al. 2016). Chemical

fusogen like poly-ethylene-glycol (Ohnishi and Kiyama 1987) and electro-fusion were used to make a successful fusion of mulberry protoplasts (Ohnishi and Tanabe 1989). Although the protoplast isolation and regeneration were successful, somatic hybrid development in mulberry was not. As a result, efforts in this direction must be continued.

4.7 Applications of Molecular Techniques in Mulberry Breeding

Application of molecular techniques for mulberry crop improvement found more specific and target oriented since last two decades. For many years, morphological or yield attributing factors have been used to evaluate mulberry germplasm and genotypes as a pre-breeding study (Vijayan et al. 1997). Mostly, the agronomical traits fluctuate substantially depending on the various developmental phases and are influenced by the environmental conditions, and on the other hand, field data collection is time-consuming and labour intensive. As a result, highly polymorphic, multiallelic, non-epistatic, neutral, and insensitive to environmental influence molecular markers have been widely utilized for genotyping, identifying desired genes of interest, and genetic evaluation of mulberry germplasm resources (Awasthi et al. 2004; Vijayan 2010). Modern molecular techniques allow for the precise identification of usable genotypes with desired agronomic traits that may be used in any breeding programme, as well as a detailed image of the parent–offspring relationship towards positive outcomes (Vijayan et al. 2006).

4.8 Use of DNA Molecular Markers in Mulberry

DNA markers are widely employed in plant kingdom for screening, genotyping, gene identification, germplasm characterisation, and other genetic investigations since they are not impacted by the environment, particularly in DNA fingerprinting, marker-assisted selection (MAS), and genome mapping (Vos et al. 1995; Yildirim and Kandemir 2001). DNA markers have acquired favour among conservationists, geneticists, and breeders for assessing species variety because of their stability and convenience of generating maximal information in a very short amount of time (Table 10.2). Because they are immune to environmental impacts, PCR-based markers have the ability to screen a large number of samples fast to elucidate correct genetic information. Only a few papers on the genetic analysis of wild mulberry species are known. Restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RADP), amplified fragment length polymorphism (AFLP), inter simple sequence repeat (ISSR), simple sequence repeats (SSR), and single nucleotide polymorphism (SNP) are some of the DNA markers available and

found reproducible in plant genome analysis (Lusser et al. 2012). Among all, MAS has many advantages over traditional breeding methods, including the ability to choose productive hybrids at the seedling stage and the elimination of highly difficult field trials, particularly against diseases and pests. By allowing early selection of suitable plant genotypes, MAS can reduce the time necessary for mulberry genetic improvement (Vijayan 2007). Awasthi et al. (2004) used RADP and ISSR techniques to characterize 15 mulberry varieties (12 domesticated and 3 wild) where they successfully amplified a total of 128 polymorphic RAPD bands from 19 primers and 93 polymorphic ISSR bands from 4 primers, indicating that both RAPD and ISSR techniques can be used to characterize and study about the genetic diversity of mulberries. Vijayan et al. (2004) also used 15 RAPD primers and 15 ISSR primers to examine a total of 19 mulberry genotypes from different species and found that 86 percent of RAPD bands and 78 percent of ISSR bands were polymorphic. Orhan et al. in 2007 used 16 RAPD markers derived from 101 primers to identify the genetic relatedness of 15 white mulberry genotypes, claiming that genetically different genotypes might be used in future breeding experiments. Other molecular markers like SSR are used to distinguish between the Indian mulberry genotypes viz., *M. indica* L and *M. alba* L., and Japanese mulberry genotypes viz., *M. latifolia Poir* and *M. bombycis* (Vijayan et al. 2003). The genotypes *M. serrata* and *M. laevigata* could also be differentiated using these markers; however, the genotypes of *M. indica* and *M. alba* could not be split into discrete groups; this could be due to the mingling of gene pools of these two species (Vijayan et al. 2006). Progeny selection is usually a tough process because the expression of agronomic traits varies and it takes at least 10 years for the plant to fully express all of its traits (Vijayan et al. 2006). The RADP technique may be used to determine the parent–offspring connection in both inter-specific and intra-specific mulberry hybrids, as well as analyse genomic DNA differences in breeding materials and make selections from both inter-specific and intra-specific hybrid materials (Tikader and Kamble 2009). This RAPD approach allows for precise genotype identification in breeding programmes (Orhan and Ercisli 2010). SSR and SNP markers are the most suitable molecular markers for the genetic characterization of mulberry germplasm due to their repeatability, robustness, and informative contents (Tikader and Kamble 2009). Mulberry is heterozygous; therefore, hybrids show a lot of variation. Using molecular markers in conjunction with traditional breeding should allow for a more precise evaluation and selection of superior hybrids (Tikader and Kamble 2009).

4.9 Transgenesis in Mulberry

The use of good genes and insertion into the mulberry towards the development of qualitative traits was initiated by Sugimura et al. 1999, where they successfully established the transformation process in mulberry by introducing *GUS* marker gene (GUS: β-glucuronidase) into protoplast. Later on, several researchers worked on different genes in mulberry like *AlaBlb*-soyabean glycine gene; *bch-L* inhibitor

Table 10.3 Transgenesis in mulberry (*Morus* spp.) (Source: Vijayan et al. 2011a, 2011b)

Sl. No.	Gene	Expression profile	Reference
1.	*WAP21*[a]	Cold tolerance	Ukaji et al. (2001)
2.	*GUS*	GUS incorporated into the protoplast through electroporation	Sugimura et al. (1999)
3.	*Agrobacterium rhizogenes*	Hairy roots	Oka and Tewary (2000)
4.	*COR*	Cold tolerance	Ukaji et al. (2001)
5.	*AlaBlb*	Salinity tolerance	Wang et al. (2003)
6.	*OC*	Insect resistance	Wang et al. (2003)
7.	*SHN 1*	Drought tolerance	Aharoni et al. (2004)
8.	*HVA1*	Drought and salinity stress	Lal et al. (2008)
9.	*bch1*	High-temperature tolerances	Das (2009)
10.	*NHX*	Drought and salinity stress	Khurana (2010)
11.	*HAL3a, dehydrin*	Abiotic stress	Das et al. (2013)

[a] *AlaBlb* soyabean glycine gene; *bch-L* inhibitor 2-aminobicyclo-(2, 2, 1)-heptane-2-carboxylic acid; *COR* cold on regulation; *GUS* β-Glucuronidase; *HVA1 Hevea brasiliensis* abiotic stress gene; *NHX* Na+/H+ exchanger; *OC* osteocalcin; *Osmotin* osmotic stress-induced gene; *SHN 1* schnurri from *Drosophila melanogaster*; *WAP21* water allocation plan

2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid; *COR*-cold on regulation; *HVA1*-Hevea *braziliensis* abiotic stress gene; *NHX-Na+/H+* exchanger *OC*-osteocalcin osmotin-osmotic stress-induced gene; *SHN 1*-schnurri from *Drosophila melanogaster*; *WAP21*-water allocation plan (Ukaji et al. 2001; Oka and Tewary 2000; Wang et al. 2003; Aharoni et al. 2004; Lal et al. 2008; Khurana 2010; Das et al. 2013) (Table 10.3). Though these experiments were successful, no transgenic mulberry is commercially available in the market to date as field trials need huge time to release them as varieties.

5 Sustainability and Eco-friendly Mulberry Cultivation

Rapid industrialization, combined with rising population, has had a negative impact on the environment by means of population density. Natural calamities, global warming, disasters such as floods and droughts, abrupt soil erosion, and a high rate of a disease outbreaks are some of the phenomena thought to have direct links to environmental pollution. Mulberry has a number of unique characteristics that have been linked to its ability to reduce pollutants in the environment (Jian et al. 2012). Mulberry plantations, for example, have shown promising outcomes in terms of

improving air quality, boosting water retention capacity, removing heavy metals from polluted soils and the environment, and fostering healthy soil microflora and fauna (Lu et al. 2004; Yao et al. 2004; Chen et al. 2006). This section demonstrates the value of the mulberry tree in terms of reducing the problems that have arisen as a result of pollution.

5.1 Air Quality Improvement

Mulberry trees are considered as excellent carbon sink plants, which helps to improve the atmospheric air quality. Every year, a single mulberry tree is expected to take 4162 kg of carbon dioxide and emit 3064 kg of oxygen in nature. Mulberry leaves also have a high absorption capacity of air contaminants such as chlorine, hydrogen fluoride, and sulphur dioxide, among others. Mulberry leaves were shown to be unaffected by greater levels of chlorine pollution in the atmosphere, indicating that the mulberry plant has a natural resistance to chlorine (Lu et al. 2004).

5.2 Adaptability to Soil and Water Conservation

Mulberry trees have a high level of soil adaptation capabilities. They may grow in deep, permeable, fertile soil as well as barren, nutrient-depleted soil too (Han 2007). Mulberry has a well-developed deep and branched root system where it forms a tangled and dense network in the soil environment. Mulberry trees are excellent in reducing sand storms and conserving water and soil. In comparison to the typical planting pattern system, it is projected that the runoff water co-efficient in mulberry cultivation can be lowered by 10–20%. Mulberry tree hedgerows were also found to have a significant impact on overall runoff and nutrient enrichment ratios (Du et al. 2001; Shi et al. 2005).

5.3 Water Requirement and Genetic Plasticity

Mulberry, for example, may thrive in arid and semi-arid deserts with an annual rainfall of less than 300–600 mm. According to Dai et al. (2009), mulberry has a stronger ability to adapt in desert areas even in annual rainfall less than 150 mm. According to research reports, adult mulberry trees were shown to be able to withstand a 20 days' flood during their growth stage. Among other xerophytic plants, this is extremely rare. Mulberry trees have a high resistance to water logging when they are under dormant conditions. They have been shown to have a high level of genetic plasticity, allowing them to adapt to a variety of agro-climatic conditions even the most resistant to cold are dormant mulberry trees (Yao et al. 2004; Chen

et al. 2006). Mulberry's hardiness is demonstrated by its distribution pattern worldwide, which ranges from temperate to tropical regions.

5.4 *Role of Mulberry in Phytoremediation*

One of the most successful strategies for removing heavy metals from soils is phytoremediation. Mulberry has the capacity to clean up soils that have been contaminated with heavy metals (Zhou et al. 2015). An established mulberry's root system aids in the absorption of soil nutrients and is also conducive to the absorption of metals in the soil to some extent (Jothimani et al. 2013). Heavy metals like lead, cadmium, and copper are mostly remediated by mulberry species. It has been reported that the deposition of mercury heavy metal in leaf, stem, and root sections of *Morus nigra* has been examined by Hashemi and Tabibian (2018). There was a higher level of mercury absorption in root components than in leaf and stem sections.

5.5 *Eco-Friendly Activity*

Mulberry, being a perennial crop with good leaves, contributes to soil conservation and offers greenery, making sericulture an environmentally acceptable activity. Silkworm waste can be recycled and used as garden fertilizer. Mulberry plantation development programmes are mostly focused on upland locations where underutilized cultivable land is turned into productive land.

6 Mulberry in Economic Empowerment Through Income Generation

In terms of job creation, sericulture is a component of the country's agricultural activity where moriculture, i.e. mulberry cultivation and foliage production is a major part. Compared to the other agricultural industries, it creates more job prospects, particularly in rural and semi-urban areas. Sericulture, in all of its forms, is a labour-intensive enterprise that starts from mulberry cultivation to raw silk production. For every kilogramme of raw silk production, it can employ up to 11 skilled people where more than half of them are women (Rama Lakshmi 2007). Indirectly or directly, mulberry plantations create jobs for farmers. Mulberry is not only used in sericulture, but also other industries for employment and money transduction. It has been reported that the leaf, stem, and root components of the mulberry are used in animal husbandry, food processing, cosmetics, dyeing, and

pharmaceutical industries and thus huge employment opportunities are made for the youth.

6.1 Integrated Farming System with Mulberry

To boost up food production for human consumption, the integrated farming system integrates mulberry sericulture to agriculture, animal husbandry, poultry, and fish aquaculture (Astudillo et al. 2014). In many agricultural practices, grass can be grown as an intercrop to provide feed and fodder for domesticated animals, resulting in meat and milk production (Doran et al. 2007). Likewise, mulberry can also be intercropped with other cash crops, vegetable crops, and medicinal plants to generate additional benefits. The horticultural crop mushrooms can be produced using dried leaves of mulberry trees as a substrate during the autumn seasons (Hugar et al. 2016). As a protein supplement, dried leaf powder of mulberry is used for poultry feedings and even used for biofuel production in India (Guha and Reddy 2013). Mushroom, fodder, veggies, etc. are all products of the integrated farming system associated with the mulberry tree. These benefits include increased food production, a boost to the rural economy and an increase in household nutrition. The system also has the potential to create new jobs across the rural sectors (Reddy et al. 2008). Such an integrated farming system (IFS) has a specific significance in India because of its potential to improve the socio-economic condition of economically weaker parts of the society and rural populations, especially tribal groups, which depend heavily on natural resources.

6.2 Low Gestation

Sericulture activities demand a small initial expenditure to get started. Mulberry needs only 6 months to develop for silkworm rearing, and once planted, mulberry will continue to support silkworm rearing year after year for 15 to 20 years, depending on the management practices. A farmer can earn up to Rs. 30,000 per acre per year by following the prescribed package of practices. These include the use of disease-pest resistant saplings for mulberry cultivation, timely irrigation, fertilizer application, pruning and foliage harvesting. Because of the low gestation and higher yields, sericulture is an ideal programme for the poorer sections of society. In the state's sericulture sector, acres of mulberry garden and silkworm rearing can reduce labour costs and save pay.

6.3 High Returns and Women Empowerment

Sericulture offers enormous economic prospects to the women in rural sectors, particularly in the mulberry production, and reeling tasks. In India, 60 percent of women work in sericulture's downstream activities. In rural India, women engage in a wide range of economic occupations. No caste, creed, gender, or religion has been shown to be discriminated against by sericultural activities. Rich and poor sericulture farmers obtain the same money from this industry, which is a notable aspect. A key role in sericulture for women gives chances and makes them independent on a social, economic, political, and other level (Geetha and Indira 2010, 2011; Goyal 2007; Pillai and Shanta 2011; Sengupta et al. 2020).

6.4 Income Generation

Sericulture is an income-generating agro-enterprise in the countries where agriculture is the main domain of economic development like India. It aims to reduce poverty by increasing rural women's employment and income in every steps. The source of income is not only from raw silk production, but also for mulberry plant selling to the pharma industries for drug, supplements, and cosmetics production. Thus, solely one can be economically benefitted from mulberry production even without rearing of silkworm. As a result, it is a prospective agricultural practice for improving the economic condition of the rural farmers to generate foreign revenue (Thapa and Shrestha 1999).

7 Prospect and Future Scope

Mulberry plant has been widely recognized as a feeding source for silkworms (*Bombyx mori* L.) for hundreds of years. However, as previously stated, this plant is now regarded as a multipurpose plant, as it is used in environmental safety management, human health benefits, and animal husbandry through excellent milk production and better meat production. Mulberry has a significant role in environmental clean-up through bioremediation of polluted environmental components like land, air, and water and carbon sequestration. As a result, the respective authorities should recommend this plant species for plantation drives across cities/urban areas (as it can be cultivated globally) along with road sides and in social forestry for increasing green cover and reducing pollution. So, as ecological safety and sustainable development have become increasingly significant components for global security, use of mulberry trees should be considered in addressing such ecological challenges. Similarly, academics and industrialists should place a greater emphasis on exploiting the mulberry on a broad scale for human health advantages, economic

development, and environmental protection. By taking into account all of the facts about mulberry, such as its role in the sericulture industry, human health promotion, soil conservation, ecoremediation of degraded lands, bioremediation of polluted spheres, carbon sequestration, animal husbandry, nanoparticle synthesis, industrial utilization, and people's economic empowerment, it can be considered a valuable resource.

Being an economically important plant, mulberry should be improved through biotechnological approaches. Few works have been done so far, but still many more to be explored. Qualitative and quantitative improvement through molecular breeding or even the development of transgenic is very highly essential at the commercial point of view. Pharma industries prefer tissue culture and micropropagation to produce an optimum number of disease-free plantlets within a shorter period of time. Unfortunately, commercial transgenic mulberry is not viable in the global market and hence focus should be on it.

8 Conclusion

Sericulture has risen to prominence as the most important cash crop, with little initial investment, a short gestation period, strong employment potential, and a high return on investment. As a result, one of its primary potentials, particularly for the rural people, is livelihood generation and upliftment of economic condition. Mulberry cultivation and foliage production are simple for everyone in the society, whether a large farmer or a landless elderly person, male or woman can engage themselves. It entails simple technology to comprehend and implement if the formers follow the recommended package and practices of the Central Silk Board (CSB), Ministry of Textiles, Govt of India. So, it has generated downstream employment and income in rural and semi-urban areas, with a high participation rate among low-income and socially disadvantaged populations.

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Chapter 11
Assessing the Genetic Stability of In Vitro Raised Plants

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Abstract Stem cuttings-based vegetative propagation is considered to be genetically clonal in nature; but due to lower rooting abilities in most of the plant species, it has become much difficult to propagate the commercial/important plants through stem cuttings. Because of this limitation; in vitro micropropagation of medicinal and commercial plants has gained popularity. But during in vitro culture, the explants are cultured on artificial media with synthetic growth substances and treated with artificial hormones, there are chances of genetic changes as the cells of callus are fragile in nature leading to the production of genetical variants. Hence, it has become a mandatory step in tissue culture-based propagation to confirm the genetic fidelity of regenerated plantlets using different types of markers (phenotypical, biochemical, physiological, and molecular markers). The biochemical and physiological markers are not much reliable as one has to wait for regenerated plants to gain maturity to express biochemical and physiological characters. Whereas the molecular markers-based genetic stability assessment is considered as an advanced, accurate, and reliable method. Moreover, it can be carried soon after hardening or acclimatization of micropropagated plantlets. This chapter deals with the use of different kinds of conventional and advanced molecular markers which are being utilized for assessing the genetic fidelity of commercially propagated in vitro plantlets of horticulture and

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plantation crops such as coffee, tea, blueberry, banana, papaya, sweet potato, mulberry, pineapple, date, grapes, cocoa, arecanut, cashewnut, sugarcane, potato, eucalyptus, poplar, pine, rubber tree, cassava, and oil palm.

Keywords Acclimatization · Genetic stability · Hardening · Micropropagation · Molecular markers

1 Introduction

In vitro culture-based micropropagation of plants has gained a lot of importance over the last three decades as this technique is considered as a viable tool for large-scale production of commercially important plants of medicinal, horticulture, plantation crops, and pharmaceutical importance (Thorpe 2007). Similarly, several plant species which are under vulnerable, threatened, and endangered status are being multiplied and conserved through the in vitro culture-based techniques. Advantages of tissue culture-based propagation include raising of plants under aseptic and controlled conditions with respect to temperature, pH, light, and humidity. The controlled conditions of in vitro propagation make available the culture a specific environment favorable for the growth and development of plantlets in a faster and easier way than the multiplication under in vivo or natural conditions (Garcia et al. 2010).

In vitro culture-based propagation is also considered as a good alternative to conventional propagation as the rate of multiplication is higher under in vitro conditions. Since the in vitro propagation is carried out in closed vessels or culture tubes under sterile and controlled conditions, the incidence of diseases and pests are rare (Naik and Buckseth 2018). The major advantage of this technique is the production of large number of plants in a limited space and time using culture vessels and artificial media possessing essential minerals and growth-promoting substances such as plant growth regulators (Akin-Idowu et al. 2009). Millions of plantlets could be raised using small explants around the year irrespective of the season and outside conditions via plant tissue culture techniques.

Other applications of tissue culture-based propagation includes the production of virus-free plants through meristem tip culture (Garcia et al. 2010; Chatenet et al. 2001), somatic embryogenesis-based mass propagation (Xiangqian et al. 2002; Rohela et al. 2016), production of disease-free plantlets (El-Dougdoug and El-Shamy 2011), and preservation and multiplication of plant genetic resources in the form of synthetic seeds (Kulus 2019; Sharma et al. 2019a, b). Apart from in vitro propagation and conservation, tissue culture is also playing a vital role in the production of medicinally important phytochemicals and secondary metabolites through cell suspension cultures-based precursor, treatment-based adventitious root cultures, and utilization of omics-based tools (genomics, proteomics, and transcriptomics) in tissue cultures of model plants for identifying important genes of morphogenesis and somatic embryogenesis (Paul and Kumaria 2020; Rohela et al. 2021; Gautam et al. 2021).

In micropropagation studies, any plant part which is used as a sample for raising the plantlets under in vitro conditions are known as explants. Among the various types of explants, the somatic tissue-based explants are considered as ideal explants as they possess meristematic cells which adopt well under artificial conditions, respond well to the artificial media and perform the cell division in lesser time (Hussain et al. 2012). They also show the morphogenetic response of caulogenesis and rhizogenesis with the amended of plant growth hormones in culture media (Sagare et al. 2000). Among the various types of plant growth regulators, cytokinins and auxins are most widely used for obtaining morphogenetic response of shoot regeneration and root induction. Higher concentrations of cytokinins usually result in the formation of shoots and the high concentrations of auxins help in root formation (Ravi et al. 2012; Sujatha et al. 2013; Rohela et al. 2018b).

In many commercially important plants, there are specific genotypes that are high yielding, but their seed-based progeny is lacking those economical traits. In order to make clonal propagation of those economically important genotypes, usually stem cuttings-based clonal propagation is mostly recommended (Waman et al. 2019). But, most of those genotypes are genetically poor rooters and hence stem cuttings-based propagation is very difficult; under these circumstances, plant tissue culture-based clonal propagation through the leaf, nodal, and shoot tip-based explants has gained more importance over the last three decades (Hussain et al. 2012; Khan et al. 2015). Explants like leaf, node, internode, petiole, and shoot tips are mostly chosen by plant biotechnologists for micropropagataion studies, as these explants-based micropropagation results in the production of plantlets which are most probably genetically identical to the mother plants. However, it is an essential step to confirm the genetical stability of micropropagated plantlets (Martins et al. 2004).

With much progress in in vitro culture-based micropropagation of plants, several new types of synthetic chemicals are being utilized as plant growth regulators in promoting the cell divisions and to carry out efficient regeneration. However, the introduction of newly introduced synthetic chemicals in plant tissue culture may pose problems with regard to the genetical clonal nature of in vitro propagated plantlets. Hence, there is a much need to carry the assessment of genetic stability of in vitro raised plantlets to ensure the production of genetically true clones of commercial plants.

2 Need for Genetic Fidelity Assessment of In Vitro Micropropagated Plants

In recent years, there are several reports about the abnormality of in vitro raised plants with that of mother plants with regard to their genomic integrity (ploidy level, aneuploidy, and somaclonal variations with altered gene sequences). In tissue culture studies when explants and callus cells are passaged through the regeneration phase, there is a chance of easy entry of synthetic chemicals into the fragile natured callus

cells due to which the resulting in vitro-derived plantlets are more likely to develop epigenetic changes which results in mutations or change in ploidy level or induce aneuploidy. These chromosomal or genetic abnormalities in in vitro culture-derived plantlets arise due to chemical-induced molecular phenomenons like gene rearrangements, point mutations, DNA methylation, mitotic abnormalities such as abnormal mitotic phases, chromosomal misalignments during divisions, chromosomal breakups, and failure of spindle fibers (Phillips et al. 1994; Truta et al. 2011).

Certain synthetic chemicals and plant growth regulators which are amended with plant tissue culture media are reported to cause the mutations and result in genetical changes of regenerated plantlets (Zheng 1991; Cecchini et al. 1992). Among the different plant growth regulators used by the researchers, 2,4-dichlorophenoxy acetic acid (2,4-D) was mostly reported to induce abnormalities in the regenerated plants at gene and chromatin level as well (Kumari and Vaidyanath 1989; Pavlica et al. 1991; Pescador et al. 2008; Ozkul et al. 2016; Garcia et al. 2019). Other plant growth regulators like 1-naphthalene acetamide are also reported for their genotoxicity effect on the in vitro regenerated plants (Kocaman and Güven 2015).

The in vitro culture also involves exposure of explants and callus to an artificial light sources which is also a potential source of inducing the alterations in the genetic makeup of plant cells. Due to the above facts, the micropropagated plantlets are likely to contain genetic alterations; hence, they are no longer genetically true copies of donor genotypes (Rani and Raina 2000). Genetic instability is considered as one of the major drawbacks of in vitro culture-based propagation (Ray et al. 2006). In in vitro cultural studies, for mass propagation of commercial plants, the efficiency of regeneration is of prime importance but perhaps even more important is maintaining the genetic integrity and homogeneity of in vitro regenerated plants with donor genotypes (Haisel et al. 2001).

Even though the regenerated plantlets may be phenotypically similar to donor plants, it doesn't necessarily imply their genetic similarity with mother plants (Larkin and Scowcroft 1981; Bahmankar et al. 2017) and therefore it is necessary to assess the genetic fidelity of in vitro raised plantlets at the molecular level by using various techniques. Hence, it has become an essential step to carry the genetic homogeneity studies of regenerated plants to determine their genetical clonal nature with that of mother plant (Alizadeh and Singh 2009). The genetical characterization of tissue culture-derived plantlets will ensure the production and supply of true clones of the commercial plant varieties to farming communities at commercial level.

To assess the genetic stability of in vitro micropropagated plantlets, several techniques are in usage such as phenotypical, biochemical, physiological, and molecular markers (Miguel and Marum 2011; Bandupriya et al. 2021). The phenotypical variations obtained in the regenerated plants may not be exact due to genotypical changes, and it may also be due to epigenetic changes that occurred during the in vitro cultural stages such as callus and somatic embryogenesis (Vogt et al. 2008). The biochemical and physiological markers are also not much reliable; as one has to wait for regenerated plant's maturity to express biochemical and physiological characters. Whereas the molecular markers-based genetic stability

assessment is considered as most advanced, accurate, and reliable type. More importantly, it can be carried at an early stage soon after hardening or acclimatization of micropropagated plantlets (Sharma et al. 2014; Korra et al. 2017).

On the other hand, the genetic fidelity analysis by molecular markers-based polymerase chain reaction methods are mostly in use recently (Mujib et al., 2017; Raji et al. 2017; Zafar et al. 2019; Malik et al. 2020; Syeed et al. 2021). Compared to phenotypical, biochemical, and physiological markers, molecular markers-based genetic fidelity studies are more accurate and reliable. In this chapter, the different types of molecular markers used for the assessment of genetic fidelity are discussed in detail. Molecular markers such as randomly amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSR), internal transcribed space (ITS), starting codon targeted (SCoT), directed amplification of minisatellite DNA (DAMD), ribulose-1,5-bisphosphate carboxylase (rbcL), and maturase k (matK) are discussed.

3 Assessing the Genetic Fidelity of In Vitro Raised Plants by RAPD Markers

Among the various types of molecular markers used for assessing the genetic fidelity of in vitro regenerated plantlets; initially randomly amplified polymorphic DNA (RAPD) markers are used a lot and gained much importance to differentiate the genetic clones from somaclonal variants (Heinze and Schmidt 1995). The RAPD marker-based genetic analysis of tissue cultured plantlets involves the utilization of short primers consisting of random sequences usually in the range of 8 to 16 nucle nucleotides in length (Agarwal et al. 2008). The RAPD primers are initially utilized as genetic markers for identifying the genetic diversity among the progeny resulting from a parental cross (Williams et al. 1990). Later RAPD primers also made their way for testing the genetic trueness of in vitro regenerated plantlets (Heinze and Schmidt 1995). The RAPD primers with arbitrary nucleotide sequence bind randomly to a specific sequence of genome and result in random amplification of DNA segments (Wilde et al. 1992). After amplification with RAPD primers, based on the genomic sequence among the regenerated plantlets, it will result either in the generation of monomorphic or polymorphic DNA bands (Tikendra et al. 2021).

In testing the genetic fidelity of in vitro generated plantlets, initially DNA is to be isolated from leaves of the mother plant and 6–8 randomly selected in vitro raised and acclimatized plantlets. The isolated DNA is assessed for their quality and concentration by using different molecular techniques. After the quality check, the isolated DNA is amplified by using a PCR machine with different RAPD primers by following three steps of denaturation, annealing, and polymerization with specific duration and temperatures for each step of the PCR cycle. The PCR reaction is usually carried for 30–40 cycles to make repeated amplifications, for generation of DNA bands and for easy detection of genetic homogeneity or diversity among the

in vitro raised plantlets. While carrying the amplifications, the annealing temperature was set based on the nucleotide sequence (GC + AT content) of RAPD primers. Based on the nucleotide sequence complementarity, RAPD primers will anneal to various regions of plant genomic DNA and after amplification, it results in the generation of PCR products with DNA bands of either similar patterns or complex patterns (Kumar and Gurusubramanian 2011; Prasad et al. 2013).

After amplification, if the generated pattern of DNA bands is monomorphic across the regenerated plantlets with that of the mother plant, then the micropropagated plantlets are considered as genetically clonal in nature (Korra et al. 2017; Faisal et al. 2018a). On the other hand; if the generated DNA banding patterns are diverse, then the regenerated planttets are assumed to possess the alteration in the genomic sequence and are called somaclonal variants (Munthali et al. 1996).

The RAPD markers are used successfully to assess the genetic homogeneity of regenerated plantlets of several plant species (Table 11.1). The utilization of RAPD markers in genetical fidelity/diversity studies has both advantages and disadvantages. Advantages of RAPD includes; it is a quick method for confirming the genetical clones and distinguishing the genetically diverse lines of plants (Dewir et al. 2005; Yadav et al. 2012). Poor reproducibility is the major disadvantage of the RAPD markers. They are dominant in nature, and hence it may show different results in heterozygous plant populations based on the annealing of RAPD primers either to dominant or recessive locus (Malviya and Yadav 2010; Bhagyawant 2016). Sometimes, the slight mismatch between DNA template and RAPD primer may not result in successful amplification (Wilkie et al. 1993) and incorrect results may be generated among the different lines of the same population with similar genomic sequence (Fernandez et al. 2002). Other applications of RAPD markers include the identification of specific gene markers responsible for disease resistance (viral, bacterial, and fungal), pest resistance, yield and productivity traits (Da Silva et al. 2000; Kobayashi et al. 2000; Ferreira et al. 2000; Priya et al. 2013). The RAPD markers are also used for molecular taxonomic studies (Hadrys et al. 1992).

The limitations of the RAPD technique can be overcome by the recent developments taking place in the RAPD technique; such as sequence-related amplified polymorphism (SRAP), DNA amplification fingerprinting (DAF), random amplified hybridization microsatellites (RAHM), random amplified microsatellite polymorphism (RAMPO), cleaved amplified polymorphic sequences (CAPS), sequence characterized amplified region (SCAR), and arbitrarily primed polymerase chain reaction (AP-PCR) (Babu et al. 2021). In the future, these RAPD-derived techniques will be highly useful for determining the diversity and fidelity studies.

Table 11.1 Genetic fidelity analysis of in vitro regenerated plantlets carried out using RAPD markers

Plant species	Explant	Mode of regeneration	Year	Molecular marker	Reference
Butea monosperma	Cotyledonary node	Direct regeneration	2013	RAPD	Yarra et al. (2016)
Capparis spinosa	Unfertilized ovules	Indirect regeneration	2012	RAPD	Carra et al. (2012)
Cassia alata	Nodal	Direct regeneration	2017	RAPD	Ahmed et al. (2017)
Chlorophytum borivilianum	Shoot base	Direct regeneration	2010	RAPD	Samantaray and Maiti (2010)
Citrullus colocynthis	Shoot tip	Direct regeneration	2010	RAPD	Verma et al. (2013)
Citrullus lanatus	Cotyledonary node	Direct regeneration	2017	RAPD	Vasudevan et al. (2017)
Citrus Limon L.	Nodal	Direct regeneration	2013	RAPD	Goswami et al. (2013)
Dendrobium chrysotoxum	Unripe green capsules	Direct regeneration	2019	RAPD	Tikendra et al. (2019)
Dendrocalamus asper	Nodal	Direct regeneration	2013	RAPD	Singh et al. (2013)
Dendrocalamus strictus (Roxb.)	Nodal	Direct regeneration	2015	RAPD	Goyal et al. (2015)
Exacum bicolor	Nodal	Direct regeneration	2015	RAPD	Ashwini et al. (2015)
Gerbera jamesonii bolus	Capitulum	Direct regeneration	2011	RAPD	Bhatia et al. (2011)
Jatropha curcas L.	Leaf	Indirect regeneration	2012	RAPD	Maharana et al. (2012)
Ledebouria revoluta	Bulb	Indirect regeneration	2016	RAPD	Haque and Ghosh (2016)
Phoenix dactylifera L.	Shoot tip	Indirect regeneration	2010	RAPD	Kumar et al. (2010)
Pisum sativum L.	Cotyledonary node	Direct regeneration	2019	RAPD	Ajithan et al. (2019)
Punica granatum	Leaf	Indirect regeneration	2017	RAPD	Guranna et al. (2017)
Ranunculus wallichianus	Leaf	Indirect regeneration	2021	RAPD	Srinivasan et al. (2021)
Rauwolfia tetraphylla	Stem	Indirect regeneration	2019	RAPD	Rohela et al. (2019)
Rumex nepalensis	Rhizome bud	Indirect regeneration	2017	RAPD	Bhattacharyya et al. (2017a, b)
Sapindus mukorossi	Rachis	Indirect regeneration	2016	RAPD	Singh et al. (2016)
Sapindus trifoliatus L.	Nodal	Direct regeneration	2011	RAPD	Asthana et al. (2011)

(continued)

Table 11.1 (continued)

Plant species	Explant	Mode of regeneration	Year	Molecular marker	Reference
Simmondsia chinensis	Nodal segments	Direct regeneration	2011	RAPD	Kumar et al. (2011)
Solanum erianthum	Leaf	Indirect regeneration	2020	RAPD	Sarkar and Banerjee (2020)
Solanum melongena L.	Hypocotyl	Direct regeneration	2013	RAPD	Mallaya and Ravishankar (2013)
Solanum viarum	Leaf	Direct regeneration	2020	RAPD	Pandey et al. (2020)
Spilanthes calva DC.	Nodal	Direct regeneration	2013	RAPD	Razaq et al. (2013)
Stevia rebaudiana	Nodal	Direct regeneration	2015	RAPD	Modi et al. (2012)
Tecoma stans L.	Shoot tip	Direct regeneration	2020	RAPD	Hussain et al. (2020)
Terminalia arjuna	Nodal	Direct regeneration	2014	RAPD	Gupta et al. (2014)
Terminalia bellerica	Nodal	Direct regeneration	2014	RAPD	Dangi et al. (2014)
Valeriana officinalis L.	Leaf	Indirect regeneration	2014	RAPD	Ghaderi and Jafari (2014).
Viola patrinii	Petiole	Indirect regeneration	2012	RAPD	Chalageri and Babu (2012)
Vitex trifolia	Nodal	Direct regeneration	2013	RAPD	Ahmad et al. (2013)

4 Genetic Fidelity Analysis of In Vitro Raised Plants Using ISSR Markers

The DNA-based molecular markers are widely utilized in plant tissue culture studies for the assessment of genetic fidelity of regenerated plantlets, genomic diversity among the mother plants and somaclonal variants. Similarly in plant breeding, ISSR markers are also utilized to map genomic regions associated with desired traits, etc. Among the molecular markers, RAPD, AFLP, and SSR markers are frequently used PCR-based markers and are generally employed for the marker-assisted breeding programs in different crop plants due to their characteristics. These markers are utilized in several areas like genomic diversity assessment, genetic fidelity, gene tagging and mapping, evolutionary studies, and phylogenetic relationship among crop plants (Reddy et al. 2002).

The inter simple sequence repeat (ISSR) is a PCR-based molecular marker first used by Zietkiewicz et al. (1994) using (CA)n repeat as a primer for the amplification of mammalian DNA sample. This marker amplifies the genomic region betwixt the two amplified microsatellite repeat region which are in opposite directions (Reddy et al. 2002; Alizadeh et al. 2015; Amom and Nongdam 2017). In simple words, as it

is a multilocus marker it mainly amplifies the inter-SSR region. The amplified products resulted from inter-SSR PCR only when the SSRs are found on opposite orientation within a PCR amplifiable distance with flanking sequences matching the oligonucleotide primers. Like RAPD marker where a single primer acts as both forward and reverse primer, ISSR used a single primer of 16–25 base pair length (Reddy et al. 2002; Alizadeh et al. 2015; Amom and Nongdam 2017). Similar to SSR, the di-, tri-, tetra, and penta-nucleotide repeats are used as a primer. Zietkiewicz et al. (1994) first utilized the anchored 3′ or 5′ end with (CA)n repeats as primer. Therefore, for ISSR amplification the primer can be used either unanchored or anchored at 3′ or 5′ having a 1–4 degenerated base. Due to its multilocus pattern of fingerprinting, ISSR can be utilized in various genetic studies such as genetic identity, parentage, and clone and strain identification. Besides these uses, it can also be beneficial in gene mapping studies, testing stability of in vitro propagated plants (Amom and Nongdam 2017). Due to the advantages concerned with ISSR markers over the RAPD in terms of its reproducibility and requirement of little DNA, ISSR was now-a-days a preferred molecular marker in carrying the genetic fidelity analysis of in vitro regenerated plantlets.

The ISSR marker is almost similar to RAPD with a difference in primer length which is designed from SSR regions. The marker has a high rate of stringency due to longer primer of 16–25 base pairs which allows the amplification at an annealing temperature of 45–60 °C (Reddy et al. 2002). In literature, ISSR is reported as both dominant (Gupta et al. 1994; Tsumura et al. 1996; Ratnaparkhe et al. 1998; Wang et al. 1998) and co-dominant (Wu et al. 1994; Akagi et al. 1996; Wang et al. 1998; Sankar and Moore 2001) marker, as it follows the simple Mendelian genetics. The rate of polymorphism exhibited by the ISSR marker relies on various factors such as nature of primer used (unanchored, 3′ anchored, or 5′ anchored), the motif of the repeat targeted (di-, tri-, tetra-, penta-nucleotides of higher), the sequence of the primer (AT rich, GC rich or balanced), length of the primer, and extant of the optimization in the PCR, method of detection (agarose gel with ethidium bromide or PAGE with silver staining/radioactive detection) (Reddy et al. 2002). ISSR marker along with the combination of other molecular markers would be an ideal strategy for carrying the molecular analysis of plants.

The ISSR fingerprinting is developed in such a way that no sequence knowledge is required for primer designing, it requires a low quantity of DNA template (5–50 ng per reaction), has high polymorphism rate, distributed at random across the genome, produces several informative bands, and is amenable to automation. On the other hand, this technique also has some limitations of the dominance of alleles, similar-sized fragments may not be homologous to each other (Singh et al. 2009). In *Flemingia macrophylla* (Fabaceae), Sirikonda et al. (2020) used ISSR and RAPD marker and confirmed that the in vitro propagated clones are true-to-type. Rohela et al. (2019) also confirmed the in vitro propoagted plantlets of *Rauwolfia tetraphylla* as true-to-type by employing SCoT, ISSR, and RAPD markers and showed no difference between regenerated plants and mother plant. The regenerated in vitro plants of endemic species *Reseda pentagyna* are also tested with ISSR marker and validated the genetic integrity (Al-Qurainy et al. 2018). Khatun et al. (2018) verified

the genetic fidelity of in vitro generated *Aloe vera* plants using ISSR and RAPD markers. Mulberry, a genetically heterozygous tree plant is checked for genetic homogeneity using ISSR markers by Rohela et al. (2018a, b), and no polymorphism was obtained in between the clones and the mother plants. ISSR is widely utilized for many genetic studies and especially for the assessment of genetic stability of in vitro propagated plants in several economically important plants (Table 11.2).

5 Analysis of Genetic Fidelity of In Vitro Raised Plants by SCoT Markers

Start codon-targeted (SCoT) is a novel and advanced molecular marker, first employed by Collard and Mackill (2009) in rice which amplifies the short-conserved genic sequence adjoining the translation initiation code, ATG (Joshi et al. 1997; Sawant et al. 1999). Similar to RAPD and ISSR marker, single primer acts as both forward and reverse in SCoT marker (Gupta et al. 1994; Williams et al. 1990). The primer for SCoT analysis is designed based on the consensus sequence neighboring the start codon. The DNA nucleotides A, T, G, and C have different variant combinations such as at +1, +2, and + 3 position and the ATG sequence followed by G, C, A, C, C at +4, +5, +7, +8, and + 9 position. Generally, molecular markers have the problem of reproducibility which rely on several factors such as primer length, annealing temperature, and magnesium chloride co-factor of Taq polymerase enzyme. The SCoT primer of 18-mer length is suitable for obtaining the amplification of a large number of markers. For SCoT marker annealing temperature of 50 °C and extension of 2 min are most convenient. The PCR amplification of SCoT primer generates two to six products of a size range of 200–1500 base pairs. The confirmation of SCoT results reproducibility comprised of two phases, i.e., comparison of PCR amplification results between duplicate samples of particular genotype followed by reconfirmation of results through different PCR machines on different days. The results could be visualized through agarose gel electrophoresis and other staining. Other than these factors, the reproducibility depends on the GC content of primers. The higher the GC content, the more reproducible will be the marker. SCoT marker is most suitable for assessment of genetic diversity in crop plants, bulked segregant analysis, and QTL mapping (Collard and Mackill 2009). It has also been used for testing the genetic integrity of in vitro plants (Table 11.3).

Chirumamilla et al. (2021) confirmed the genetic fidelity of micropropagated *Solanum khasianum* plants through SCoT markers. *Annona reticulata* L. is an important medicinal plant first time propagated through nodal segment explant, and the regenerated plants are tested with molecular markers SCoT and ISSR molecular markers (Kudikala et al. 2020). The molecular evaluation revealed monomorphism between the in vitro propagated plantlets and true-to-type to the mother plant (Kudikala et al. 2020). In mulberry, Rohela et al. (2020) confirmed the genetic

Table 11.2 Genetic fidelity assessment of in vitro raised plantlets carried using ISSR markers

Plant species	Explant	Mode of regeneration	Year	Molecular marker	Reference
Abutilon indicum L.	Leaf	Indirect regeneration	2019	ISSR	Seth and Panigrahi (2019)
Achras sapota L.	Cotyledon node	Direct regeneration	2018	ISSR	Chittora (2018)
Andrographis alata	Nodal	Direct regeneration	2021	ISSR	Kadapatti and Murthy (2021)
Andrographis echioides	Leaf	Direct regeneration	2020	ISSR	Savitikadi et al. (2020)
Annona reticulata	Nodal	Direct regeneration	2020	ISSR	Kudikala et al. (2020)
Artemisa vulgaris	Nodal	Direct regeneration	2020	ISSR	Jogam et al. (2020)
Bacopa monnieri	Shoot tip	Indirect regeneration	2021	ISSR	Pramanik et al. (2021)
Bambusa balcooa	Nodal	Direct regeneration	2020	ISSR	Rajput et al. (2020)
Cicer arietinum	Cotyledon node	Direct regeneration	2020	ISSR	Sadhu et al. (2020)
Corallocarpus epigaeus	Nodal	Direct regeneration	2020	ISSR	Vemula et al. (2020)
Corynandra chelidonii	Nodal	Direct regeneration	2020	ISSR	Sirangi et al. (2021)
Cucumis melo L.	Cotyledon	Direct regeneration	2021	ISSR	Raji and Farajpour (2021)
Dioscorea deltoidea	Nodal	Direct regeneration	2020	ISSR	Nazir et al. (2021)
Ficus carica	Stem	Indirect regeneration	2020	ISSR	Abdolinejad et al. (2020)
Finger millet	Shoot tip	Direct regeneration	2018	ISSR	Babu et al. (2018)
Flemingia macrophylla	Cotyledon node	Direct regeneration	2020	ISSR	Sirikonda et al. (2020)
Morus alba	Nodal	Indirect regeneration	2020	ISSR	Rohela et al. (2020)
Muntingia calabura	Nodal	Direct regeneration	2021	ISSR	Vankudoth et al. (2020)
Origanum majorana	Nodal	Direct regeneration	2021	ISSR	Sandhya et al. (2021)
Paederia foetida	Nodal	Direct regeneration	2018	ISSR	Behera et al. (2018)
Phoenix dactylifera L.	Shoot tip	Direct regeneration	2019	ISSR	Mazri et al. (2019)
Prunus salicina	Nodal	Direct regeneration	2021	ISSR	Thakur et al. (2021)

(continued)

Table 11.2 (continued)

Plant species	Explant	Mode of regeneration	Year	Molecular marker	Reference
Ranunculus wallichianus	Leaf	Indirect regeneration	2021	ISSR	Srinivasan et al. (2021)
Rauwolfia tetraphylla	Leaf	Indirect regeneration	2013	ISSR	Rohela et al. (2013)
Saccharum officinarum L.	Leaf	Indirect regeneration	2017	ISSR	Thorat et al. (2017)
Santalum album	Nodal	Direct regeneration	2021	ISSR	Shekhawat et al. (2021)
Sapindus mukorossi	Rachis	Indirect regeneration	2016	ISSR	Singh et al. (2016)
Scaevola taccada	Nodal	Direct regeneration	2021	ISSR	Shekhawat et al. (2021)
Solanum khasianum	Nodal	Indirect regeneration	2020	ISSR	Chirumamilla et al. (2021)
Solanum trilobatum	Nodal	Indirect regeneration	2020	ISSR	Pendli et al. (2019)
Tecoma stans L.	Nodal	Direct regeneration	2019	ISSR	Hussain et al. (2019)

fidelity of in vitro regenerated plants of *Morus alba* L. cv. Chinese white through SCoT markers.

6 Assessment of Genetic Fidelity of In Vitro Raised Plantlets Using DNA Barcoding Markers

Other than regularly used molecular markers such as RFLP, RAPD, and ISSR, recently the DNA barcoding markers are also increasingly used for assessing the genetic fidelity of in vitro regenerated plantlets. Initially, DNA barcoding markers are more commonly used for plant species identification at molecular level. Now these are used in confirming the genetic homogeneity of regenerated plantlets by targeting important DNA barcoding genes of plants. The important DNA barcoding genes of plants include large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcL*), cytochrome c oxidase I (*CO1*), maturase K (*matK*), internal transcribed spacer 1 (*ITS-1*), internal transcribed spacer 2 (*ITS-2*), and photosystem II protein D1-tRNA-His (*psb*A-*trn*H) (Techen et al. 2014; Umdale et al. 2017). Among the list of important plant DNA barcodes, mostly maturase K (*matK*) and large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcL*) genes of chloroplast genome are the most commonly exploited for identifying the genetic similarity with regard to these important genes (Hollingsworth et al. 2009).

Recently, DNA barcode primers based on genetic homogeneity analysis of in vitro regenerated plantlets are carried in several plant species of commercial and

Table 11.3 Analysis of genetic fidelity of tissue culture raised plantlets by SCoT markers

Plant species	Explant	Mode of regeneration	Year	Molecular marker	Reference
Abutilon indicum L.	Leaf	Indirect regeneration	2017	SCoT	Seth et al. (2017)
Aenhenrya rotundifolia	Juvenile axillary bud	Direct regeneration	2020	SCoT	Sherif et al. (2020)
Albizia julibrissin	Leaf	Direct regeneration	2016	SCoT	Rahmani et al. (2016)
Aloe vera L.	Shoot tip	Direct regeneration	2021	SCoT	Hamdeni et al. (2021)
Ansellia africana	Nodal	Direct regeneration	2017	SCoT	Bhattacharyya et al. (2017a, b)
Aranda Broga	Protocorm like bodies	Direct regeneration	2020	SCoT	Khor et al. (2020)
Asystasia gangetica	Leaf	Indirect regeneration	2021	SCoT	Dilkalal et al. (2021)
Atropa acuminata	Root	Indirect regeneration	2020	SCoT	Rajput and Agrawal (2020)
Bauhinia racemosa	Nodal	Direct regeneration	2019	SCoT	Sharma et al. (2019a, b)
Cannabis sativa L.	Nodal	Direct regeneration	2016	SCoT	Lata et al. (2016)
Chlorophytum borivilianum	Flower stalk	Direct regeneration	2021	SCoT	Kaushal et al. (2021)
Citrus x meyeri	Shoot tip	Direct regeneration	2021	SCoT	Haradzi et al. (2021)
Crinum malabaricum	Bulb	Direct regeneration	2020	SCoT	Priyadharshini et al. (2020)
Crocus sativus L.	Shoot bud	Direct regeneration	2021	SCoT	Gautam and Bhattacharya (2021)
Curcuma longa L.	Shoot bud	Direct regeneration	2021	SCoT	Pittampalli et al. (2021)
Dendrobium thyrsiflorum	Nodal explants	Direct regeneration	2015	SCoT	Bhattacharyya et al. (2015)
Helicteres isora L.	Cotyledonary node	Direct regeneration	2020	SCoT	Muthukumar et al. (2020)
Helicteres isora L.	Leaf	Indirect regeneration	2016	SCoT	Muthukumar et al. (2016)
Nardostachys jatamansi	Rhizome	Direct regeneration	2021	SCoT	Dhiman et al. (2021)
Nyctanthes arbor-tristis L.	Nodal	Direct regeneration	2020	SCoT	Rath et al. (2020)
Pittosporum eriocarpum	Nodal	Direct regeneration	2016	SCoT	Thakur et al. (2016)
Saccharum officinarum L.	Immature leaf roll	Direct regeneration	2018	SCoT	Sathish et al. (2018)

(continued)

Table 11.3 (continued)

Plant species	Explant	Mode of regeneration	Year	Molecular marker	Reference
Salvia sclarea L.	Nodal	Direct regeneration	2020	SCoT	Erişen et al. (2020)
Santalum album	Nodal	Direct regeneration	2021	SCoT	Manokari et al. (2021a, b)
Simmondsia chinensis	Nodal	Direct regeneration	2015	SCoT	Bekheet et al. (2015)
Solanum trilobatum L.	Nodal	Direct regeneration	2021	SCoT	Shilpha et al. (2021)
Spondias pinnata	Leaf	Indirect regeneration	2021	SCoT	Jaiswal et al. (2021)
Taraxacum pieninicum	Shoot tip	Direct regeneration	2019	SCoT	Kamińska et al. (2020)
Tecomella undulata	Nodal	Direct regeneration	2017	SCoT	Chhajer and Kalia (2017)
Vanilla planifolia	Nodal	Direct	2021	SCoT	Manokari et al. (2021a, b)
Withania somnifera L.	Leaf	Direct regeneration	2021	SCoT	Kaur et al. (2021)

medicinal importance. Jogam et al. (2020) has used the *rbcLa* DNA barcoding primers and confirmed the genetic stability of micropropagated plantlets of *Artemisia vulgaris* L.. Similarly, *rbcLa* primers are utilized for the genetic fidelity analysis of in vitro raised medicinal plantlets of *Andrographis echioides* (L.) (Savitikadi et al. 2020). A combination of two DNA barcoding primers, i.e., *trnH–psbA* and *rbcL* are employed to confirm the genetic clonal nature of in vitro raised plantlets of two important medicinal plant species of *Dioscorea hirtiflora* Benth and *Dioscorea bulbifera* L. (Adeniran et al. 2018). Plastid genome-based *ycf1*b DNA barcoding primers are used for confirming the genetic stability of in vitro raised plantlets of *Asystasia gangetica* (*L.*) (Dilkalal et al. 2021). In the future, DNA barcoding primers based on genetic fidelity assessment of regenerated plantlets of medicinal and commercial importance will gain more importance as most of these primers are targeted for the amplification of photosynthetically important genes encodes by chloroplast gnome.

Due to certain disadvantages concerned with the usage of regular DNA molecular markers and uncertainty about single marker-based genetic assessment studies, it is necessary to carry the genetic fidelity studies by using combination of one of the regular molecular markers (RAPD, ISSR, AFLP, RFLP) and one of the DNA barcoding-based markers (*rbcL*, *CO1*, *matK*, *ITS-1*, *ITS-2*, *ycf1*b). As stated above, already there are certain specific reports about the combined use of one regular molecular marker with DNA barcoding-based marker (Jogam et al. 2020; Savitikadi et al. 2020; Dilkalal et al. 2021). Adeniran et al. (2018) used the combination of SCoT and *ycf1*b DNA barcoding primers for confirming the genetic stability of in vitro raised plantlets of *Asystasia gangetica*. Jogam et al. (2020) has used the

combination of ISSR and *rbcLa* DNA barcoding primers to confirm the genetic stability of in vitro micropropagated plantlets of *Artemisia vulgaris* L. Similarly, Savitikadi et al. (2020) also used combination of ISSR and *rbcLa* DNA barcoding primers to confirm the genetic stability of in vitro micropropagated plantlets of *Andrographis echioides*.

The advantage of use of DNA barcoding-based genetic fidelity studies is; it confirms the genetic trueness of regenerated plants with regard to important genes related to photosynthesis and respiration. In DNA barcoding-based genetic homogeneity studies, there are different steps of DNA isolation, purity, and concentration check of isolated DNA, DNA barcoding primers-based PCR amplification and assessment of genetic fidelity of regenerated plantlets with that of mother plant by comparing the number and size of DNA bands with DNA ladder (Jogam et al. 2020). The DNA barcoding-based approach has been widely accepted for confirming the genetic trueness of regenerated plants with regard to plant-based important genes, especially related to chloroplast genome carrying the vital steps of photosynthesis. Usually, short DNA sequence of <1000 bp are targeted for amplification and generation of DNA bands in DNA barcoding-based studies (Hebert et al. 2003) which is an advantage over the conventional DNA markers. In recent years, DNA barcoding-based approach is also widely accepted for testing and validating the molecular identification of medicinal plants (Roy et al. 2010; Chen et al. 2010; Fu et al. 2011; Schori and Showalter 2011; Stoeckle et al. 2011; Techen et al. 2014; Gantait et al. 2014; de Vere et al., 2015; Mishra et al. 2016; Liu et al. 2017; Malik et al. 2019; Sherif et al. 2020; Feau et al. 2021).

7 Analyzing the Genetic Fidelity Using Other Molecular Markers

Apart from RAPD, ISSR, RAPD, SCoT, and DNA barcoding-based markers, other markers of restriction fragment polymorphism (RFLP), amplified fragment length polymorphism (AFLP), and directed amplification of minisatellite DNA (DAMD) markers are also utilized for assessing the genetic stability of in vitro regenerated plantlets (Devarumath et al. 2002; Singh et al. 2002; Singh et al. 2014; Othmani et al. 2010; Yin et al. 2013; Dhiman et al. 2021). In RFLP-based technique, the genomic DNA was isolated from the mother plant and randomly selected regenerated plantlets, the isolated genomic DNA was digested by a set of restriction endonucleases and based on the nucleotide sequence similarity of the restriction site, it will generate either DNA fragments of the same length or different lengths. The generated DNA fragments were separated on the agarose gel electrophoresis to visualize the pattern of DNA bands under UV transilluminator to ascertain the genetic stability of regenerated plants in comparison to mother plants. The genetic stability of in vitro regenerated plantlets of *Camellia assamica* ssp. *assamica* (Assam-India type) and *Camellia sinensis* (China type) were evaluated using

RFLP markers (Devarumath et al. 2002). Similarly, other researchers also utilized the RFLP markers to assess the genetic fidelity of in vitro regenerated plants of commercial and medicinal importance (Table. 11.4).

A similar procedure to RFLP is followed initially in AFLP-based technique. Still, after the digestion of genomic DNA by restriction endonucleases, the generated DNA fragments were selectively amplified using specific primers carrying polymerase chain reaction-based amplification to generate the DNA bands. Based on the genomic sequence similarity, it will result either in the generation of monomorphic DNA bands (genetic fidelity) across the regenerated plantlets and mother plant or it generates the polymorphic DNA bands (genetic diversity). The AFLP-based genetic fidelity tests are carried out successfully to determine the genetic stability of regenerated plants of *Azadirachta indica* A. Juss. (Singh et al. 2002), *Phoenix dactylifera* L. (Othmani et al. 2010), *Gentiana pannonica* Scop. (Fiuk et al. 2010), *Lilium* spp. (Yin et al. 2013), etc. Other reports of using AFLP markers for the genetic assessment of in vitro regenerated plantlets are represented in Table 11.4**.**

In recent days, an advanced marker known as directed amplification of minisatellite DNA (DAMD) is also in use to assess the genetic stability in tissue cultured plantlets.. Compared to RAPD, RFLP, and AFLP markers, DAMD markers are more efficient in generating the results with a reproducibility nature. This is due to the fact that RAPD, RFLP, and AFLP markers-based amplicons are generated by carrying the amplification or restriction digestion of entire genomic regions, whereas DAMD markers carry the amplification of only microsatellite regions of the genome (Kumar et al., 2014). The DAMD markers along with ISSR and RAPD are recently started utilizing as single primer-based amplification reaction (SPAR) method for determining the genetic relatedness among the different species of a genus or among the regenerated plantlets of a species (Bhattacharya et al. 2005). These three markers (DAMD, ISSR, and RAPD) can collectively prove to provide comprehensive details about the genetic relatedness (diversity and fidelity studies) among the plant population (Bhattacharya et al. 2005; Ranade et al. 2009; Sharma et al. 2011a, b).

Due to the advantages concerned with DAMD markers, it is used either singly or in combination with other SPAR-based markers, i.e., ISSR and RAPD markers for the genetic stability analysis of tissue cultured plantlets. DAMD markers are utilized successfully for the genetic fidelity analysis of in vitro regenerated plantlets of *Nepenthes khasiana* (Devi et al. 2014), *Mentha arvensis* (Faisal et al. 2014), *Withania somnifera* L. (Fatima et al. 2015), *Henckelia incana* (Prameela et al. 2015), *Avicennia marina* (Alatar et al. 2015), *Bacopa monnieri* L. (Largia et al. 2015), *Solanum lycopersicum* (Alatar et al. 2017), *Saccharum officinarum* (Kumari et al. 2017), *Hibiscus sabdariffa* L. (Konar et al. 2019), *Nardostachys jatamansi* (Dhiman et al. 2021), *Ficus carica* var. black jack (Parab et al. 2021), and *Crocus sativus* L. (Gautam and Bhattacharya 2021) (Table 11.4). When the genetic fidelity assessment was carried with more than one marker, especially by the SPAR method using single primer amplification reaction utilizing DAMD, ISSR, and RAPD markers, it can eliminate any false results. When all the three primers-based genetic fidelity analyses resulting in generation of monomorphic DNA bands across the regenerated plantlets with that of mother plant, the plantlets will be assumed to be

Table 11.4 Analysis of genetic fidelity of in vitro propagated plantlets using RFLP, AFLP and DAMD markers

Plant species	Explant	Mode of regeneration	Year	Molecular marker	Reference
Camellia sinensis	Node	Direct regeneration	2002	RFLP	Devarumath et al. (2002)
Bambusa nutans wall	Node	Direct regeneration	2011	AFLP	Mehta et al. (2011)
Coffea arabica	Embryogenic suspensions	Indirect regeneration	2013	AFLP	Bobadilla et al. (2013)
Freesia hybrida	Flower	Direct and indirect regeneration	2010	AFLP	Gao et al. (2010)
Zea mays	Callus	Indirect regeneration	1991	RFLP	Brown et al. (1991)
Gentiana pannonica Scop	Somatic embryo	Indirect regeneration	2010	AFLP	Fiuk et al. (2010)
Jatropha curcas	Node	Direct regeneration	2011	AFLP	Sharma et al. (2011a, b)
Azadirachta indica	Node	Direct regeneration	2002	AFLP	Singh et al. (2002)
Lilium spp.	Leaf	Indirect regeneration	2013	AFLP	Yin et al. (2013)
Phoenix dactylifera L.	Leaf	Indirect regeneration	2006	AFLP	Saker et al. (2006)
Camellia assamica	Node	Direct regeneration	2002	RFLP	Devarumath et al. (2002)
Phoenix dactylifera L.	Somatic embryo	Indirect regeneration	2010	AFLP	Othmani et al. (2010)
Nardostachys jatamansi	Rhizome	Direct regeneration	2021	DAMD	Dhiman et al. (2021)
Nepenthes khasiana hook.	Leaf	Indirect regeneration	2014	DAMD	Devi et al. (2014)
Ficus carica	Node	Direct regeneration	2021	DAMD	Parab et al. (2021)
Bacopa monnieri L.	Node	Direct regeneration	2018	DAMD	Faisal et al. (2018b)
Mentha arvensis	Node	Direct regeneration	2014	DAMD	Faisal et al. (2014)
Solanum lycopersicum	Cotyledonary leaf and Cotyledonary node	Direct and indirect regeneration	2017	DAMD	Alatar et al. (2017)
Withania somnifera L.	Axillary bud	Direct regeneration	2015	DAMD	Fatima et al. (2015)
Henckelia incana	Leaf	Indirect regeneration	2015	DAMD	Prameela et al. (2015)

(continued)

Table 11.4 (continued)

Plant species	Explant	Mode of regeneration	Year	Molecular marker	Reference
Avicennia marina	Node	Direct regeneration	2015	DAMD	Alatar et al. (2015)
Hibiscus sabdariffa L.	Root	Indirect regeneration	2019	DAMD	Konar et al. (2019)
Bacopa monnieri L.	Leaf	Indirect regeneration	2015	DAMD	Largia et al. (2015)
Crocus sativus L.	Node	Direct regeneration	2021	DAMD	Gautam and Bhattacharya (2021)
Saccharum officinarum L.	Node	Direct regeneration	2017	DAMD	Kumari et al. (2017)
Chlorophytum borivilianum	Flower stalk	Indirect regeneration	2021	DAMD	Kaushal et al. (2021)
Solanum trilobatum L.	Node	Direct regeneration	2014	DAMD	Shilpha et al. (2014)

genetically true and clonal in nature. In the future, the combined SPAR-based markers will be used in lot to carry out the genetic relatedness studies in plants for ascertaining the diversity or uniqueness of different plant populations.

8 Conclusions and Future Prospectives

With the introduction of new types of plant growth regulators in plant tissue culture media, always there is a chance of some of the newly introduced chemicals that might act as a mutagen and induce the mutations more likely at callus and regeneration stages. Beacuse of this, it is essential to continuously examine the in vitro regenerated plantlets for their genetic stability using advanced markers developed over the period. Similarly now-a-days inorder to provide faster and easy growth of plants under in vitro conditions, different types of artificial light sources were introduced in the plant growth chambers or culture rooms. These light sources emit radiations which may also act as mutagen. Due to the use of new chemicals in tissue culture media, new types of light sources, and the fragile nature of callus, there is always a chance of having genetic variation in tissue-cultured plantlets. Thus, it is essential to carry out genetic fidelity tests through molecular marker-assisted techniques to provide genetically uniform clones to the end-users. The content mentioned in this book chapter shows that there is a difference in the usage of different molecular markers for assessing the genetic stability of regenerated plantlets over the years. This is mainly due to the disadvantages of costly and non-reproducibility nature concerned with the conventionally used molecular markers and, on the other hand, due to the cost-effectiveness,

reproducibility, and accurate results provided by the advanced markers. Most of the researchers are using advanced markers like ISSR, SCoT, and DAMD recently to know the genetic stability of in vitro regenerated plantlets of horticulture and plantation crops such as coffee, apple, tea, blueberry, banana, papaya, grapes, sweet potato, cassava, potato, mulberry, pineapple, cocoa, arecanut, date, cashewnut, sugarcane, eucalyptus, poplar, pine, rubber tree, and oil palm. The combination of three markers (ISSR, SCoT, and DAMD) would be more beneficial in accurately predicting the genetic uniformity of the plants raised through tissue culture.

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Chapter 12
Improvement of Plant Survival and Expediting Acclimatization Process

Kumari Shiwani, Dushyant Sharma, and Arun Kumar

Abstract Plant propagation by tissue culture is one of the crucial modern breeding technologies and an efficient method of clonal plant propagation which allows for increased production of horticulture and forest crops. The success of plant tissue culture depends on its potential to transfer plants out of culture vessels into the field conditions for large-scale production with a high survival rate. Micropropagated plants lack cuticle layer, stomata closure and have poorly developed roots leading to transpiration losses when transferred to ex vitro conditions. This chapter focuses on the diverse methods that can be used for the improvement of the tissue-cultured plant's survival and for accelerating the acclimatization process. In vitro priming of propagules done with the help of growth regulators, simple changes to the growing environment and in vitro and ex vitro biotization with competent plant growth-promoting microorganisms (PGPMs) for improving plant survival/performance under the acclimatization process and consequently enhancing yield are some of the approaches which have been dealt upon in detail in this chapter.

Keywords Micropropagation · Plant survival · Acclimatization · Biopriming · Biotization · PGPMs

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1 Introduction

Micropropagation is a technique that allows speedy production of uniform, disease-free and high-quality planting material. Speedy production through micropropagation vegetables, ornamentals, fruit and forest trees has developed new possibilities in worldwide trading of the planting material and for generating rural employment for farmers and nursery owners. Explants (seeds, roots, shoots) grown in vitro are constantly subjected to an entirely different microenvironment which has been selected to provide optimum growing conditions with minimal stress for plant multiplication. Tissue-cultured plantlets are grown aseptically in culture vessels under conditions like low light and high humidity and medium containing sufficient sugar and nutrients. It produces a culture-induced phenotype which are when directly places in a greenhouse or field, cannot withstand the ex vitro conditions due to higher light and low levels of relative humidity with septic environmental conditions that are stressful for micropropagated plants propagules as compared to in vitro conditions. The low survival rate of micropropagated plantlets in natural conditions has made the use of micropropagation methods economically unviable for many plants including horticulture and forest species (Nguyen et al. 2001). To overcome this issue, numerous methods to acclimatize tissue-cultured plantlets in vitro as well as ex vitro propagation can be utilized thereby facilitating the survival rate of tissue-cultured plantlets in the soil.

Priming of in vitro and ex vitro propagules/plantlets priming refers to the alterations of growing conditions prior to or during transplanting, which is an emerging and integral part of tissue culture propagation. Priming for shooting, rooting and efficient photosynthesis can be achieved with the help of growth regulators or by simple changes to the growing conditions that affect the post-transplanting establishment of the plant propagules. Plantlets grown in a photoautotrophic system on culture media with or without sucrose, high CO_2 level, high light intensity, good gas exchange and low humidity are more vigorous, have larger root system and are less susceptible to microbial contamination. Plants adapted slowly to the ex vitro conditions have a high survival rate when transfer to the soil. Different types of microorganisms, including fungi, bacteria and archaea residing in the plant-roots zone and cater on abolished plant parts and roots exudates are used as plant growth-promoting microorganisms (PGPMs) in horticulture and forestry plant species (Al-Ani 2019). As the importance of PGPMs for enhancing plant growth and development, alleviating abiotic and biotic stresses, preventing deadly diseases and restoring soil health has been confirmed, attention is also been given to favourable uses of these microorganisms in plant micropropagation. Referring to this, the suitable utilization of plant growth-promoting microorganisms under both in vitro and ex vitro conditions was examined and referred to as ‘biopriming’ or ‘biotization’. Plant growth-promoting microorganisms (PGPMs) can assist in shoot elongation, effective rooting and the success of acclimatization of micropropagation plantlets. They can protect against different types of stresses (biotic and abiotic) that occur during acclimatization and hardening phase of in vitro propagation. This chapter focuses on the

effective methods and techniques that can be used for the improvement of plant survival and speeding up the acclimatization process with special emphasis on the potentiality of biopriming in plant tissue culture.

2 In Vitro Acclimatization of Plant Propagules

The practice of hardening of plantlets can be started in in vitro which can further speed up the acclimation in ex vitro conditions.

2.1 Abiotic Techniques/Approaches for Acclimatization

Changing in vitro environmental conditions of the culture vessels to have healthy plantlets with no morphological or physiological aberrations and with a high acclimatization rate is tremendously crucial for horticulture and forest industry. The following major approaches have emerged to set off phenotype with improved, photosynthetic competence, fluid retention and storage capacity thus facilitating ex vitro transplantation.

2.2 Role of Sugar in the Medium

In culture media, the adhering/persistent leaves, crammed with carbohydrates, glycoproteins and minerals (storage compounds) which further contribute more after transplantation of plantlets in ex vitro. The nutrient status of persistent leaves would possibly maximize with the increase of sugar concentration in the medium. The photosynthetic capacity of plantlets seems to vary with the plant. High fresh and dry weight and shoot length of plants species *Ficus lyrata* and *Potentilla fruticosa* were observed by Wainwright and Scrace (1989) in ex vitro condition when formerly treated with sucrose (2% or 4%). After incorporation of sucrose to the medium, direct increase in plants constituents like starch and total chlorophyll which increases subsequent ex vitro survival and growth was revealed by Hazarika et al. (2006). Different concentrations of sucrose in medium affect the survival rate of ex vitro plants. Kumar et al. (2001) obtained 68.8% ex vitro survival of Kinnow mandarin by in vitro regeneration from epicotyl segment of the plant using 3% sucrose in the medium. Induction of multiple shoots of gerbera plants was obtained when supplemented with sucrose (3%) and other phytohormones in medium, resulting in complete survival rate (100%) upon transfer to the field conditions (Aswath and Choudhury 2002). Mehta et al. (2000) found increased shoots formation in tamarind explants from 34% to 48% with the increase in sucrose concentration (from 2% to 4%) in the medium. Misra and Dutta (2001) showed that bulblets

were grown profusely in a liquid medium that contained sucrose (9%) and other phytohormones while working on acclimatization of Asiatic hybrid lilies under stress conditions.

2.3 Promotion of Autotrophic Mode of Nutrition

Autotrophic growth (increased photosynthetic efficiency) of the plantlets has the advantage of better acclimatization under ex vitro conditions than conventional methods. The objective of this approach is to alter the culture to encourage the phenotypes towards the autotrophic mode of nutrients, for this oxygen concentration in the culture environment is reduced which in turn reduced the rate of photorespiration (Shimada et al. 1988). Another method of promoting autotrophy in plant tissue culture is partially or completely elimination of sugars (Kozai 1988) while increasing the photosynthetic photon flux (PPF) and CO_2 concentration in the medium. Alternatively, for increasing CO_2 concentration and light penetration in culture vessel and decreasing O_2 level and relative humidity of the vessel, a clear plastic gas permeable film is used as a vessel closure which improved gas exchange in culture, which lately has been proposed as a method of lowering the production cost micropropagation process (Kozai et al. 1987). Photoautotrophic growth of Chrysanthemum meristems could be enhanced by culturing them in a sugar-free medium. Chrysanthemum plants cultured under this mechanism manifested a high rate of photosynthetic activity as compared to those found in seedlings (Short et al. 1987). Therefore, this procedure can make it possible to use a large culture vessel without the risk of increasing the loss of plantlets due to contamination. Furthermore, this approach can be used to expedite the effective transfer of tissue-cultured plants propagules to ex vitro conditions.

2.4 Reduced Relative Humidity

This approach presumes that plantlets grown under low relative humidity will have low translocation and transpiration rates in ex vivo conditions. Decreased relative humidity in the culture vessels can be an effective way of accelerating their survival in greenhouse or field conditions. Lower deposition of surface wax, stomatal abnormalities and a non-continuous cuticle are typical anatomical features of herbaceous plants growing under abundant moisture conditions. This characteristic in vitro anatomy can be prevented by increasing the vapour-pressure gradient between the leaf and the atmosphere. Various techniques for lowering the relative humidity in culture vessels, such as sheathing the medium molten lanolin layer, increasing the air ventilation, using desiccants and saturated salt, hanging silica gel bag in the culture vessels, water vapour permeable lid and using a bottom cooling technique (Asayesh et al. 2017). Osmotic agents such as polyethylene glycol or increasing the agar or

sugar concentration of the medium will also serve the purpose of desiccant as it lowers the relative humidity in some cases (Leshem 1983).

Many inventive culture vessels have been developed by many researchers to ease the growth and development of tissue-cultured plants in in vitro which are having low levels of relative humidity. In this lane, Tanaka et al. (1992) developed a disposable gas permeable, fluorocarbon polymer film culture vessel which facilitated reduction in relative humidity. Novel micropropagation vessels have been shown to effectively acclimatize plantlets in vitro without the need for a post ex vitro acclimatization step. Several plant species (ornamental, vegetable and woody) have already been successfully acclimatized in vitro using this novel system, which despite its price constraints, allow for the *en masse* production of morphologically sturdier plants, (photosynthetically, physiologically and genetically stable) under photoautotrophic conditions (Teixeira da Silva et al. 2006). These vessels allow for gaseous exchange, eliminate the negative effects of ethylene accumulation and remove abnormal morphologies associated with hyperhydricity.

2.5 Growth Retardants/Regulators

In natural/in vivo environment, plant growth retardants generally induce many effects on higher plants including shortening of internode, thickening of roots, reduction of leaf size and escalation of green leaf pigment. Besides this, various growth retardants when use in vitro prevent damage caused by wilting of plantlets without any adverse effects. Therefore, knowing the importance of role of growth retardants in in vitro, Smith et al. (1991) studied the effects of anti-gibberellin and paclobutrazol in rooting media of Chrysanthemum plantlets in hardening process of these plants. Furthermore, Wang and Steffens (1987) showed that in vitro treatments of plantlets with paclobutrazol upshot shift partitioning of assimilates from the leaves to the roots, increased carbohydrates, soluble protein and minerals in leaf tissues, raise chlorophyll content and root respiration rate, alleviating cell wall polysaccharides and water loss and assembling of stress hormone (abscisic acid, ABA). High ex vitro survival rate of citrus plantlets was achieved with the help of paclobutrazol preconditioning of media by Hazarika et al. (2000a). Introduction of paclobutrazol in culture medium produced stomata unlike normal stomata due to a general reduction in cell expansion caused by anti-gibberellin activity (Hazarika et al. 2001). Use of saturated KCl solution in culture vessels to reduce the relative humidity (RH) decrease the stomatal aperture and density was explained by Asayesh et al. (2017). Triadimefon increases both stomatal resistance (*Lycopersicon esculentum*) and shoot water potential (*Lycopersicon esculentum* and *Phaseolus vulgaris*) as reported by Smith et al. (1991). The strategies discussed above, advocated to acclimatize plantlets during in vitro culture by exposing them to plant growth retardants or by reduced humidity.

IBA (Indole-3-butyric acid) is a promising growth regulator used widely or large scale in plant tissue culture for root induction. Bari Banana 1 cultivar showed

maximum survival up to 95–100% after 15–20 days of in vitro culture on IBA (0.4–0.6 mg/L) with 7 days hardening at room temperature (Molla et al. 2004). In another study, IBA treated apple shoots followed by acclimatization in plastic-covered pots gave 70%–100% vigorously growing shoots in the greenhouse (Bolar et al. 1998). Application of two derivatives of auxin (1.0 mg/L NAA + 1.5 mg/L IBA) together in the culture enhanced in vitro rooting, survival rate and shoot growth during the acclimatization stage in citrus cultivars (Soares and Miranda 2016). Polyethylene glycol (PEG) acts as an osmotic agent to the tissue culture media during the rooting stage of plantlets, which further induces water stress conditions before transferring to the ex vitro environment. PEG helps in increasing the epicuticular wax deposition and cause stomatal closure, reducing the water loss which further increases the survival rate of cultured plants. Zein El Din et al. (2020) reported a 90% ex vitro survival rate of date palm (*Phoenix dactylifera*) when preconditioned with 20 g L^{-1} PEG in vitro.

3 Biotic Techniques/Approaches for Acclimatization

In natural conditions, each plant is colonized by specific either external or internal microorganisms. Some bacteria and fungi are reported to enhance plant performance under stress conditions and improve yield. Plants infected with microorganisms, develop systemic resistance (Induced systemic resistance or Systemic acquired resistance) and/or benefit from their antagonistic abilities towards pathogens (cross-protection). Plant tissue culture is traditionally performed in aseptic conditions thus, the surface sterilize of any explant is done to eliminate the microorganisms in in vitro cultures. The role of PGPMs in plant tissue culture has been confirmed so more attention has been paid to the favourable effects of these microorganisms in the better growth and acclimatization of in vitro plantlets. In this regard, the use of PGPMs in vitro and ex vitro cultures, in micropropagation is called as biotization. Recently, microbial inoculants, predominantly mycorrhizal and bacterial have been appraised as priming agents both as in vitro cultures and on transplanting. Standard microbiological techniques permit culturing of only a few naturally occurring microorganisms. The progress of new culture methodologies that allow the balanced union between plants and favourable microorganisms, both in vitro and ex vitro, under different environmental conditions is a major challenge of future research.

3.1 Function of Plant Growth-Promoting Fungi (PGPF) In Vitro Plant Culture

In vitro plants have a weak root system, incapable of absorption of nutrients from the soil during the early stage of the weaning step. The importance of inoculation with arbuscular mycorrhizal fungi (AMF) in vitro is a major tool to cope with this problem. Akin-Idowu et al. (2009) explained that AMF affects the ex vitro performance of tissue-cultured grown plants through biosynthesis of phytohormones by increasing nutrient availability and bring about resistance to pathogens. Phytohormones including auxins, cytokinins, abscisic acid, gibberellic acids, ethylene, jasmonic acid and salicylic acid are produced by some of the fungi which further activate signalling pathways during biotic and/or abiotic stresses and control plant development accordingly (Streletskii et al. 2019). A problem like the weak root system of tissue-cultured plants can be overcome by AMF through their arbuscules and hyphae which transfer nutrients from the soil to the plant (Chen et al. 2018).

Many times transferred plants are unable to find their natural microsymbiont partner which ultimately causes poor establishment and lower survival rate of tissue-cultured plants in ex vitro conditions. In this context, one of the studies carried out on Hydrangea plant by Varma and Schuepp (1994) showed that in vitro plants inoculated with the Arbuscular Mycorrhizal Fungi (AMF), *Rhizophagus intraradices* were strongly mycorrhized at the acclimatization stage with 100% survival rate. In the same lane of work, Diez et al. (2000) reported the increased formation of secondary roots and survival after acclimatization of cork oak plantlets when in vitro mycorrhized with *Pisolithus tinctorius* and *Scleroderma polyrhizum* strains. Improved post-transplantation survival rate (90%) of micropropagated tobacco (*Nicotiana tabacum* L.) and brinjal (*Solanum melongena* L.) plants was observed when inoculated with endophytic fungus, *Piriformospora indica* (Sahay and Varma 2000). Some reports on successful biotization with endophytic fungi are listed in Table 12.1.

It is broadly accepted that deforestation and forest degradation occurring at faster rate than their natural regenerated or artificial replanted. The benefits of in vitro plant regeneration in forestry as in afforestation and reforestation programmes have been recognized. In natural conditions, most of the forest trees species depends on the symbiotic roots association with ecto and/or endomycorrhizal fungi. Due to the absence of mycorrhizal fungal symbionts, forest trees are often failed to establish at new sites. Therefore, it is important to allow the in vitro plants to form an effective mycorrhizal association, especially for tree species having a high mycorrhizal dependency to maximize the benefits of micropropagation. Biotization approach can be widely applicable to these forest tree species with little or no modification as explained by Ba et al. (2010).

Various findings by different researchers authenticate the use of in vitro mycorrhization for several forest spices such as *Quercus suber* L. and *Eucalyptus spp.* (Di-Gaudio et al. 2020), *Castanea sativa* (Martins 2008), *Cistus* spp. (Quatrini et al. 2003) and *Helianthemum* spp. (Morte et al. 1994). Success in developing

Table 12.1 Effects of growth regulators produced by microorganisms on plant development and morphology

Bacteria/fungi	Microbial phytohormones	Observed effects on explant
Bacillus megaterium MiR-4	Auxins	Root elongation in *Vigna radiata*
Azospirillum brasilense SR80 Sp245 and *A. halopraeferens*	Auxins (IAA)	Increases the effectiveness of clonal micropropagation of potato (*Solanum tuberosum* L.)
Pseudomonas, Arthrobacter and *bacillus Azospirillum*	Cytokinins (IBA and NAA)	Stimulated root biomass of *Platycladus orientalis*
Azospirillum lipoferum	Gibberellins	Elongate the stem and shoots of *Alnus glutinosa*
Streptomyces sp. strain DBT204	IAA and kinetin (KN)	Enhancing growth of tomato (*Solanum lycopersicum* L.) and chilli (*Capsicum annuum* L.) seedlings
Fusarium strain	Auxin	Significant increase in growth and all tested growth parameters for *Euphorbia pekinensis*

Source: Soumare et al. (2021). *IAA* indole-3-acetic acid; *KN* kinetin; *IBA* indole-3-butyric acid; and *NAA* naphthalene acetic acid

adventitious roots in hypocotyl cuttings of Scots pine (*Pinus sylvestris*) with two ectomycorrhizal fungi, *Pisolithus tinctorius* and *Paxillus involutus* was experienced by Niemi and Scagel (2007).

3.2 Function of Plant Growth-Promoting Bacteria (PGPB) In Vitro Plant Culture

In the year 1987, the first in vitro bacterization with favourable effects of *Pseudomonas putida* and *Pseudomonas fluorescens* on rooting and acclimatization of Primula micro shoots was reported by Digat et al. 1987. Generally, plant growth-promoting bacteria (PGPB) enhances growth by releasing phytohormones required for in vitro propagation. Phytohormones produced by microorganisms play a major role, especially when tissue-cultured plants are unable to produce endogenously under sub-optimal environmental conditions. Different strains belonging to *Bacillus*, *Pseudomonas*, *Rhizobium*, *Methylobacterium*, *Microbacterium, Bradyrhizobium*, *Enterobacter*, *Acinetobacter*a and *Rhodococcus*, and their PGRs have been characterized, quantified and tested in plant tissue culture. Auxins and cytokinin's biosynthesis are widespread among rhizobacteria, and different biosynthesis pathways have been identified by Amara et al. (2015). As explained by Zakharchenko et al. (2011) *Pseudomonas aureofaciens* gives better growth in potato (*Solanum tuberosum* L.) and strawberry (*Fragaria* × *ananassa*) plantlets at acclimatization, when inoculated before rooting. *Pseudomonas* strain promoted root growth of *Citrullus lanatus* (Nowak 1998) and some bacteria have also exhibited good potential for application

in vitro orchid cultivation, Bezerra et al. (2020) reported a positive impact of rooting-derived bacterial isolate in in vitro culture and plantlet acclimatization of *Oncidium varicosum*. Increased photosynthetic efficiency and biomass production in bacterial biotized tissue-cultured plantlets was shown by Elmeskaoui et al. (1995).

4 Ex Vitro Acclimatization of Plant Propagules

4.1 Abiotic Factors

Acclimatization of in vitro cultured plantlets to in vivo or greenhouse/field conditions is a crucial step in micropropagation. Successful acclimatization contributes towards optimum growth, establishment and survival of tissue-cultured plants. Conventionally, the acclimatization conditions are modified to wean the in vitro plants towards ambient relative humidifies and light levels. Whereas, novel techniques of ex vitro acclimatization include CO_2 enrichment and/or supplementary lighting. This reduces the acclimatization period under in vivo or greenhouses conditions. The most advanced ex vitro acclimatization procedure includes the use of 'acclimatization units' or climate-controlled greenhouses. The computer-controlled acclimatization units can regulate and control almost every aspect of the environment. All the aspects of the environment can be made to change from simulated in vitro conditions at transplantation to that of the greenhouse or open field, even weeks later. At the beginning of the hardening process, changes are made in small inclusion which is later increased. The main emphasis of any hardening process is on minimizing the water stress in the early stages of acclimatization in ex vitro. Acclimatization units escalate both the survival and growth rate of the transplant.

4.1.1 Anti-transpirants

The use of anti-transpirants to decrease the water loss during the acclimatization process has mixed results in the literature. Many leaf-surface coating agents (paraffin, glycerol and grease) promoted ex vitro survival of numerous herbaceous plants species (Selvapandiyan et al. 1988). Phenylmercuric acetate (PMA) and 2-chloroethyl trimethyl ammonium chloride (CCC) induced stomatal closure and delayed wilting in tomato plants as repo (Rao 1985). Other agents such as B-9 and PMA as reported by Amaregouda et al. (1994) showed high stomatal resistance in plantlets, while others like alachlor, sunguard, China clay and silica powder maintained moderate stomatal resistance. The relative water content followed the opposite trend in these treatments. Hazarika et al. (2000b) reported that 8 HQ 2 mL/L was effective in controlling water loss from in vitro-grown citrus plantlets and subsequently helps in ex vitro survival. Latex polymer in acclimatization of tissue-cultured walnut plantlets was used by Voyiatzis and McGranahan (1994) and found

that the survival rate of plants dipped in latex was higher than that of the control. Plantlets treated with latex accumulated significantly more dry matter, apparently because their newly formed leaves were able to photosynthesize under favourable conditions of the open environment, longer than the control.

ABA acts as an anti-transpirant and reduces the relative water loss from the leaves during the process of acclimatization of cultured plantlets even in the presence of non-functional stomata (Pospisilova et al. 2007). Pospisilova et al. (2007) also claimed that acclimatization was improved by the positive effect of ABA on chlorophyll *a* content and other photosynthetic parameters as well as on plant growth. Wardle et al. (1979) successfully reduce the stomatal transpiration of micropropagated cauliflower (*Brassica oleracea* var. *botrytis*) plants with ABA (10 mM, leaf spray). Other reports also document the significant role of ABA in the acclimatization of tissue culture-raised plants (Hronkova et al. 2003).

4.2 Simultaneous Rooting and Acclimatization

Approximately 35–75% of the total cost of micropropagation has been estimated to account for the process of in vitro rooting. As per the reports by Thomas and Ravindra (2002), the natural environment with reduced light intensity could be used for the acclimatization of tissue-cultured plants thus reducing the cost of production and benefiting a tissue culture establishment with poor infrastructure and limited resources. Sorbarods (cellulose plugs) facilitates the proper handling of micro shoots and helped in the production of larger roots which enhances establishment of plantlets. Douglas et al. (1989) reported the improved rooting of micropropagated plants in compost when precultured for 2 weeks in vitro on sorbarods (soaked in a liquid medium containing IAA). Roat et al. in two different studies Rout et al. (1989a, b) reported 92–98 percentage survival of micropropagated hybrid roses cultivars when transferred to the greenhouse at 82–85% relative humidity and planted in earthen pots containing a mixture of sand, soil and cow dung manure (2:1:1). It is observed that micropropagated saplings of in vitro propagated red banana may be hardened by using red soil + sand + coco peat (1:1:1) combination (Uzaribara et al. 2015) which resulted in maximum survival up to 95%. Grand Naine, a well-known cultivar of banana reported the highest survival rate (100%) of micropropagated plants during acclimatization with the potting mixture soil: sand: FYM (2:1:1) and covering the plants with glass beakers (Ahmed et al. 2014). In grapevine, the hardening of plants using potting mixtures has been demonstrated for survival (Dev et al. 2019). The most suitable potting mixture for the hardening of micropropagated grape cultivars constituting coco peat + vermiculite + perlite (2:1:1) resulted in the highest survival (85.97%) of the plants within a period of 24 days.

4.3 Ex Vitro Biopriming

To follow the certification requirements of micropropagation, commercial plant tissue culture laboratories are hesitant to introduce microorganisms into their in vitro cultures. One approach is to wean plants in the rhizosphere with both beneficial bacteria and mycorrhizal fungi. In potatoes, the tissue-cultured plantlets are readily infected with bacteria present in the transplanting medium, both in external and internal tissues. Though attractive, this approach needs to address the problem of maintaining stable populations of the introduced microorganisms. The advantageous outcome of bacterial inoculation on tree species seedlings (Enebak et al. 1998) and greenhouse-produced potato tubers (Nowak et al. 1999) show the potential of post-*vitro* bacterization of tissue-cultured propagules. The post-*vitro* mycorrhization and bacterization of micropropagated potato, strawberry and azalea with certain combinations of bacteria and mycorrhiza-enhanced greenhouse production of mini-tubers, and a mixture of three strains of rhizobacteria improved the ex vitro performance of strawberries (Vosatka et al. 2000).

4.4 Mycorrhization

The inoculation with endomycorrhizal fungi was found beneficial to tissue-cultured plantlets and subsequent plant growth. Plant root colonization with vesicular-arbuscular mycorrhizae (VAM) improves water management, nutritional status and disease resistance. A variety of phenylpropanoid compounds induced by ectomycorrhizal inoculants have also been identified in several conifers. Such compounds are associated with biotic and abiotic stress management in plants. To improve the performance of tissue-cultured plantlets, rooted shoots are inoculated with mycorrhiza at the transplanting stage. The benefits of mycorrhization depend on the plant mycorrhizal species, growing medium and on the degree of root colonization. Murphy et al. (1997) observed a better establishment rate in mycorrhized plants with more runners in strawberry (*Fragaria* × *ananassa*) plants. Improved growth was observed after post-vitro transplant inoculation in garlic (*Allium sativum*) by Lubraco et al. (2000). Several studies have demonstrated concerted effects of VAM fungi and diazotrophic bacteria on the growth and nutrition of various crops (Paula et al. 1991). The creation of defined tissue culture micro-ecosystems could allow the study of complexed plant–microbial environment interactions, which could refine and improve our traditional in vitro propagation methods and prime the propagules for ex vitro environments.

5 Conclusions

Plant tissue culture is an efficient and quick method of clonal propagation that increases the production of important horticulture and forest crops. However, the process is complex due to the high mortality rates during the process of acclimatization. Micropropagated plants lack the cuticle layer, stomata closure and have poorly developed roots which result in transpiration loss when transferred to ex vitro conditions. For improvement of tissue-cultured plant survival and expediting, the acclimatization process various in vitro and ex vitro approaches are developed which includes in vitro priming of propagules with growth regulators and simple adjustments to the growing environment and in vitro and ex vitro biotization with competent plant growth-promoting microorganisms (PGPMs). The use of microorganisms used in in vitro plant propagations requires careful monitoring for endophytic microbes' communities, especially in plants species that are consumed as raw (in the case of salad vegetables) because some pathogenic strains which are harmful to humans can be steadily cultivated in cultured tissues and ex vitro plants. Further research is needed to identify the most appropriate candidates in plant growth-promoting microorganisms and to develop suitable bioformulations and their effects on plant tissue culture.

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Chapter 13
Evaluation of Genetic Stability of In Vitro Raised Orchids Using Molecular-Based Markers

Priyanka Mohapatra, Asit Ray, and Sudipta Jena

Abstract Orchids comprise some of the world's most valuable floricultural and ornamental plants. Apart from their beauty, they also possess valuable phytochemicals like alkaloids, flavonoids, bibenzyl derivatives, and phenanthrenes that are responsible for their medicinal properties. Unfortunately, several orchid species are considered to be threatened due to over-exploitation by orchid lovers and pharmaceutical companies. The conventional mode of propagation is not suitable for conserving orchids because of their delicate seeds. Therefore, micropropagation serves as an age-old reliable alternative method for propagating orchids. However, in vitro regeneration's major concerns are genetic instability of the in vitro regenerants, which might be due to somaclonal variation. Several factors such as media composition, the concentration of plant growth regulators, and culture duration might induce somaclonal variation. Therefore, evaluation of genetic stability of in vitro raised orchids is necessary for large-scale cultivation. DNA-based molecular markers such as RAPD, RFLP, AFLP, and ISSR are commonly used markers to evaluate the genetic stability of the in vitro regenerants. With the advancement of molecular techniques, advanced markers such as mitochondrial and chloroplast-based microsatellites and retrotransposon markers are being used as they reveal genetic information through increased genome coverage. Nowadays, flow cytometry analysis is also being used to ascertain the genetic fidelity of in vitro raised plants by evaluating the ploidy level and genome size of in vitro propagated plants. This chapter summarizes the employment of different molecular markers used for assessing the genetic fidelity of in vitro raised orchids.

Keywords Molecular-based markers · Somaclonal variation · RAPD · ISSR · Flow cytometry

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1 Introduction

Floriculture is a division of horticulture that deals with the planting of flowering and ornamental plants and involves cut flowers, flowering plants, potted plants, cut greens, and houseplants. Orchids hold the top position among all flowering plants valued for floricultural (cut flowers) and ornamental (potted) plants (Kumar et al. 2018a, b). They belong to the family Orchidaceae which is one of the most diverse and largest families approximately comprising of 30,000–35,000 species under 850 genera (Hossain et al. 2013). They are found mostly in all places around the planet excepting hot deserts and severely cold places like Antarctica, but their maximum varieties are found in the countries having tropical and sub-tropical climate (Hossain 2011). The exotic beauty and the fragrance, varieties in color, structure, range in sizes, and shapes available for orchids serves as an attraction for botanists and horticulturists alike (Dressler 1993).

Currently, orchids monopolize the international floriculture market for cut flower trade due to its long-lasting charm, right seasonal blooming, high productivity, convenient packaging, and transportation (De et al. 2014). Countries like Taiwan, Brazil, Italy, Thailand, Japan, New Zealand, and the UK are the biggest exporters of potted orchids whereas the United States stands first in import (Chugh et al. 2009). In the year 2005, in the United States alone the wholesale value of potted orchids was US$ 144 million (U.S. Department of Agriculture 2006). Thailand earns US$ 30 million every year and is ranked sixth among the world's largest exporter of orchids, while the earnings of Singapore was reported to be US$ 16 million per year only from orchid exports (Reddy 2008). In 2012, the annual business of the global orchid trade was reported to be US$ 504 million involving more than 40 exporting and 60 importing countries worldwide (De 2015).

In addition to their high commercial value orchids are also used as herbal medicines and food by various tribes and many cultures (Arditti 1992). Orchids are known to contain phytochemicals such as alkaloids, flavonoids, bibenzyl derivatives, phenanthrenes, glycosides, and triterpenoids, (Singh and Duggal 2009) which are responsible for exhibiting antimicrobial, antitumor, anti-inflammatory, and antiviral activities (Singh et al. 2012a, b). Apart from that orchids have been used to treat many diseases and ailments such as chest pain, arthritis, tuberculosis, paralysis, cholera, stomach disorders, acidity, jaundice, syphilis, eczema, piles, inflammations, boils, tumor, diarrhea, blood dysentery, menstrual disorder, muscular pain, hepatitis, malaria, dyspepsia, bone fractures, rheumatism, earache, sexually transmitted diseases, wounds, asthma, and sores. But unfortunately, they are considered to be the most vulnerable of all flowering plants because of rampant collection by growing ayurvedic medicine companies, orchid lovers, deforestation, illegal trade, depletion of pollinator, genetic drift, population fragmentation, and human-induced pressures (Hossain 2011). Some orchids species like *Aphyllorchis gollani, Anoectochilus rotundifolius, Coelogyne treutleri, Pleione lagenaria,* and *Paphiopedilum charlesworthii* are already extinct from the Indian haunts, while many species like *Rhynchostylis and Paphiopedilum delanti have* become rare and

endangered (Chugh et al. 2009). The major reason for the dwindling orchid population is over-exploitation.

Conventional methods of propagation are not practiced to grow orchids because the seeds although formed in huge amounts in each capsule are very friable and contain practically no reserved food or endosperm (Mitra 1971). Another reason is that orchids are inherently slow growers, and their development is determined by specific microclimatic conditions and floristics canopy protecting them in their natural habitats (Chugh et al. 2009). Moreover, in the case of horticultural plants in vitro propagation using seedlings is not preferred due to the lengthy juvenile period prior to flowering (Decruse et al. 2003). In fact, it takes approximately 2 years for an orchid plant to reach its flowering stage in the tropical climates. Therefore, in vitro propagation has opened many possibilities for the commercial utilization of orchids as it can produce disease-free, high-yielding, and genetically uniform orchids throughout the year (Vij 2002). However, the greatest drawback for in vitro propagation is the development of somaclonal variation that alters the true-to-type nature of in vitro raised plants (Krishna et al. 2016). Thus, assessing the genetic variability of the tissue culture regenerants periodically is crucial for the mass-scale production of true-to-type plants (Larkin and Scowcroft 1981). Different strategies like morphophysiological, cytological, and biochemical approaches are available to evaluate the genetic stability of micropropagated plants (Alizadeh et al. 2015). Thus, the aim of this chapter is to present an elaborate account of potential applications of different markers for the assessment of genetic stability of in vitro propagated orchids.

2 Importance of Orchids

Orchids are known to be among the top selling flowers in the world market for potted plant trade and orchids capture 10% share of the international floricultural market for fresh cut flowers trades. In the year 2015, Netherlands alone exported potted orchids worth approximately €500 million, this serves as a significant global trade for any country. The biggest producers of orchids in the world, Thailand, Taiwan, Netherlands, and Japan, are growing their economic value every year as the demand for both cut flowers and potted plants is increasing globally (Hinsley et al. 2018).

Orchids are valued not only for their ornamental values but also for their medicinal properties worldwide (Bhattacharyya et al. 2015). Many orchid species are used in various countries for their therapeutic properties (Hossain 2011). Orchids have been used both in the Indian and Chinese systems of traditional medicine since Vedic periods (Kimura and Migo 1936; Lüning 1974; Kaushik 1983). Orchids are a rich source of phytochemicals such as anthocyanins, flavonoids, alkaloids, carotenoids, and sterols which are responsible for their medicinal properties (Hossain 2011). In various parts of Malaya, the liquid squeezed from the boiled leaves of *Nervilia aragoana* is given to the women to drink immediately after childbirth to prevent possible sickness (Duggal 1971). In Africa, the leaves and pseudo bulbs of *Ansellia*

africana are mixed together to form a paste which is put inside an amulet to use as a short-term contraceptive (Berliocchi 2004; Saleem 2007). The hot juice extracted from roasted fruits of *Bulbophyllum vaginatum* is used to treat earache (Lewis 1886). *Goodyera pubescens* known as "Downy Rattlesnake Orchid" found in North America were used to treat the bite of a mad dog and to cure scrofula (Duggal 1971). In North America, many species of *Cypripedium* were used to counter insomnia, spasms, and nervous tension (Wilson 2007). Different species of Eulophia were used to cure barrenness and prevent miscarriage by the Africans also, powdered *Eulophia flaccida in* were put over cuts made on the skin of painful limbs to give fast relief from pain (Hossain 2011). In Malawi, *Cyrtorchis arcuata* is used to treat diabetes and skin infections and *Eulophia cucullata* is used to cure epilepsy (Davenport and Bytebier 2004). Leaves and roots of *Habenaria intermedia* found in the Eastern Himalayan region are used to treat diseases of the blood (Singh 2006). In the southern part of Indian sub-continent, powdered flowers of *Vanda spathulata* are used for treating asthma and mania (Khory 1982).

In addition to its medicinal properties, orchids are also consumed by humans all over the world, certain edible products, like vanilla flavorings and several eatable products, are utilized universally. *Vanilla planifolia* Andrews is the main species followed by the hybrid *Vanilla* × *tahitensis* J.W. Moore that ranks second most cultivated species of vanilla used for food trade (Lubinsky et al. 2008a, b). The tubers of orchids that are rich in polysaccharides are employed to prepare a healthy beverage called salep, in Turkey, and Mediterranean countries. More than 35 species of orchids such as Anacamptis Rich, Steveniella Schltr, Dactylorhiza Neck. ex Nevski, and Himantoglossum Spreng are traded for preparing salep powder (Hinsley et al. 2018). The fragrant leaves of *Jumellea rossii Senghas* and *Jumellea fragrans* (Thouars) Schltr. are employed in Creole medicine (Longuefosse 2006), and they have been traded for a long time to flavor rum and produce "Bourbon tea" or "Madagascar tea" in certain islands of the Indian Ocean (Decary 1955).

Since ancient times orchid flowers are marketed for their use in a broad array of cultural and religious ceremonies. In Sri Lanka, flowers of *Dendrobium maccarthiae Thwaites* are offered as a special offering in temples, whereas flowers and pseudo bulbs of *Laelia* Lindl are offered in Mexican Day of the Dead ceremonies (Duggal 1971). Orchid flowers are also called the national flower of Myanmar, while species like *Bulbophyllum auricomum* Lindl and *Bulbophyllum sukhakulii* Seidenf are commonly used to decorate women's hair (Goh 2013).

3 Somaclonal Variation and Significance of Genetic Stability in Tissue Cultured Plants

Orchid propagation in the industries is mostly done by two approaches one by tissue culture (flower stalk nodal culture or meristem culture or by axillary shoot tip culture) and another approach is in vitro seed germination. The multiplication rate

is higher for the tissue culture method but with the risk of somaclonal variation (Chen et al. 1998). Somaclonal variation is known as variation seen in plant variants originating from cell or tissue cultures (Larkin and Scowcroft 1981). These kinds of variations may occur spontaneously and are largely uncontrolled is of two different types viz., changes caused by genetic (permanent genetic change undergone by cells) and those induced by temporary changes undergone by cells or tissues and are stimulated either by genetic or environmental conditions (Pierik 1987; Karp 1994). Certain factors like application of plant growth regulators (PGR) in higher amounts, maintaining cells and tissues under microenvironment for prolonged periods, and the stress caused during regular subculturing of cultured cells and tissues are responsible for inducing somaclonal variation in the in vitro regenerants (Rani and Raina 2000; Jain 2001). Besides that, the kind of explants utilized for culture initiation likewise plays a crucial role in inducing somaclonal variations in tissue cultured regenerants. Generally, plants propagated via intermediate callus phase are believed to be most susceptible to somaclonal variation (Nookaraju and Agrawal 2012). Several studies proposed that plantlets regenerated via axillary branching are least vulnerable to genetic changes that happens under in vitro conditions and maintain genetic integrity and therefore are considered to be reliable for clonal propagation (Negi and Saxena 2010; Singh et al. 2012a, b).

The occurrence of cryptic genetic defects due to somaclonal variation in the in vitro regenerants pose a threat to the vast utility of micropropagation systems (Peredo et al. 2009). In order to preserve an elite genotype, it is very important to maintain identical nature of the in vitro raised plants with their mother plant to maintain high degree of genetic uniformity among the micropropagated plants. The development of somaclonal variation poses a demerit for both in vitro cloning and preservation of germplasm. Hence, it is very important to confirm the genetic uniformity of micropropagated plants beforehand (Hammerschalg 1992). Some significant variations induced by somaclonal variations are growth rate and modifications of reproductive apparatus (sterility, internodal length, precocious flowering, and flower abnormalities), and leaf (albino, variegation, chlorotic, etc.), fruit color, increased salt resistance and thornlessness, and isoenzymatic activity changes. Therefore, explant selection, culture conditions, culture age, genotype, and method of plant regeneration are significant factors that determines genetic stability of the regenerated plant (Rout et al. 2006).

4 Tools for Evaluating Somaclonal Variation

Somaclonal variation is defined by complex changes, which are shown at several levels like phenotypic, cytological, genetic/epigenetic, and biochemical (Kaeppler et al. 2000). Thus, the approach for identifying somaclones has to depend on such manifestations. Several tools are available for the identifying and characterizing somaclonal variants which are basically based on differences in morphological,

cytogenetical, and molecular markers (Krishna et al. 2016). Some of these tools have been shown in Fig. 13.1.

4.1 Morphological Markers

This is one of the primitive and widely used methods to identify variants in tissue cultured plants. Somaclonal variants can be easily differentiated according to the variation in leaf morphology, plant height, canopy structure, and pigmentation abnormality (Israeli et al. 1991). But morphological features used for phenotypic characterization are minimal in number, and frequently affected by environmental factors, which many times do not show the true genetic make-up of a plant (Cloutier and Landry 1994). Moreover, the detection based on the morphological traits of off-types among in vitro raised plants is time-taking and is not suitable for perennial crops, where several observations are noted until maturity. Apart from this, all the genetic changes may not appear in the phenotypic changes that are observed leading to incorrect interpretation.

At times to improve efficiency for identification of clonal variants morphologically and physiological traits are also taken into consideration. Features such as diameter and thickness of leaf vascular tissue, leaf aquiferous parenchyma, stoma diameter, number of stomata per square millimeter, thickness of the leaf photosynthetic parenchyma, photosynthetic rate, chlorophyll pigment contents, transpiration rate, water use efficiency, and internal leaf CO_2 concentration are also taken into account (Alizadeh et al. 2015). Faria et al. (2004) reported a reduction in plant height, number of shoots, and root length of *Dendrobium nobile* plants when (photoheterotrophic) culture medium was not supplemented with sucrose and white fluorescent lamps were used for culture. Jeon et al. (2005, 2006) examined certain factors like the morphology, photosynthesis, and growth parameters of *Doritaenopsis* for 4 months post acclimatization. It was observed that highest value for area, length, chlorophyll a/b ratio, and fresh and dry weight of leaves were obtained when acclimatization took place under intermediate photosynthetically active radiations (PAR) (270 μmol m^{-2} s^{-1}) when compared to low (175 μmol m^{-2} s^{-1}) PAR and high (450 μmol m^{-2} s^{-1}) PAR. Moreover, net transpiration, CO_2 assimilation, and stomatal conductance were higher when plants were acclimatized under higher light conditions. Furthermore, highest wax formation and thick velamen layer of leaves were found in plants acclimatized at high PAR as indicators of adaptation to ex vitro conditions. Phenotypical alterations (elongated and fascinated leaves) were observed in the in-vitro raised *Dendrobium* (Second Love) during the culture period (Ferreira et al. 2006).

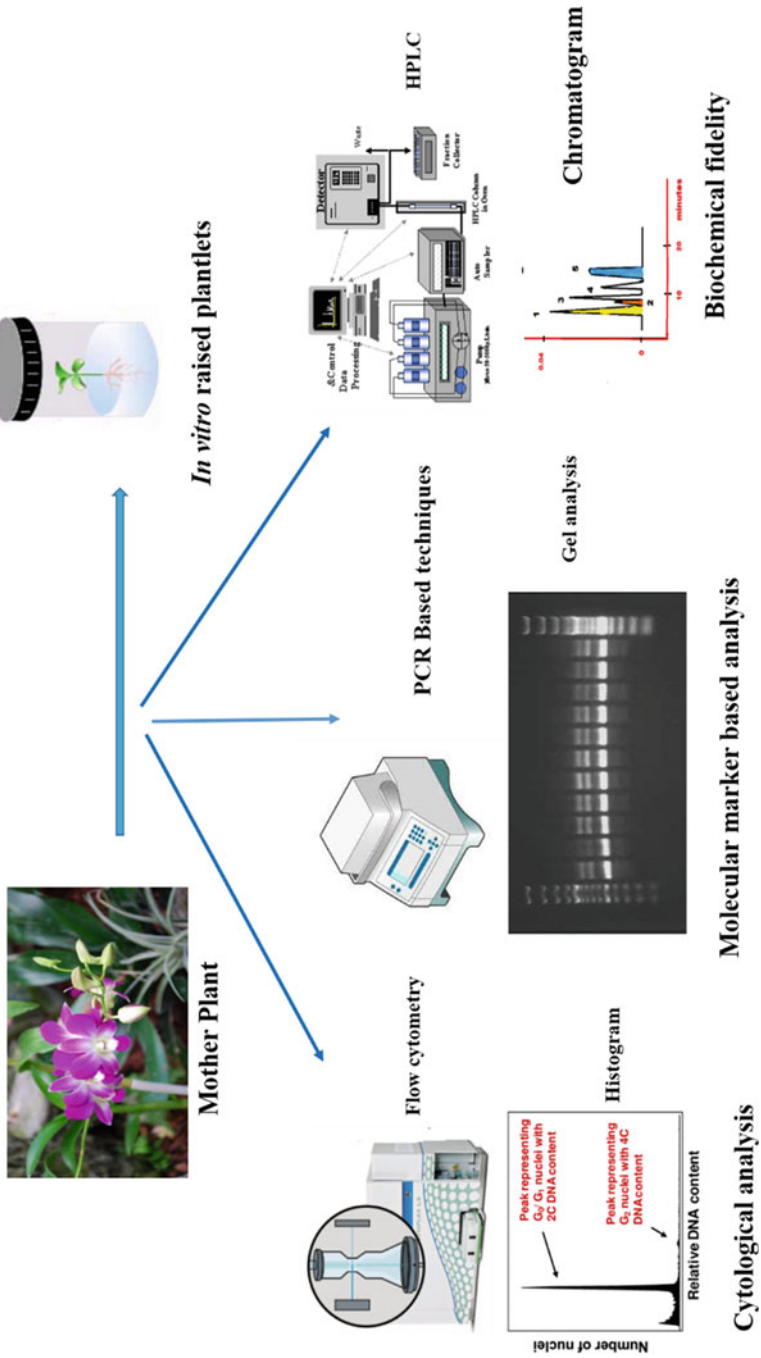

Fig. 13.1 Tools for evaluating somaclonal variation of orchids

4.2 Cytological Analysis

Cytological markers are markers that are associated with variations found in the size, shape, numbers, orders, banding patterns, and position of chromosomes or it indicates to the chromosomal banding generated by different stains such as G banding (Jiang 2013). Several workers have used the observations based on oil immersion, conventional staining, light microscopy, and other complex microscopy techniques to carry out cytological analysis (Bennici et al. 2004; Ryynänen and Aronen 2005). But these are accompanied by various limitations like generally inconvenient to observe when chromosome number is high or smaller in size (Rani and Raina 2000). Nowadays, flow cytometry has replaced the conventional method of counting chromosomes (Dolezel et al. 2004).

4.2.1 Karyotyping

The karyotype is the complete set of chromosomes present in a species; it is arranged with respect to size, shape, and number of a cell chromosome complement, wherein single chromosomes can be identified (Gianfranco et al. 2008). In the past, generally it was assumed that variants among somaclones resulted due to gross karyotype changes like aneuploidy or polyploidy. Changes due to gross karyotype alterations have been frequently observed in several in vitro propagated plant cells (Larkin and Scowcroft 1981). Cytological analysis was carried out for assessing cytogenetic stability of *Spathoglottis plicata* Blume-derived mother plant and artificial seed-derived plants. The mother plant and artificial seed-derived plants were reported to have no ploidy change during in vitro dedifferentiation, redifferentiation, and encapsulation as there was no change in the chromosome number ($2n = 40$), this confirmed that all the encapsulated protocorm-like body (PLB)-derived plants were cytologically stable (Haque and Ghosh 2017). A study was undertaken by Meesawat et al. (2008) to evaluate the DNA content of the selected *Dendrobium crumenatum* popularly known as pigeon orchid from both mother and in vitro raised plants. The mother plant and the first system tissue cultured plants of *D. crumenatum* were observed to be diploid ($2n = 38$). The experiment demonstrated stability and were concluded to be an efficient estimation method to check ploidy level to establish genetic stability of tissue cultured plants, but variation was observed in the chromosome number due to its subculture for prolong period and the presence of growth regulators. In *Bulbophyllum auricomum* genetic stability the chromosome number analysis of micropropagated clones of each seed-derived tissue cultured plant showed the occurrence of same chromosome number as their respective mother plants (Than et al. 2011).

4.2.2 Flow Cytometry

Somaclonal variation is known to cause variations in chromosomal rearrangements and polyploidization or aberrations, and mutations in in vitro propagated plants. Generally, chromosome counting is used to evaluate ploidy, but it is time-taking and counting gets difficult specifically when the chromosomes are large in number or small in size; therefore, this method may be substituted by flow cytometry (FCM) (Sliwinska 2018). Nowadays, FCM is widely used in tissue culture of horticultural plants to evaluate plant genome size stability following tissue culture and compare the nuclear DNA content of tissue cultured plants with its donor plant to ascertain genetic stability (Choudhury et al. 2014). Some authors have reported the use of FCM to ascertain genetic stability in case of several in vitro-raised orchid species (Table 13.1).

4.3 Biochemical Markers

They are mainly classified as protein-based markers or phytochemical markers (Kumar et al. 2019).

Table 13.1 Assessment of genetic stability using flow cytometry

Orchid species	Variation detected	Reference
Oncidium flexuosum Sims	No changes in ploidy level were found in seedlings that developed from cryopreserved seeds	Galdiano et al. (2013)
Dendrobium crumenatum	There was no variation in the 2C DNA content ($p < 0.05$) between mother plants (greenhouse grown) and the first in vitro raised plants in the culture system (2.30 ± 0.14 pg $2C^{-1}$)	Meesawat et al. (2008)
Eulophia nuda Lindl	The results showed that there was no variation in the ploidy levels of seeds germinated and in vitro raised plants with their mother plants	Nanekar et al. (2014)
Spathoglottis eburnea Gagnep	There was no change in the ploidy level in the tissue cultured plants and mother plant	Pornchuti et al. (2016)
Dendrobium Swartz. Hybrid	FCM analysis showed that even seedlings regenerated from seeds exposed to a plant vitrification solution 2 for 6 h showed no changes in ploidy and thus, confirmed the genetic stability of plants	Galdiano et al. (2014)
Malaxis wallichii	No significant difference in the 2C DNA content between the mother (2.76 ± 0.02) and in vitro-derived plants (2.75 ± 0.02)	Bose et al. (2017)
Coelogyne cristata	Same ploidy levels between mother plants grown in the greenhouse and tissue culture-generated plants	Naing et al. (2011)
Catasetum pileatum	Flow cytometry histogram showed two peaks of nuclear DNA content, which concluded that the plantlets were mixoploids	Kazemi and Kaviani (2020)

4.3.1 Protein Markers

Isozymes are the generally used tools in protein-based marker. Isozymes are protein markers that differ in amino acid sequence but catalyze the same chemical reaction. The variation in somaclonal variants can be identified on the basis of three categories as loss/gain of protein bands, modified electrophoretic mobility, and modified level of specific protein. Chen et al. (1998) evaluated the isozyme patterns in normal and variant somaclones of Phalaenopsis. They screened 11 enzymes, out of which 2 (aspartate aminotransferase (AAT) and phosphoglucomutase (PGM)) revealed clear and consistent isozyme pattern. AAT and PGM isozyme bands that were expressed in normal somaclones were more in number than in variant, indicated that there was repression of genes in the variant somaclones that were normally present in the normal somaclones.

4.3.2 Phytochemical Markers

Orchids contain a broad array of bioactive compounds. Generally orchid phytochemicals are polyphenols, flavonoids, carotenoids, alkaloids, and sterols (Mahendran and Bai 2016). Tissue culture techniques are known to enhance the production of various bioactive components like flavonoids, tannins, phenols, and triterpenes under in vitro conditions which significantly results in bio-enrichment of plants (Rajput and Agrawal 2020). Therefore, evaluation of bioactive compounds of micropropagated plants is very essential. The advancement of modern techniques likes liquid chromatography and mass spectrometry has made the evaluation of bioactive compounds very convenient. Several authors have evaluated the presence of these bioactive compounds in several in vitro raised orchid species (Table 13.2).

4.4 DNA-Based Molecular Markers

DNA sequences that can be recognized, found at particular site of the genome, and transmitted from one generation to the next in accordance with the laws of inheritance are called as molecular markers (Semagn et al. 2006). They may or may not correspond with phenotypic expression of a trait. Markers that show polymorphisms at the DNA level are DNA-based markers. (Kumar 1999). They consist of unlimited number of primers which can scan coding along with the non-coding domains of the genome (Rani and Raina 2000). The major advantage of DNA markers is that they are expressed in all tissues, not affected by the environment, and may be scored at all phases of plant growth (Agarwal et al. 2008). By using DNA markers the occurrence of somaclonal variation can be detected if present in the nuclear, chloroplast, and mitochondrial genome separately. DNA markers can be further classified into two groups: hybridization-based polymorphisms and PCR-based polymorphisms

Table 13.2 Assessment of genetic stability using biochemical markers

Orchid species	Variation detected	Reference
Habenaria edgeworthii	HPLC analysis showed the occurrence of two phenolic compounds (gallic acid and hydroxybenzoic acid). A threefold higher amount of hydroxybenzoic acid (20.7 mg/100 g DW) was noted in in vitro raised tuber as compared to wild (7.6 mg/100 g DW). But negligible difference was observed in the amount of gallic acid in both types of tubers	Giri et al. (2012)
Aphyllorchis Montana	The phenolics content of the wild grown plant (121.34 ± 1.23 mg/g) was significantly higher than that of the in vitro grown plant (61.89 ± 3.19 mg/g). The flavonoid content of the methanol extracts indicated that wild grown plant (93.62 ± 1.17 mg/g) had significantly higher flavonoid content than tissue culture regenerated plants (34.59 ± 1.14 mg/g)	Mahendran (2014)
Aerides odorata	The screening of the extracts showed that leaves (111.62 ± 2.40 mg/g dry extract GAE) and roots (189.65 ± 3.75 mg/g dry extract GAE) of in vitro calli contain higher phenolic content than mother plant	Prasad et al. (2016)
Herminium lanceum	The occurrence of gallic acid, p-hydroxybenzoic acid, syringic acid, and caffeic acid was found in leaves and tubers of both in vitro and in vivo plants. The leaves and tubers of in vitro plants contained higher content of gallic acid and syringic acid than those of in vivo plants	Singh and Babbar (2016)
Dendrobium fimbriatum	The HPLC analysis of the tissue culture-generated plantlets of *D. fimbriatum* (leaves, roots, and stems) revealed that the leaves contained the highest amount (5.89 mg/g) of β-sitosterol as compared to the stems and roots. Also, β-sitosterol content was higher in in vitro raised plants compared to the control	Paul et al. (2017)
Ansellia africana	Ultrahigh performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) analyses revealed that meta-topolin derivatives and thidiazuron significantly improved the synthesis and bioaccumulation of both benzoates and cinnamates in the cultured protocorm—like—bodies biomass in comparison to control	Bhattacharyya et al. (2019)
Bulbophyllum odoratissimum	HPLC analysis revealed that phytochemical components detected tissue culture regenerated plants were similar to those found in mother plant and there was negligible difference	Prasad et al. (2021)
Satyrium nepalense	HPLC analysis of therapeutically important phenolic acids of leaves and tubers of in vitro regenerated and in vivo plants showed the occurrence of higher amount of selected phenolic acids in in vitro regenerated plants than their mother plants	Singh et al. (2021)

(Kumar et al. 2019). An array of DNA-based markers have been used to evaluate the genetic stability of several orchid species which have been given below.

4.4.1 Hybridization-Based Polymorphisms

Restriction Fragment Length Polymorphism (RFLP) is the most extensively used hybridization-based molecular marker. The method depends on restriction enzymes that show difference in the pattern between sizes of the DNA fragment in individual organisms. The changes in nucleotide sequences due to mutations that causes shift in fragment size or any changes at the recognition sites of restriction enzymes events are detected by RFLP (Tanksley et al. 1989). RFLP markers are preferable because they are co-dominant, highly reproducible, have high locus-specificity and prior sequence information is not required. But there are some disadvantages of RFLP like it is time-taking, laborious, requires large amount of DNA and the need of each point mutation to be analyzed individually (Kumar et al. 2018a, b).

RFLP was used to estimate the genetic stability of tissue cultured orchid hybrid *Aerides vandarum*× *Vanda stangeana*. They amplified a DNA fragment with an approximate length of 930 bp extracted from the donor plant and each of its in vitro regenerants, representing the complete nrITS region containing of ITS1, ITS2, and 5.8SrDNA. There was no variation in length for the nrITS regions between the donor plant and the in vitro regenerants. Out of the nine restriction enzymes used, four viz., TaqI, AluI, HaeIII, and EcoRV had restriction sites in the ITS region. TaqI restricted the ITS region amplified by PCR into two parts (400 and 148 bp), AluI restricted into two parts (710 and 218 bp), HaeIII restricted into four fragments (250, 200, 181 and 111 bp), and EcoRV restricted into two fragments (520 and 410 bp). It was observed that for each of the four restriction enzymes there was no difference among the RFLPs of the amplified nrITS regions of the donor plant and its regenerants.

The matK region was amplified using the primers matK1 and matK1r, and no length variation was found for the matK regions among the donor and in vitro regenerants. The matK region was restricted into two parts (1222 and 719 bp) by AluI, three fragments (800, 539, and 422 bp) by TaqI, into two parts (872 and 486 bp) by DraI, into three parts (800, 700, and 365 bp) by EcoRI, and into two parts (968 and 852 bp) by EcoRV. Similarly, RFLPs of the PCR amplified matK region of the donor plant and tissue culture regenerants were recorded for each of the five restriction enzymes.

Similarly, six restriction enzymes viz., (EcoRV, HaeIII, AluI, DraI, EcoRI, and TaqI) were employed to restrict the PCR amplified trnL-F regions of the donor plant and its regenerants and the obtained RFLPs of the amplified regions were found to be identical (Kishor and Devi 2009).

4.4.2 PCR-Based Techniques

PCR is a molecular biology technique used for enzymatically amplifying small amounts of DNA without involving a living organism. After its invention in 1983, by Kary Mullis several approaches were detailed for generating PCR-based molecular markers, mainly because of its simplicity and reproducibility. There are two sub-categories of PCR-based techniques (1) sequence nonspecific techniques or arbitrarily primed PCR-based techniques (RAPD, ISSR, AFLP) (2) gene-targeted PCR-based techniques (IRAP, SCoT) (Semagn et al. 2006). In the following section, all the PCR-based markers used for evaluating the genetic stability of various orchid species has been given.

Arbitrarily Primed PCR-Based Techniques

Random Amplified Polymorphic DNA (RAPD)

RAPD depends on the PCR amplification of random segments of DNA with universal primers also known as random nucleotide sequences as no prior information of the genome to be analyzed is required. The two primers attach to complementary DNA sequences close enough for a successful PCR reaction. This is followed by visualization of amplified DNA products by gel electrophoresis (Kumar et al. 2018a, b). In case of RAPDs, the polymorphisms give no information about the heterozygocity but only detects the presence or absence of a band having a certain molecular weight (Jiang 2013). Several researchers have used RAPD to assess the genetic fidelity of orchids that have been given in Table 13.3.

Inter Simple Sequence Repeat (ISSR)

ISSRs marker is mostly used to evaluate the variation or genetic homogeneity in plant biology, due to its low cost and high reproducibility (Dangi et al. 2014). They are found in both nuclear and organellar DNA and are highly polymorphic (Pinheiro et al. 2012). ISSR primers are (15–30 primers) long and use high annealing temperature (45–60°C) (Tiwari et al. 2013). ISSR primers employed in this method are called as microsatellite and are available as di-, tri-, tetra-, or penta-nucleotide repeats, no prior information of DNA sequences is required. DNA segments present in the middle of two identical but oppositely oriented microsatellite repeat regions are amplified by the ISSR primers, and the amplified products (200–2000 bp) long and can be seen using agarose or PAGE (Nadeem et al. 2018). ISSR has been used to evaluate the genetic stability of various orchids species that have been shown in Table 13.4.

Table 13.3 Assessment of genetic stability using RAPD

Orchid species	Variation detected	References
Phalaenopsis (true lady "B79–19")	The RAPD data revealed that normal and variant somaclones were not genetically similar	Chen et al. (1998)
Dendrobium (second love)	RAPD analysis did not show any polymorphism	Ferreira et al. (2006)
Dendrobium nobile	No genetic variation was observed in the in vitro shoots derived from *D. nobile* (callus) that had been subcultured for as many as 15 cycles	Song et al. (2007)
Aerides vandarum×*Vanda stangeana*	Multiple shoots of the orchid hybrid regenerated by employing 2 mgl^{-1}TDZ was observed to be genetically stable	Kishor and Devi (2009)
Cymbidium giganteum	Emergence of new bands in the in vitro regenerants revealed the presence of genetic changes which indicated the development of new binding sites in the in vitro regenerants	Roy et al. (2012)
Cymbidium sp.	High variability was induced by tissue culture	Sorina et al. (2013)
Dendrobium nobile	11.11% polymorphism was observed among the regenerated orchids and mother plant	Bhattacharyya et al. (2014)
Paphiopedilum niveum	As revealed by the similar banding patterns, no genetic variation was detected between the in vitro-derived mother plants (V1) obtained from the original protocorm and the in vitro regenerant clones (V2 and V3) obtained from primary and secondary somatic embryos, respectively	Soonthornkalump et al. (2019)
Dendrobium moschatum	Low degree of polymorphism (4.8%) among the mother plant and the tissue culture regenerants	Tikendra et al. (2019a, b)
Dendrobium chrysotoxum	Low polymorphism (1.19%) among the regenerated orchids and mother plant	Tikendra et al. (2019a, b)
Rhynchostylis retusa	The amplified bands of the mother plant and all the samples of in vitro raised plants were identical and no polymorphism was recorded	Oliya et al. (2021)

Amplified Fragment Length Polymorphism (AFLP)

AFLP technology was developed to reduce the constraints of reproducibility linked with RAPD (Vos et al. 1995). It produces fingerprints of any DNA irrespective of its origin, and prior information of DNA sequence is not required (Agarwal et al. 2008). A study was conducted by Xiang et al. (2003) to assess the genetic relationship of well-known Dendrobium hybrids (used commercially) with AFLP analysis guide the breeders select parental plants for breeding. Their other aim was to assess the practicability of employing AFLP to generate a fingerprint profile of the orchid species that can be used to protect new plant varieties. Commercial Dendrobium hybrids were collected from many orchid farms across Singapore. Tissue culture mutants (two) along with their mother plant were also collected. They were similar to their mother plants in all other features except that they had less anthocyanin synthesis revealed by the presence of white colored or lighter colored flowers. The

Table 13.4 Assessment of genetic stability using ISSR

Orchid species	Variation detected	References
Aerides vandarum×*Vanda stangeana*	Monomorphic banding pattern obtained for both the mother plant and in vitro regenerants revealed genetic stability	Kishor and Devi (2009)
Anoectochilus formosanus	Polymorphism 2.76%	Zhang et al. (2010)
Habenaria edgeworthii	No variations in PCR product profile (pattern) observed for in vitro regenerated and mother plant for all primers tested	Giri et al. (2012)
Phalaenopsis gigantea	Monomorphic pattern of band for secondary protocorm-like bodies produced during the process of subculturing and its mother plant	Samarfard et al. (2013)
Dendrobium thyrsiflorum	4.76% variability in case of indirect shoot organogenesis and no variability in case of direct shoot organogenesis was observed	Bhattacharyya et al. (2015)
Dendrobium crepidatum	None was found to be polymorphic	Bhattacharyya et al. (2016a, b)
Anoectochilus elatus	Polymorphism was recorded to be 5.77 (direct somatic embryogenesis) and 6.90% (indirect somatic embryogenesis), respectively	Sherif et al. (2018)
Dendrobium aphyllum	4% polymorphism noted	Bhattacharyya et al. (2018a)
Dendrobium Sabin blue	Highest polymorphism (16.39%) was observed when Protocorm-like bodies were subcultured on culture media supplemented with kinetin	Chin et al. (2019)
Dendrobium moschatum	Polymorphism observed was 2% between mother plant and tissue cultured regenerants	Tikendra et al. (2019a, b)
Dendrobium chrysotoxum	Polymorphism noted was 2.53% between the mother plant and in vitro regenerants	Tikendra et al. (2019a, b)
Aenhenrya rotundifolia	1.41% polymorphism	Sherif et al. (2020)
Bulbophyllum odoratissimum	Low degree polymorphism	Prasad et al. (2021)

tissue culture procedure may have introduced an epigenetic modification in the genes that were involved in synthesis of anthocyanin. As there was no change in the DNA sequence, AFLP is not so effective in detecting such alterations.

Gene-Targeted PCRbased Techniques

Start Codon-Targeted Marker (SCOT)

SCoT is a simple, unique, and gene-targeted DNA marker that depends on the short conserved region flanking the ATG translation start codon in plant genes. Although this method is identical to RAPD or ISSR as a single primer is employed as the forward and the reverse primer (Collard and Mackill 2009), they are more efficient

Table 13.5 Assessment of genetic stability using SCOT markers

Orchid species	Variation detected	References
Dendrobium nobile	3.5% polymorphism was observed between donor mother plant and in vitro raised plants	Bhattacharyya et al. (2014)
Dendrobium thyrsiflorum	3.22% variability in case of direct shoot organogenesis and 8% clonal variability in case of indirect shoot organogenesis-derived plants	Bhattacharyya et al. (2015)
Dendrobium crepidatum	Polymorphism 10%	Bhattacharyya et al. (2016a, b)
Dendrobium nobile	Polymorphism 7.01%	Bhattacharyya et al. (2016a, b)
Ansellia africana	Polymorphism 7.14%	Bhattacharyya et al. (2017)
Ansellia africana	Polymorphism 7.27%	Bhattacharyya et al. (2018a, b)
Aenhenrya rotundifolia	0.51% polymorphism between donor mother plant and in vitro raised plants	Sherif et al. (2020)

than RAPD and ISSR in terms of their reproducibility, it has also been suggested that annealing temperature and primer length are not the only factors that determines the reproducibility of SCoT (Gorji et al. 2011). These markers are visualized by using the basic agarose gel electrophoresis which makes this method suitable for application in many of plant research labs having normal equipment (Collard and Mackill 2009). SCoT has been utilized to evaluate genetic stability of several orchid species shown in Table 13.5.

Inter-Retrotransposon Amplified Polymorphism (IRAP)

The presence of somaclonal variations may be associated with the movement of the transposable element (Larkin and Scowcroft 1981). Therefore, to investigate and identify the movement of retrotransposon in the genome, IRAP is a common technique which amplifies genomic distances between two long terminal repeats (LTR) which is a part of subclass of retrotransposons (Kalendar and Schulman 2006). Bhattacharyya et al. (2016a, b) evaluated the genetic stability of *Dendrobium nobile*, by screening 7 IRAP primers out of which 5 IRAP primers produced 26 amplifiable bands and polymorphism was recorded to be 7.69%. *Dendrobium aphyllum* was propagated using transverse thin cell layers as explant, genetic stability was estimated by screening 10 IRAP primers out of which 5 IRAP primers generated 26 reproducible bands. Variability was observed to be 7.26% (Bhattacharyya et al. 2018a, b). Similarly, IRAP primers were utilized to estimate the genetic stability of *Ansellia africana,* the variability between the mother plant and regenerants was found to be 7.69% (Bhattacharyya et al. 2018a, b).

5 Genetic Stability of Cryopreserved In Vitro Raised Plants

Cryopreservation is a potential method for storage of orchid plant material for a long period of time and can facilitate in the conservation of endangered orchids (Vendrame 2015). The purpose of cryopreservation is to inhibit the major biologic reactions in liquid phase (−196°C) of nitrogen or vapor (−150°C), facilitating the maintenance of genetic fidelity of vast range of plant explants such as seeds, in vitro buds, zygotic and somatic embryos, and suspension cultures without somaclonal variation for an infinite period (Kırdök et al. 2016).

Immature seeds of *Cyrtopodium hatschbachii* were cryopreserved utilizing encapsulation-dehydration technology. After 195 days in vitro regenerated plants from encapsulated and cryopreserved seeds along with in vitro regenerated plants from encapsulated seeds but not cryopreserved (control) were obtained. Genetic stability was established by observing the morphology and chromosome number (Surenciski et al. 2007). The effects of cryoprotectants such as Supercool X-1000Ò and phloroglucinol in the vitrification solution and the exposure time were examined to develop a cryopreservation protocol for mature seeds of *Oncidium flexuosum.* The genetic stability for plants regenerated from both cryopreserved seeds and untreated seeds were evaluated employing flow cytometry analysis. The resulting histograms were similar thus confirming no changes in ploidy level in seedlings developed from cryopreserved seeds (Galdiano et al. 2013). Targeted region amplification polymorphism (TRAP) and SCoT DNA markers were found to be effective techniques to assess potential genetic stability of cryopreserved protocorm-like bodies of *Dendrobium bobby* Messina (Antony et al. 2015). Cryopreservation by vitrification was found to be an efficient method for the preservation of immature seeds of the *Cattleya tigrina* and *Cattleya harrisoniana* × *Cattleya walkeriana* hybrid with high water contents and mature seeds of *Cattleya guttata* with a low water content, direct immersion in plant vitrification solution was not required. Genetic stability of the plants originated from non-cryopreserved seeds (control) and cryopreserved seeds was estimated by flow cytometry after 1 year of in vitro growth (Vettorazzi et al. 2019). RAPD and SCoT markers confirmed the genetic fidelity of regenerated protocorm-like bodies (cryopreserved) generated from *Aranda broga* Blue orchid hybrid (Khor et al. 2020). A flowchart showing the steps for assessing genetic stability of cryopreserved in vitro raised orchids has been shown in Fig. 13.2.

6 Conclusion

The major commercial tissue culture laboratories around the globe are associated with orchid micropropagation, which shows the popularity of the flower. The increasing popularity of this flower has generated a huge demand for orchids in the world market. Thus, the production of orchids commercially has become a lucrative industry in several parts of the world. Therefore, producing true-to-type

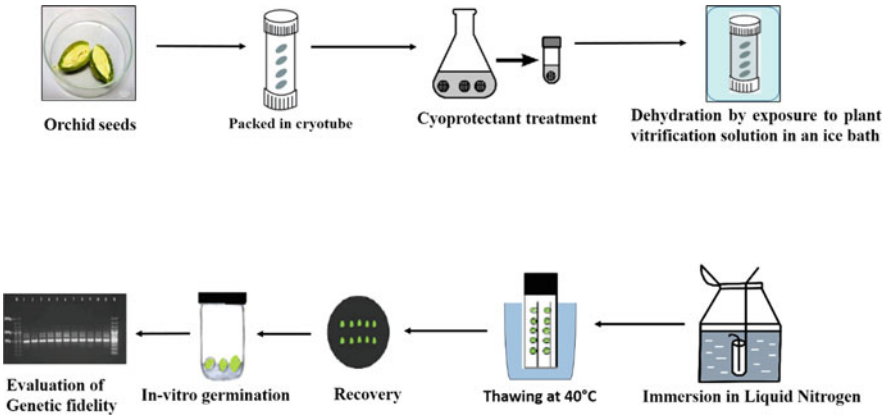

Fig. 13.2 Flowchart of assessing genetic stability of cryopreserved in vitro raised orchids

plant materials is an essential step in micropropagation so that it can be used for commercial utilization. Various approaches have been used to confirm the genetic stability of the tissue culture raised progenies. Though morphological and cytological assays remain the basic assay for detecting stability related to the generation of clonal plants. The effectiveness of the molecular tools with reference to their speed, sensitivity, and accuracy has facilitated the detection of somaclonal variants at an early stage. Studies in the past revealed that utilizing more than one DNA amplification method is more beneficial to assess somaclonal variations in orchids. This chapter summarizes all the assays used so far for evaluating the genetic fidelity of micropropagated orchids to the best of our knowledge.

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Chapter 14
Recent In Vitro Propagation Advances in Genus *Paphiopedilum*: Lady Slipper Orchids

Reema Vareen Diengdoh, Meera Chettri Das, Alvareen Nongsiang, and Suman Kumaria

Abstract *Paphiopedilum* is a popular and horticulturally important orchid and is also known as lady's slipper orchid. The genus is widely traded as cut flowers and potted plants due to its spectacularly beautiful flowers with a long shelf-life. Due to market demands, the orchids of this genus has been collected excessively leading to endangerment to its wild populations. Propagation of this orchid for commercialization through the conventional method has a slow growth rate, low seed germination rate and requires the association of mycorrhizal fungi for germination. Hence, in vitro propagation or micropropagation provides an alternative approach in meeting the needs for sustainable commercial demand and also in the conservation of wild slipper orchids. This chapter aims to discuss the latest research progress on in vitro propagation and acclimatization of *Paphiopedilum* orchids.

Keywords In vitro propagation · TDZ · Commercialization · Paphiopedilum

1 Introduction

The genus name Paphiopedilum was established by Ernst Hugo Heinrich Pfitzer in 1886 and is derived from 'Paphos' (a city in Cyprus, a place sacred to Aphrodite) and ancient Greek pedilon "slipper'. Ironically, no *Paphiopedilum* occurs in the family of Cyprus as the genus to date. It was often mixed up with its Holo-arctic relative *Cypripedium*, along the Mediterranean region. *Paphiopedilum* was finally decided to

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be a valid taxon in 1959, but the use of this taxon was restricted to eastern Asian *Paphiopedilum* species.

Paphiopedilum spp. are known as lady's slipper orchids and have high horticultural value. China is one of the main origins of the genus with 27 species (Chen et al. 2004). It comprises 96–100 species extending from India eastwards across southern China to the Philippines, South-east Asia, the New Guinea to the Malay Archipelago, and the Solomon Islands (Chen et al. 2004; Zhang et al. 2015; Clements et al. 2016) widely distributed as terrestrial, lithophytic, or epiphytic orchids of this Genus. Liu et al. (2009) described *Paphiopedilum* spp. as deceptive orchids with a trap-lip pollination system since most species of the tropical Asian *Paphiopedilum* are pollinated by hoverflies (Shi et al. 2009). Flowers of these species have aphid-like spots that attract gravid female hoverflies that normally lay their eggs in aphid colonies (Cribb 1998). Combinations of visual cues located on the staminode, lateral petals and sepals in association with scent cues are considered critical in determining which mode of pollination-by-deceit is employed by a *Paphiopedilum* species (Shi et al. 2009). The genus comprises some 80 accepted taxa including natural hybrids. These orchids under this genus have captured the interest of many orchid growers and hobbyists and are one of the most popular and rare orchid genera being sold and exhibited today (Cribb 1998; Liu et al. 2009; Kaur and Bhutani 2016).

2 Economic Importance and Conventional Method of Propagation

Paphiopedilum species are marketed globally as cut flowers for boutonnieres, floral arrangements, and bouquets; as potted flowering plants and as bedding or aerial plants due to their long shelf-life of 60 to 90 days (Diengdoh et al. 2017; Yuan et al. 2021). There is an increasing demand in the production of *Paphiopedilum* hybrids as many hybrids of this species with complex parentage are found to exist in the international orchid markets (Sun et al. 2011). There are also few reports on ethnobotanical utilization of *Paphiopedilum* as medicine in curing stomach ailments (Friesen and Friesen 2012).

The conventional propagation method for species is through the division of the axillary bud from the mother plant. This is very inefficient and time-consuming because only one new growth can be obtained per year after a mature plant has flowered (Zhang et al. 2015). Biotic stress like low frequency of fruit set, limited evidence of prolific seed germination, influences of ecological connections like mycorrhizal fungi and pollinators (Shi et al. 2009; Khamchatra et al. 2016) adds to slow growth of more than 2 years for plantlets to reach the flowering stage in their natural habitats. While abiotic stress like over-collection for commercial demands, habitat destruction and degradation, and changed abiotic conditions (e.g. soil and hydrology), often induced by human activities (Swarts and Dixon 2009) have further significantly reduced the species abundance in the wild population. Currently, many

species of this genus are listed as endangered in the Red Data Book prepared by International Union for Conservation of Nature and Natural Resources (IUCN) (Rankou and Kumar 2015; http://www.iucnredlist.org/) and are also included in Appendix-II of Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), where the international trade is strictly controlled and monitored.

3 Need for In Vitro Propagation of *Paphiopedilum*

The natural mode of multiplication in these orchids is restrained by many aspects. The orchid–pollinator relationship shows only 67% of 456 orchid species to have known pollinators, thereby making pollinator specificity act as the main mechanism of pre-mating reproductive isolation for orchids (Cozzolino et al. 2006). Another fascinating aspect of orchid biology is their dependence on mycorrhizal fungi for symbiotic germination (Rasmussen 1995; Sathiyadash et al. 2020). Orchids are optimally adapted to wind dispersal as they produce a large number of minute seeds (Arditti and Ghani 2000). The developing seedling remains dependent on fungal sugars for several years, a strategy called myco-heterotrophy (Rasmussen 1995). The multiplication via. Axillary bud division from the mother plant is slow and reduced. This hampers the time taken for flowering in *Paphiopedilum* species as plantlets take more than 4 years to mature.

In situ conservation approach would have been an ideal (Tandon 2004). However, due to the factors like habitat loss, maintenance of genetic resources through in situ is not safe hence genetic materials may be lost through disease, weather, natural disasters, etc. and so with this regard, ex situ collections should be maintained in many cases (Chugh et al. 2009; Engelmann 2011). In order to device sustainable ex situ conservation of these rare, endangered and threatened (RET) plants especially orchids, in vitro technologies play an important role (Bhattacharyya et al. 2013; Diengdoh et al. 2019).

Paphiopedilum species are the only commercially grown orchid which cannot be cloned since explants from mature plants are recalcitrant to shoot induction and plant regeneration (Chugh et al. 2009). Plant tissue culture technique proved highly successful in enabling the seeds of *P. armeniacum* to germinate, develop into seedlings and reintroduced to the farmers as reported by Zhang et al. 2015. Further, it is difficult to establish in vitro culture from explants of mature plants due to severe endogenous bacterial contamination. Till date, due to the limited success in tissue culture protocols, commercial *Paphiopedilum* propagation by growers had still been entirely through an asymbiotic seed germination under in vitro propagation (Hong et al. 2008; Zeng et al. 2015; Diengdoh et al. 2017). In Table 14.1, explants sources have been mentioned that have been used for in vitro propagation along with the most optimal hardening compost mixture as the success rate of an in vitro propagation is species reaction specific. As there are no reports on more commercialization of this genus as their species are mostly listed in appendix I of CITIES. It is all the

Table 14.1 In vitro propagation of *Paphiopedilum* orchids

Species/hybrid	Explant details	Culture medium	Culture conditions	Type of respond	Root/plantlet development medium	Acclimatization (survival %)	References
Paphiopedilum Armeni White	Cross-sectioned flower buds (between 1.5–3.0 cm)	¼ MS medium + 2% sucrose + 0.8% agar + 4.43 μM BAP + 4.52 μM 2,4-D	25 ± 1 °C with 55–60% RH, 16-h photoperiod at 30 μmol/m^2/s	Multiple shoots	¼ MS medium + 2% sucrose + 0.8% agar + 1 g/L AC	New Zealand sphagnum, maintained in a greenhouse at 27 ± 2 °C with 70 ± 10% RH	Liao et al. (2011)
Paphiopedilum callosum	Seedlings	1/2 MS medium + 0.5 μM TDZ or 10 μM BAP	25 ± 2 °C, 16/8-hour photoperiod at 30 μmol/m^2/s	Multiple shoots	1/2 MS media without PGR	NR (not reported)	Wattanawikkit et al. (2011)
Paphiopedilum Deperle	Cross-sectioned flower buds (between 1.5 and 3.0 cm)	¼ MS medium + 2% sucrose + 0.8% agar + 44.39 μM BAP + 26.85 μM NAA	25 ± 1 °C with 55–60% RH, 16-h photoperiod at 30 μmol/m^2/s	Multiple shoots	¼ MS medium + 2% sucrose + 0.8% agar + 1 g/L AC	New Zealand sphagnum, maintained in a greenhouse at 27 ± 2 °C with 70 ± 10% RH	Liao et al. (2011)
Paphiopedilum rothschildianum	(1) Nodal stem (2) Callus	(1) ½ MS + 4 μM kin + 2 g/L peptone (2) ½ MS + 4 μM kin	(1) 25 ± 2 °C in dark culture (2) 25 ± 2 °C at 40 μmol/m^2/s	(1) callus (2) PLBs	½ MS medium + 20% (v/v) CW	NR	Ng and Saleh (2011)
Paphiopedilum Hsinying Rubyweb	Intact and vertically cut seedlings (2–4 cm in height)	RB medium + 10 g/L sucrose + 2 g/L AC (activated charcoal) + 1 g/L bacto-tryptone +	25 ± 2 °C, 12/12-h (day/night) photoperiod at PPF of 40 ± 10 μmol/m/s	Multiple shoots	RB medium + 10 g/L sucrose + 2 g/L AC + 1 g/L bacto-tryptone + 50 g/L potato extract + 25 g/L	NR	Udomdee et al. (2012)

		50 g/L potato extract + 25 g/L banana + 5 g/L sigma agar + 1 g/L Gelrite			banana + 5 g/L sigma agar + 1 g/L Gelrite		
Paphiopedilum wardii	Seeds (180 DAP)	½ MS + 0.5 mg/L NAA + 10% coconut water + 1.0 g/L AC	25 ± 1 °C, 16-h photoperiod PPFD (photo-synthetic photon flux density) of ca. 45 μmol/m/s	Protocorms with 65.33% germination percentage	Hyponex N016 medium + 1.0 mg/L NAA + 1.0 g/L peptone + 100 g/L BH and 1.0 g/L AC	2:1:1ratio of Zhijing stone for orchids: Sieved peat: Shattered fir bark (92.33%)	Zeng et al. (2012)
Paphiopedilum 'Delrosi'	Shoots (with three leaves)	Hyponex medium + 100 g/L PH + 2 g/L pep-tone + 2.5 g/L sucrose + 1 g/L AC + 2.5 g/L gelrite + 0.45 μM TDZ + 4.52 μM 2,4-D	24 ± 1 °C, 16-h photoperiod at 35–40 μmol/m^2/s provided by cool-white fluo-rescent lamps	Multiple shoots (90%)	Hyponex medium + 100 g/L PH + 2 g/L pep-tone + 2.5 g/L sucrose + 1 g/L AC + 2.5 g/L gelrite + 2.25 μM TDZ + 13.56 μM 2,4-D	NR	Thongpukdee et al. (2013)
Paphiopedilum hangianum	Seeds (180 DAP)	H026 medium + 0.5 mg/L NAA + 10% CW + 1.0 g/L AC	25 ± 1 °C, 16-h Photoperiod with PPFD of ca. 45 μmol/m/s	Protocorms with 72.67% germination percentage	Hyponex N016 medium + 1.0 mg/L NAA + 100 g/L BH	2:1:1 (v/v) Zhijing stone for orchids: sieved peat: shattered fir bark (88.5%)	Zeng et al. (2013)
Paphiopedilum spicerianum	Seeds	BM1 medium + 0.2% AC	25 ± 2 °C, con-tinuous darkness of 0/24 h light/dark	Protocorms with 62.75% germination percentage	BM1 medium + 0.2% AC + 1.5 mg/L NAA (12/12 h	NR	Kaur and Bhutani (2013)

(continued)

Table 14.1 (continued)

Species/hybrid	Explant details	Culture medium	Culture conditions	Type of respond	Root/plantlet development medium	Acclimatization (survival %)	References
					light/dark photoperiod)		
Paphiopedilum armeniacum	Seeds (1) 95 DAP (2) 110 DAP	(1) 1/ 8 MS + 1.0 g/ L AC + 20 g/L sucrose + 10% CW + 0.5 mg/L NAA + 5.2 g/L agar (2) ¼ MS+ 1.0 g/ L AC + 20 g/L sucrose +10% CW + 0.5 mg/L NAA + 5.2 g/L agar	25 ± 1 °C, 16-h photoperiod with PPFD of ca. 45 μmol/m^2/s	Protocorms with germination percentage of 94.3% (95 DAP) and 75.0% (110 DAP)	Hyponex N026 medium + 1.0 g/L peptone + 1.0 mg/ L NAA + 20 g/L sucrose + 1.0 g/ L AC + 50 g/L BH	Zhijing stone for orchids Substrate in greenhouse with <800 μmol/m^2/s natural light (85.3%)	Zhang et al. (2015)
Paphiopedilum delenatii	Stem nodes exposed to monochromatic blue LED (100B)	Liquid SH medium + 30 g/L sucrose + 1.0 mg/ L TDZ + 0.3 mg/ L NAA with cotton wool plug as the substrate	16–25 °C (day and night), with 30 μmol/m^2/s PPF under 24 h photoperiod	Multiple shoots with regeneration rate of 45%	SH medium + 0.5 mg/L BA + 0.5 mg/L NAA + 30 g/L sucrose + 1 g/ L AC	Fern fibres, under natural light with PPFD <200 μmol/m^2/s ^{1} using sunshade nets, 16–25 °C, 60–90% RH	Luan et al. (2015)
Paphiopedilum liemianum	Seeds (4 months old)	VW medium + 2.5 μM NAA + 2 g/L peptone	23 ± 2 °C, 16/8 h light/dark photoperiod	Protocorms with 72.8% germination percentage	VW medium + 4 μM TDZ	Plastic pots (10 cm diameter) containing sphagnum moss at 26 ± 2 °C,	Utami et al. (2015)

						light intensity 3700 lux, 14 h photoperiod (76%)	
Paphiopedilum niveum	(1) 3–4 mm PLBs (8 weeks old) (2) 5 months old PLBs	(1) Solidified VW medium + 10% CW + 0.2% Phytagel (2) Modified VW solid medium + 15 g/L sucrose + 1 g/L Phytagel + 5.5 g/L agar + 2 g/L AC + 0.5 mg/L 2,4-D + 0.1 mg/L TDZ	(1) 25 ± 1 °C, 16/8 h light/dark photoperiod light intensity of 23 µmol/m^2/s (2) 25 ± 1 °C under dark condition (2 months), 16/8 h light/dark photoperiod light intensity of 23 µmol/m^2/s	(1) Shoots (2) Callus	Modified VW solid medium + 15 g/L sucrose + 10% CW	NR	Chaireok et al. (2016)
Paphiopedilum spicerianum	Seeds (356 DAP) pretreated with 1% NaOCl for 40 min	1/4 strength modified MS + 6 g/L agar + 20 g/L sucrose + 10% CW	24 h dark photoperiod for approximately 30 days, 25 ± 2 °C, 45% RH and light intensity of 200 µmol/m/s	Protocorms with 79 ± 8% germination percentage	3.0 g/L Hyponex No 1 + 1.0 mg/L NAA + 0.5 g/L AC + 10% BH + 1.0 mg/L 6-BA	NR	Chen et al. (2015)
Paphiopedilum SCBG red Jewel	Seeds (180 DAP)	Hyponex N026 medium + 0.5 mg/L NAA + 10% CW + 1.0 g/L AC	25 ± 1 °C with a 16-hour photoperiod PPFD of approx. 45 µmol/m/s	Protocorms with 39.9% germination percentage	NR	NR	Jiang et al. (2016)

(continued)

Table 14.1 (continued)

Species/hybrid	Explant details	Culture medium	Culture conditions	Type of respond	Root/plantlet development medium	Acclimatization (survival %)	References
Paphiopedilum venustum	Seeds	Modified terrestrial orchid medium in 12-h photoperiod	25 °C, 12-h photoperiod at 40 µmol/m/s light intensity	Protocorms with 82.7% germination percentage	BM-1 medium + peptone at 1.0 g/L	Community pots filled with 1:1 ratio of soil and leaf litter (80%)	Kaur and Bhutani (2016)
Paphiopedilum exul	Seeds (5 months old)	½ MS medium + 20 g/L sucrose/ pH 5	(1) Cultured in dark condition for 2 months (2) 25 ± 3 °C under light intensity of 37 µmol/m/s for 16 h/day	Protocorms with germination percentage of 24.97% in 20 g/L sucrose and 28.0% at pH 5	NR	NR	Imsomboon et al. (2017)
Paphiopedilum callosum var. *sublaeve*	Shoot tip-derived transverse thin cell layer (tTCL)	MVW solid medium + 0.2% AC (activated charcoal) + 5.5 g/L agar + 1 g/L Phytagel + 1.0 mg/L TDZ	25 ± 2 °C, 16-h photoperiod at irradiance of 23 µmol/m2/s provided by cool-white fluorescent tubes	PLBs with percentage induction of 46.67%	MVW medium + 1.0 mg/L TDZ	3-inch pot containing sphagnum moss (80%)	Wattanapan et al. (2018)
Paphiopedilum 'Delrosi'	(1) Nodes (2) Basal stem tissue	(1) Modified Hyponex medium + 20 g/L sucrose (2) Modified Hyponex medium + 20 g/L maltose + 0.45 µM TDZ + 4.52 µM 2,4-D	24 ± 1 °C, 16-h photoperiod at 35–40 µmol/m^2/s	Shoot induction	(1) Modified Hyponex medium + 20 g/L sucrose (2) Modified Hyponex medium + 20 g/L maltose + 0.45 µM TDZ + 4.52 µM 2,4-D	NR	Obsuwan et al. (2018)

Paphiopedilum vietnamense	Axillary buds	½ MS medium + 2.0 mg/L BA + 1.0 mg/L NAA	NR	Multiple shoots	½ MS medium + 0.5 mg/L NAA + 1.0 g/L AC	NR	Nguyen et al. (2018)
Paphiopedilum rothschildianum	(1) Seeds (2) Calli induced from seed explant	(1) ½ semi-solid MS media + 22.6 µM 2,4-D + 4.54 µM TDZ (2) Liquid culture RITA® system vessel containing liq. ½MS + 58 mM sucrose +2.27 µM TDZ + 12.0 µM BAP	(1) 25 ± 2 °C in darkness (2) 25 ± 2 °C in a 16 h photo period	(1) Calli with percentage induction of 77.0% (2) PLBs with 190 PLBs per gram calli	NR	NR	Masnoddin et al. (2018)
Paphiopedilum villosum var. *boxallii*	Seeds	MS medium + 3% sucrose + 4 µM NAA + 8 µM BA	25 °C, 20 µmol/m/s light intensity	PLBs with germination percentage of 60%	MS medium + 3% sucrose + 4 µM NAA + 8 µM BA	1:1:1:1:1 ratio of sand, organic decaying matter, brick pieces, charcoal chunks, and dried cow dung (65.1%)	Deb and Jakha (2019)
Paphiopedilum callosum	Internode segments	SH medium + 30 g/L sucrose + 1 mg/L TDZ + 1 mg/L 2,4-D	(1) Shoot explant subjected to 14 days of dark and 1 day of light condition (2) 25 ± 2 °C, RH of 50% to 55% and light	Callus and PLBs induction	Hyponex N016 medium +200 g/L PH + 1.0 mg/L NAA, 30 g/L sucrose, 170 mg/L NaH2PO4, 1.0 g/L peptone and 9 g/L Bacto agar	Fern fibre (100%)	Huy et al. (2019)

(continued)

Table 14.1 (continued)

Species/hybrid	Explant details	Culture medium	Culture conditions	Type of respond	Root/plantlet development medium	Acclimatization (survival %)	References
			intensity of 15–20 µmol/m/s				
Paphiopedilum sp.	Decapitated shoot apex and wounding shoots	Liquid SH medium + 0.4–0.6 mg/L TDZ	25 ± 1 °C, 16-h photoperiod with light intensity of 15–20 µmol/m/s	Multiple shoots	SH medium + 1.0 mg/L NAA	Plastic pots (9 cm diameter) containing peat moss, grown in Greenhouse at 16–25 °C, 60–90% RH, and PPFD less than 200 µmol/m/s (100%)	Luan et al. (2019)
Paphiopedilum delenatii	Decapitated shoot apex and wounding shoots	Liquid SH medium + 0.4–0.6 mg/L TDZ	25 ± 1 °C, 16-h photoperiod with light intensity of 15–20 µmol/m/s	Multiple shoots	SH medium + 1.0 mg/L NAA	Plastic pots (9 cm diameter) containing peat moss, grown in greenhouse at 16–25 °C, 60–90% RH, and PPFD less than 200 µmol/m/s (100%)	Luan et al. (2019)
Paphiopedilum gratrixianum	Decapitated shoot apex and wounding shoots	Liquid SH medium + 0.4–0.6 mg/L TDZ	25 ± 1 °C, 16-h photoperiod with light intensity of 15–20 µmol/m/s	Multiple shoots	SH medium + 1.0 mg/L NAA	Plastic pots (9 cm diameter) containing peat moss, grown in greenhouse at	Luan et al. (2019)

						16–25 °C, 60–90% RH, and PPFD less than 200 μmol/m/s (100%)	
Paphiopedilum 'Hsinying Maudiae Leopard'	Shoot bud with 1 leaf attached	MS medium + 20 g/L sucrose + 4 g/L agar + 2 g/L Gelrite + 13.3 μM BAP	26 ± 2 °C under a 16/8-h photoperiod at a light intensity of 30 μmol/m/s	Multiple shoot	2.5 g/L Hyponex medium + 0.3 g/L urea + 1 g/L peptone + 20 g/L sucrose + 60 g/L PH + 25 g/L BH + 0.1 g/L myoinositol + 0.5 g/L AC + 4 g/L agar	NR	Do et al. (2019)
Paphiopedilum niveum	4 months old protocorms (approx. 1–2 mm in diameter)	MVW medium + 0.1 mg/L NAA	(1) Maintained in darkness for 3 months (2) 25 ± 2 °C, 16-h photoperiod at a PPFD of 23 μmol/m^2/s^1 for 1 month	Somatic embryos (68.33%)	MVW + 2% (w/v) sucrose + 0.2% (w/v) Bacto™ peptone + 0.2% (w/v) AC + 50 g/L BH for 12 weeks	Dried sphagnum moss in greenhouse temp. of 30 ± 2 °C, RH of 60–70%	Soonthornkalump et al. (2019)
Paphiopedilum insigne	Seeds	MS medium + 3% sucrose + 2 μM NAA + 6 μM BA	25 ± 2 °C, 12/12 h photoperiod with light intensity of 40 μmol/m^2/s^1	Protocorms with 85% germination percentage	MS medium + 3% sucrose + 4 μM NAA + 4 μM BA	1:1:1:1:1 ratio of sand: organic decaying matter: brick pieces: charcoal chunks: dried cowdung (75%)	Deb and Jakha (2020)
Paphiopedilum insigne		VW medium + 0.5 g/dm^3 BA	28 °C ± 2 °C during the day,		½ MS + 30 g/dm^3 BP	NR	Poniewozik et al. (2021)

(continued)

Table 14.1 (continued)

Species/hybrid	Explant details	Culture medium	Culture conditions	Type of respond	Root/plantlet development medium	Acclimatization (survival %)	References
	Plantlets (8–12 mm in length and 1–2 roots)		24 °C ± 2 °C at night and 16-h photoperiod with light intensity of 30 µmol/m^2/s^1	Plantlets with multiplication rate of 2.92			
Paphiopedilum tigrinum	Seeds	Modified Harvais medium (mHa) + 0.5 mg/L kin + 0.1 g/L AC + 100 mL/L CW	25 ± 2 °C, 0/24 h light/dark for germination period, 12 h photoperiod at 32–40 µmol/m^2/s^1	Protocorms with 87.29% germination percentage	1/2 MS medium + 1.0 g/L AC + 0.5 g/L dolomite flour + 15 g/L PH + 30 g/L BH	NR	Yao et al. (2021)

more important to follow the in vitro protocols for mass production that can cater to the market demands of the species.

4 Recent in Vitro Propagation Protocols in Various *Paphiopedilum* Species

Asymbiotic seed germination success depends on conditions like pollination origin and maturity; germination conditions and the constituents in the growth media (Arditti 1967; Kauth et al. 2008). An in-depth review of in vitro orchid seed germination was provided by Kauth et al. (2008). Despite many successes of in vitro asymbiotic seed germination of many endangered and threatened orchid taxa (Mohanty et al. 2012; Diengdoh et al. 2017; Deb and Jakha 2019), protocol varies since the conditions of tissue culture at each step are largely species-specific (Zeng et al. 2013; Bhattacharyya et al. 2016). Cross-pollination in *Paphiopedilum* has been shown to produce seeds that have higher germination success, compared to seeds obtained through self-pollination (Stimart and Ascher 1981; Abadie et al. 2006). Several factors in terms of explant used, media composition, and growth hormones are all species response dependent and was clearly seen in Table 14.1 for the genus *Paphiopedilum*.

Growth media conditions such as composition (Imsomboon et al. 2017), carbon source, plant growth regulators, organic nutrient additives (Long et al. 2010; Yao et al. 2021), pretreatment duration (Lee 2007; Diengdoh et al. 2017), and light availability (Stimart and Ascher 1981) can also significantly influence successful germination of *Paphiopedilum* species. Luan et al. (2019) described propagation protocols of three endangered *Paphiopedilum* species (Table 14.1) by removing shoot apex and wounding method is reported in this study when decapitated shoot apex was cultured on liquid Schenk and Hildebrandt (SH) medium added with thidiazuron (TDZ) (Table 14.1).

The morphogenetic response in orchids varies depending on the explant (Hossain et al. 2013). Recently, Poniewozik et al. (2021) reported the influence of medium type, growth regulators, i.e. BAP, KN, TDZ used Paphiopedilum separately or in combinations and natural additives, i.e. coconut water, banana pulp, casein hydrolysate, on *Paphiopedilum insigne* plantlets grown in vitro. It was found out that BAP (0.5 mg/L) allowed to obtain the highest multiplication rate (Table 14.1); however, KN (1 mg/L) resulted in the formation of bigger and higher quality plantlets which is similar to the effective combination of KN and IAA on plantlet development of *P. insigne* (Diengdoh et al. 2017). Protocorms, PLBs, and seedlings in vitro are customarily used as initial explants to study *Paphiopedilum* micropropagation. Do et al. (2019) successfully reported on the use of shoot apex for in vitro propagation of *Paphiopedilum* hybrid which was similar to reports on the same explant source use which were similar to studies by Ng et al. (2010). Use of explants sources like internodes (Ng et al. 2010; Ng and Saleh 2011), PLBs (Zeng et al. 2013; Masnoddin

et al. 2018), or leaves (Chen et al. 2004; Lin et al. 2000) have also been reported. Long et al. (2010) reported that the length and number of shoots of four *Paphiopedilum* spp. were influenced by the concentration and combination of cytokinins and auxins added to the medium. Liao et al. (2011) reported that scape transverse slices of *Paphiopedilum* hybrids (*P. deperle* and *P. armeni white*) could induce adventitious buds and regenerate into whole plants on modified MS medium supplemented with 4.43 mM (1.0 mg/L) 6-benzyl aminopurine (BAP) and 4.52 mM (1.0 mg/L) 2,4-dichlorophenoxyacetic acid (2,4-D), or on modified MS medium supplemented with 44.39 mM (10.0 mg/L) BAP and 26.85 mM (5.0 mg/L) α-naphthalene acetic acid (NAA), Reports on *P. wardii* and *P. spicerianum* showed good response to the addition of NAA for root development, and activated charcoal (AC) was important for absorbing harmful chemicals produced during seedling formation. In contrast, Long et al. (2010) found that in four *Paphiopedilum* species the addition of NAA and BAP resulted in green leaf formation; however, further sub-culturing of plantlets was not successful. They also demonstrated that there was no consistency in shoot multiplication when concentrations of NAA and BAP were varied. Huy et al. (2019) recently reported on the effectiveness of TDZ was as a plant hormone for induction of protocorm-like bodies (PLBs) produced the best shoots on medium containing 0.5 mg/L NAA and 0.3 mg/L TDZ. One of the most important ex situ conservations is in vitro propagation. According to Yao et al. (2021), seed germination of *P. tigrinum* was a modified Harvais (mHa) medium supplemented with 0.5 mg/L KN, 0.1 g/L activated charcoal and 100 mL/L coconut water (CW). At the rooting stage, a 1/2 MS medium supplemented with 1.0 g/L AC, 0.5 g/L dolomite flour, 15 g/L potato homogenate, and 30 g/L banana homogenate was most suitable for the growth and rooting of seedlings (Table 14.1). Growth and development composition for in vitro protocols varies from species to species (Kauth et al. 2008; Zeng et al. 2013; Diengdoh et al. 2017).

Terrestrial orchids unlike their epiphytic counterparts are difficult to germinate in vitro and fail to establish in soil on a large scale (Batty et al. 2001; Stewart and Kane 2006; Swarts and Dixon 2009). Plantlets raised under in vitro requires a stepwise and careful procedure for the transfer to ex vitro conditions. Different combinations of compost were used for hardening and establishment of the in vitro raised plantlets of both the *Paphiopedilum* species (Diengdoh et al. 2017). The transfer of in vitro raised plants to the field requires careful stepwise procedures to enable them to harden and acclimatize to the harsh outside environment. This is due to the poorly developed cuticle, stomatal apparatus, photosynthesis ability and conducting tissues of the *in -vitro* raised plantlets (Vij 1995; Yao et al. 2021). In vitro raised plantlets are fragile and lack sufficient vigour to survive the acclimatization shock during transplantation to the potting mixture. Transplanted plantlets need to adapt to the ex vivo temperature which may be lower or higher than the optimum temperature. Hence, care must be taken not to expose the in vitro raised plantlets on transplantation directly to the outside temperature. For the purpose of hardening, several composts have been proposed and their effects on growth vary in different species (Agnihotri et al. 2009; Gill et al. 2004; Vasane and Kothari 2006). High-quality plants of *P. callosum, P. gratrixianum,* and *P. delenatii*. Were observed

when the plantlets were transferred onto peat moss with a 100% survival rate after 24 months in the greenhouse as reported by Luan et al. (2019). Different potting mixtures have been suggested for the healthy growth and development of orchids (Hajong et al. 2010; Mohanty et al. 2012; Gogoi et al. 2012). Hardening of the in vitro raised plantlets is, therefore, essential and requires careful stepwise procedures to enable them to harden and acclimatize to the harsh outside environment (Kaur et al. 2011; Zeng et al. 2012). Humidity and temperature are important for acclimatization to the outside environment.

Perforated polythene bags thermocol pots to cover and avoid direct exposure to sunlight, and high humidity is important to maintain leading to the gradual acclimatization of plantlets within 3–4 weeks of transfer (Hiren et al. 2004; Gopi et al. 2006; Lavanya et al. 2009; Gogoi et al. 2012). Seedlings were successfully acclimatized to greenhouse conditions that could be used for ornamental, eco-rehabilitation and conservation purposes (Decruse et al. 2003; Bhattacharyya et al. 2016). A good growing substratum having properties such as maximum water holding capacity, porosity, and drainage is essential for proper growth and development of in vitro raised plants. Right stage of transplants, suitable compost, moisture, and other physical factors greatly affect the survival rate of the in vitro raised plantlets (Kumaria and Tandon 1994). A mixture of low-cost substrata (sand: decaying organic matter: brick pieces: charcoal pieces: dried cow dung at 1:1:1:1:1 ratio was observed to be most effective on *Paphiopedilum* species with over 1500 regenerates established in the wild as reported by Deb and Jakha (2020). The success of any ex situ conservation attempt is ultimately accomplished with successful hardening followed by reintroduction of in vitro plantlets into their natural habitat.

5 Conclusion

Demands for orchids in the cut flower industries are increasing annually. Global orchid marketing now has moved from nurseries, orchidariums to E-commerce platforms as well. In the future, more orchid products or hybrids of the Genus like *Paphiopedilum* needs to be encouraged with non-conventional biotechnologies for better orchid production. *Paphiopedilum* although explored in the in vitro propagation technologies for mass production however, their commercialization is still lacking as compared to its other counterparts like *Phalaenopsis, Cymbidiums,* etc. To further nurture its socio-economic prospects, more cost-effective methods with high survival rates and early flowering studies need to be encouraged of the in vitro raised plantlets. This would enhance the commercial and conservational ideas for the genus.

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