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Plant Biotechnology: Principles and Applications

Springer

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Preface

The group of technologies that use biological matter or processes to generate new and useful products and processes define biotechnology. The plant biotechnology is increasingly gaining importance, because it is related to many facets of our lives, particularly in connection with global warming, alternative energy initiatives, food production, and medicine. This book, entitled *Plant Biotechnology: Principles and Applications*, is devoted to topics with references at both graduate and postgraduate levels. The book traces the roots of plant biotechnology from the basic sciences to current applications in the biological and agricultural sciences, industry, and medicine. The processes and methods used to genetically engineer plants for agricultural, environmental, and industrial purposes along with bioethical and biosafety issues of the technology are vividly described in the book. It is also an ideal reference for teachers and researchers, filling the gap between fundamental and high-level approaches.

The book is comprised of 14 chapters. The first chapter is "Historical Perspective and Basic Principles of Plant Tissue Culture." It describes the use of tissue culture as an established technique for culturing and studying the physiological behavior of isolated plant organs, tissues, cells, protoplasts, and even cell organelles under precisely controlled physical and chemical environments and a source for obtaining new variants with desirable agronomic traits. It also discusses the micropropagation of the plants and its use in conservation of endangered species and afforestation programs.

The second chapter "Plant Tissue Culture: Application in Plant Improvement and Conservation" describes the use of micropropagation for ornamental and forest trees, production of pharmaceutically interesting compounds, and plant breeding for improved nutritional value of staple crop plants, including trees. It also highlights the application of plant tissue culture in providing high-quality planting material for fruits, vegetables, and ornamental plants and forest tree species throughout the year, irrespective of season and weather, thus opening new opportunities to producers, farmers, and nursery owners.

The third chapter "Plant Genetic Resources: Their Conservation and Utility for Plant Improvement" describes biodiversity as not merely a natural resource but an

embodiment of cultural diversity and the diverse knowledge of different communities across the world. The chapter reviews the genetic diversity in plant genetic resources in India, methods of its conservation, and the utilization of plant genetic resources in crop improvement programs.

The fourth chapter "Methods in Transgenic Technology" describes genetic engineering as an imperative tool for breeding of crops. The chapter reviews transgenic-enabling technologies such as *Agrobacterium*-mediated transformation, gateway vector-based technology, and generation of marker-free transgenics, gene targeting, and chromosomal engineering.

The fifth chapter "Plant Promoters: Characterization and Application in Transgenic Technology" describes the structural features of plant promoters followed by types along with examples; approaches available for promoter isolation, identification, and their functional characterization; and various transgenic crops commercialized or in pipeline in relation to the specific promoters used in their development.

The sixth chapter "Metabolic Engineering of Secondary Plant Metabolism" describes the strategies that have been developed to engineer complex metabolic pathways in plants, focusing on recent technological developments that allow the most significant bottlenecks to be overcome in metabolic engineering of secondary plant metabolism to enhance the productions of high-value secondary plant metabolites.

The seventh chapter "Plastome Engineering: Principles and Applications" summarizes the basic requirements of plastid genetic engineering and control levels of expression of chloroplast proteins from transgenes. It also discusses the current status and the potential of plastid transformation for expanding future studies.

The eighth chapter "Genetic Engineering to Improve Biotic Stress Tolerance in Plants" reviews the genes that have been used to genetically engineer resistance in plants against diverse plant pathogenic diseases.

The ninth chapter "Developing Stress-Tolerant Plants by Manipulating Components Involved in Oxidative Stress" describes recent advances in the defense system of plants during oxidative stress and also discusses the potential strategies for enhancing tolerance to oxidative stress.

The tenth chapter "Plant Adaptation in Mountain Ecosystem" discusses the physiological, morphological, and molecular bases of plant adaptation including secondary metabolism at varying altitudes in context to representative plant species in western Himalaya.

The eleventh chapter "Drought-Responsive Stress-Associated MicroRNAs" summarizes the recent molecular studies on miRNAs involved in the regulation of drought-responsive genes, with emphasis on their characterization and functions.

The twelfth chapter "Molecular Marker-Assisted Breeding of Crops" describes the molecular markers, their advantages, disadvantages, and the applications of these markers in marker-assisted selection (MAS) in crop plants to improve their agronomic traits.

The thirteenth chapter "Plant-Based Edible Vaccines: Issues and Advantages" reviews the recent progress made with respect to the expression and use of plant-derived vaccine antigens.

The fourteenth chapter "Biosafety, Bioethics, and IPR Issues in Plant Biotechnology" reviews the IPRs, biosafety, and ethical issues arising from the research in plant biotechnology and product obtained thereof.

Each chapter has been written by one or more eminent scientists in the field and then carefully edited to ensure thoroughness and consistency. The book shall be valuable for undergraduate and postgraduate students as a textbook and can also be used as a reference book for those working as plant biologists, biochemists, molecular biologists, plant breeders, and geneticists in academia and industries.

New Delhi, India Malik Zainul Abdin
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List of Abbreviations and Symbols

ACT	Artemisinin-based combination therapy
ADS	Amorpha-4,11-diene synthase enzyme
BA	6-Benzyladenine
BAP	Benzene amino purine
BLAST	Basic local alignment search tool
bp	Base pair
cDNA	Complementary DNA
Cm	Centimeter
CPPU	N-(2-Chloro-4-pyridyl)-N'-phenylurea
C-TAB	Cetyl trimethyl ammonium bromide
cv./cvs	Cultivar/s
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylene diamine tetra acetate
g/1	Grams per liter
gfw	Gram fresh weight
HMGR	Hydroxy methyl glutaryl coenzyme A reductase
hmgr	Hydroxy methyl glutaryl coenzyme A gene
hrs	Hours
Kb	Kilobase pairs
kDa	Kilodalton
Kn	Kinetin
MemTR	Meta-methoxy topolin
MemTTHP	Meta-methoxy topolin 9-tetrahydropyran-2-yl
mg/L	Milligram per liter
min.	Minute
mM	Millimolar
ml	Millimeter
MS	Murashige and Skoog
NAA	Naphthalene acetic acid
NOS	Nopaline opine synthase

*npt*II	Neomycin phosphotransferase gene
°C	Degree Celsius
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RNase	A Ribonuclease A
rpm	Rotations per minute
RT-PCR	Real-time polymerase chain reaction
sec.	Second
sp.	Species
TE	Tris-EDTA buffer
v/v	Volume/volume
var.	Variety
w/v	Weight/volume
YEM	Yeast extract mannitol
2,4-D	2,4-Dichlorophenoxy acetic acid
2-iP	2-Isopentenyl-adenine
µM	Micromolar
µl	Microliter
%	Percent

Chapter 1
Historical Perspective and Basic Principles of Plant Tissue Culture

Anwar Shahzad, Shiwali Sharma, Shahina Parveen, Taiba Saeed, Arjumend Shaheen, Rakhshanda Akhtar, Vikas Yadav, Anamica Upadhyay, and Zishan Ahmad

Abstract In 1902 Gottlieb Haberlandt proposed the idea to culture individual plant cells on artificial nutrient medium. Although he failed to culture them due to poor choice of experimental materials and inadequate nutrient supply, he made several valuable predictions about the nutrients' requirement for in vitro culture conditions, which could possibly induce cell division, proliferation and embryo induction. Tissue culture has now become a well-established technique for culturing and studying the physiological behaviour of isolated plant organs, tissues, cells, protoplasts and even cell organelles under precisely controlled physical and chemical conditions. Micropropagation is one of the most important applications of plant tissue culture. It provides numerous advantages over conventional propagation like mass production of true-to-type and disease-free plants of elite species in highly speedy manner irrespective of the season requiring smaller space and tissue source. Therefore, it provides a reliable technique for in vitro conservation of various rare, endangered and threatened germplasm. Micropropagation protocols have been standardized for commercial production of many important medicinal and horticultural crops. Somatic embryogenesis is an extremely important aspect of plant tissue culture, occurring in vitro either indirectly from callus, suspension or protoplast culture or directly from the cell(s) of an organized structure. Advantages of somatic embryogenesis over organogenesis include several practical means of micropropagation. It reduces the necessity of timely and costly manipulations of individual explants as compared to organogenesis.

Moreover, somatic embryogenesis does not require the time-consuming subculture steps. As somatic embryos are the bipolar structures, they overcome difficulties with micropropagation of difficult to root species (mainly recalcitrant tree species). In addition to micropropagation, plant tissue culture is extensively used for the production of secondary metabolites through callus, suspension and organ culture.

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1.1 History of Plant Tissue Culture

The science of plant tissue culture originally starts from the discovery of cell followed by the concept of cell theory (Schleiden 1838; Schwann 1839). Initial attempts to demonstrate ability of plant cell to regenerate into complete plantlet (totipotency) failed due to improper selection of tissue to culture, nutrient supply and culture conditions (Haberlandt 1902). Breakthrough was achieved during 1930 with the successful culturing of fragments from embryos and roots (Kotte 1922; Molliard 1921; Robbins 1922). Auxin, indole-3-acetic acid (IAA), was the first plant growth regulator (PGR) discovered by Went (1926). In 1934, first successful continuous culture of excised tomato root tips was achieved by White on sucrose and yeast extract (YE). Later, YE was replaced by vitamin B, namely, pyridoxine (B_6) and thiamine (B_1). The same year (1934) witnessed one of the main events in the history of tissue culture, the callus induction from woody cambial explants of oak (Gautheret 1934). Later in 1939, Gautheret, White and Nobécourt independently worked for the formation of continuous callus cultures in carrot and tobacco. By adding adenine and high concentrations of phosphate, continued induction of cell division and bud formation were achieved (Skoog and Tsui 1951). Kinetin (Kn), a derivative of adenine (6-furfuryl amino purine), was isolated in 1955 (Miller et al. 1955). Miller et al. (1955), Skoog and Miller (1957) also proposed the concept of hormonal control for organ formation and suggested that high concentration of auxin is required for root induction, while for bud formation, comparatively high concentration of natural cytokinin, i.e. kinetin, is required.

The most significant success in plant tissue culture was the formulation of a defined culture medium (Murashige and Skoog 1962). Murashige and Skoog used 25 times higher concentration of salts than Knop's solution. Nowadays, Murashige and Skoog (MS) medium has been proved as the most effective culture medium for most of the plant species.

1.2 Steps Involved in Plant Tissue Culture

1.2.1 Establishment of Culture

Explants (i.e. excised plant parts), viz. nodes, shoot tips, leaves, internodes, flower buds, petioles, leaflets, etc., collected from in vivo grown sources are usually contaminated with microorganisms of different types and constitution in the form of surface contaminants. Besides these, endophytic bacteria and fungi can express themselves in culture even after years.

Washing of explants with common sterilizing agents like sodium or calcium hypochlorite (5–10 %), ethyl alcohol (50–95 %) and mercuric chloride (0.01–0.1 %) in the appropriate solution for 1–30 min, followed by several rinses in sterilized water, is suggested to exclude the surface contaminants. It should be followed by

Fig. 1.1 Agents used for surface sterilization

rigorous screening of the stock cultures for bacterial contamination (Murashige and Skoog 1962; Rout et al. 2000). The most common surface sterilizing agents along with range of exposure time are given in Fig. 1.1.

Axenic cultures are developed, mostly in tree species, in order to combat the contaminants. For this, first explants are taken from in vivo grown mature trees and, thereafter, cultured in vitro on MS basal medium to raise single or multiple axillary shoots which in turn are used as explant source. Such explants have advantage over direct explants, as there are lesser chances of infection and they are true to type.

Another technique to check out contamination is to use seedling-derived explants. A large number of plants have been propagated through this technique where seeds are either collected or purchased, from a reliable source, are surface decontaminated following a regular washing protocol and are thereafter transferred to germination media. After germination, healthy seedlings are sacrificed, and different types of explants are used for further propagation studies. Reliable protocol has been developed for micropropagation of *Gymnema sylvestre* through seedling-derived explants (Komalavalli and Rao 2000). Aseptic seedling-derived young root segments were used for in vitro propagation of *Clitoria ternatea* (Shahzad et al. 2007), while seedling-derived cotyledonary explant was used for micropropagation in *Cassia sophera* (Parveen et al. 2010). Seedling-derived nodal segment was used for somatic embryogenesis in *Hygrophila spinosa* (Varshney et al. 2009). The only problem associated with seedling-derived explants is variation (Larkin and Scowcroft 1981). Different procedures or techniques are carried out by various workers to eradicate the above-mentioned problems, while the most common protocol followed is summarized in Fig. 1.2.

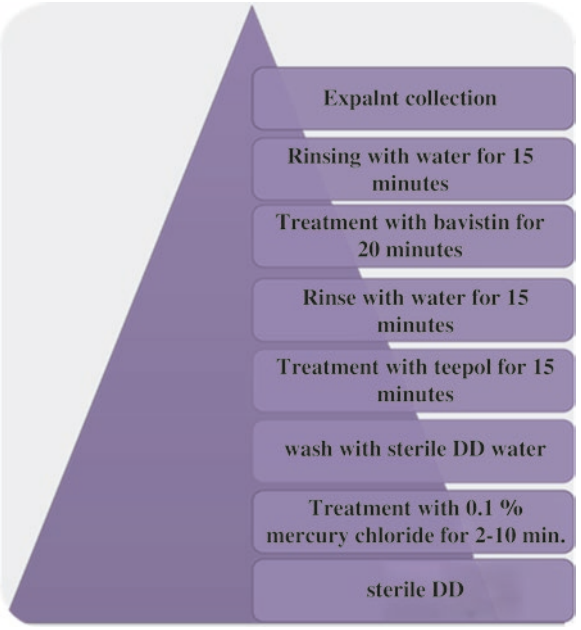

Fig. 1.2 Schematic representation of protocol for surface sterilization

1.2.2 Selection of Media

A nutrient medium consists of all the essential major and minor plant nutrient elements, vitamins, plant growth regulators and a carbohydrate as carbon source with other organic substances as optional additives. Components of media can be classified into five groups:

1. Inorganic nutrients
   (a) Macronutrients
   (b) Micronutrients
2. Organic nutrients
3. Carbon source
4. Solidifying agent
5. Growth regulators

Sucrose is generally used at a concentration of 3 % as a carbon source in plant tissue culture medium. Agar is most commonly used for preparing semisolid or solid culture media, but other gelling agents are occasionally used including gelatin, agarose, alginate and gelrite.

There are several culture media proposed from time to time for various purposes. More than 50 different devised media formulations have been used for in vitro culture of tissues from various plant species (Heller 1953; Murashige and Skoog 1962; Eriksson 1965; Nitsch and Nitsch 1969; Nagata and Takebe 1971; Schenk and

Hildebrandt 1972; Chu 1978; Lloyd and McCown 1980), but MS medium is most commonly used, often with relatively minor changes (Rout et al. 2000).

1.2.3 Selection of Plant Growth Regulators (PGRs)

Hormones are organic compounds naturally synthesized in higher plants which influence growth and development. There are two main classes of growth regulators used in tissue culture, auxin and cytokinins. The hormonal content of a cultural medium is crucial to any sustained growth of the cultures (Bhojwani and Razdan 1996). The growth regulators are required in very minute quantities (μmol l^{-1}). There are many synthetic substances having growth regulatory activity, with differences in activity and species specificity. It often requires testing of various types, concentrations and mixtures of growth substances during the development of a tissue culture protocol for a new plant species. The most important are auxins, abscisic acid, cytokinins, ethylene and gibberellins.

1.2.4 Incubation Conditions

Rout et al. (2000) stated that light, temperature and relative humidity are important parameters in culture incubation. Photosynthetic activity is not very important during initial phases of in vitro culture, but at later stages, the culture materials are induced to become autotrophic to a certain degree. Light is essential for morphogenetic processes like shoot and root initiations and somatic embryogenesis. Both quality and intensity of light as well as photoperiod are very critical to the success of certain culture experiments (Murashige 1977). An exposure to light for 12–16 h per day under 35–112 μmol m^{-2} s^{-1} provided by cool, white fluorescent lamps is usually preferred. Murashige (1977) stated that blue light promotes shoot formation, whereas rooting in many species is induced by red light. The temperature is usually maintained at 25 °C in the culture room with certain variations such as higher temperature which is usually required by tropical species (i.e. 27–30 °C; Tisserat 1981).

1.3 Micropropagation

Micropropagation is one of the most useful aspects of plant tissue culture technique. It has found widest practical application. The process of micropropagation involves the following four distinct stages (Murashige 1974). The first stage is culture initiation which depends on explant type or the donor plant at the time of excision. Explants from actively growing shoots are generally used for mass scale

multiplication. The second stage is shoot multiplication which is crucial and achieved by using plant growth regulators (PGRs) generally, auxins and cytokinins. In the third stage, elongated shoots are subsequently rooted either ex vitro or in vitro. The fourth stage is acclimatization of in vitro grown plants, which is an important step in micropropagation.

1.3.1 Organogenesis

Organogenesis, in terms of plant tissue culture, can be defined as the 'genesis' or formation of organs from unusual parts (i.e. adventitious development of organs). The adventitious origin may be attributed to either direct differentiation of cells and tissues (explants) to form an organ or via cells undergoing cycles of dedifferentiation (caulogenesis) and redifferentiation. In normal in vitro conditions and under the influence of various factors, organogenesis is a two-step process where shoots develop first and roots next, giving rise to a complete plantlet.

The tenets of organogenesis are based upon the fundamentals of in vitro cell culture which was initiated as early as 1898 by a German botanist, Gottlieb Haberlandt (1902). He isolated and cultured fully differentiated and mature cells of leaves and petiole on Knop's salt solution (1865) containing glucose and peptone, maintained under aseptic condition. His attempts were limited to the growth of cells in size and change in shape, but no growth in number of cells could be observed as none of the cells showed division. Much later, Skoog (1944) and Skoog and Tsui (1951) demonstrated callus growth and bud initiation in tobacco pith tissues in the presence of adenine and IAA. Later, Jablonski and Skoog (1954) confirmed cell division only when vascular tissues were present and pith cells alone were inefficient in inducing cell division. The technique of tissue culture relies upon certain internal and external factors which determine organogenesis. The internal factor mainly includes genotype and endogenous levels of growth regulators. Among the external factors, explant type, season of explant harvesting and culture room conditions (temperature, light, humidity, etc.) play pivotal role in overall development of cultured plants.

1.3.1.1 Effect of Plant Growth Regulators (PGRs)

PGRs play important role in cellular programming in manipulation of cell tissues in vitro (Moyo et al. 2011) through which morphogenic changes (viz. organogenesis, rhizogenesis, embryogenesis, etc.) take place. During micropropagation, the incorporation of exogenous cytokinin in the medium enhances shoot formation, and, for developing a standard plant tissue culture (PTC) protocol, the selection of cytokinin is of critical importance (Sharma et al. 2010, 2014; Sharma and Shahzad 2013; Parveen and Shahzad 2014a).

The effect of different PGRs has early been studied by Sahai and Shahzad (2013) in *Coleus forskohlii*, where BA (5 μM) in MS medium produced 13.80 ± 1.24 axillary shoots and 18.80 ± 1.59 direct adventitious shoots per explant. Rani and Rana (2010) studied the effects of Kn, BA and GA_3 in *Tylophora indica*. The shoot development showed dependency on synergistic effect of BA (2 mg/l) + GA_3 (0.2 mg/l) giving 4.86 ± 1.76 shoots/explant. Parveen et al. (2010) reported maximum shoot regeneration frequency with maximum number of shoots per explant (12.20 ± 0.73) and shoot length (6.40 ± 0.07 cm) on MS + BA (1.0 μM) + NAA (0.5 μM) through cotyledonary node explant, excised from 14-day-old aseptic seedlings. Similarly, in *Heliotropium kotschyi*, a synergistic effect of BA (8.88 μM) + IAA (5.71 μM) showed formation of 10.66 shoots per explant (Sadeq et al. 2014). Likewise, Ragavendran et al. (2014) reported 7.7 ± 1.1 shoots/explant in *Eclipta alba* in a combination of BA (0.5 mg/l) + Kn (0.3 mg/l) + GA_3 (1.5 mg/l) augmented in B_5 medium with 100 % regeneration frequency (Table 1.1).

1.3.1.2 Effect of Explant Type

The effect of explants on micropropagation has also been studied in various plant species such as *Gerbera jamesonii* (Tyagi and Kothari 2004), *Vitis vinifera* (Jaskani et al. 2008), *Citrus jambhiri* (Vijaya et al. 2010), *Stevia rebaudiana* (Sharma and Shahzad 2011) and *Tectona grandis* (Kozgar and Shahzad 2012). Explant-dependent micropropagation protocol has also been cited by many in different medicinal plants. Golec and Makowczynska (2008) studied the effects of seedling-derived explants of *Plantago camtschatica* on multiple shoot formation. Out of root, hypocotyl, cotyledon and leaf explants, they obtained best multiplication results from root explants giving out 12.7 ± 10 buds and shoots at 9.1 μM zeatin in combination with 0.6 μM IAA. In *Tectona grandis*, shoot tip proved to be the best for propagation as compared to nodal segments and cotyledonary nodes (Kozgar and Shahzad 2012). Micropropagation studies on different explants of *Bacopa monnieri* (Kumari et al. 2014) showed development of 18.8 ± 0.40 shoots per nodal explants as compared to shoot tip explants, which developed 14.6 ± 0.26 shoots per explant in MS + BA (0.5 mg/l) + Kn (0.5 mg/l) + IBA (0.25 mg/l) augmented medium. Jesmin et al. (2013) reported encouraging results from nodal explants (12.2 ± 0.32 shoots/culture) as compared to ST explants on the same medium, i.e. MS + BA (1 mg/l) showing 90 % regeneration rate in a period of only 10–11 days (Table 1.2).

1.3.1.3 Effect of Seasonal Variation

Bhatt and Dhar (2004) found that shoot collection season reduces percent browning and induces bud break in *Myrica esculenta.* The season of inoculation of explant as reported by Mannan et al. (2006) in *Artocarpus heterophyllus* describes survivability of shoot buds and their proliferation. A well-defined regeneration protocol showing seasonal variation has been discussed by Malik and Wadhwani (2009) for *Tridax*

Table 1.1 Effect of plant growth regulators

Plant	PGR	Explant	Medium	Observation	References
Coleonema album	BA,Kn, mT, MemTR, MemTTHP, TDZ	ST, young leaves, petiole of young leaves, stem cuttings	MS	Among various cytokinins tested mT (5 µM) supplemented in MS medium produced 14.5 shoots/ST explant, surpassing the other PGRs tested. The effects of KIN didn't influence organogenesis much when compared to the control	Fajinmi et al. (2014)
Dendrobium chrysanthum	BA, TDZ, 2,4-D	Axenic nodal segments	MS	Among all the concentrations and combinations of PGRs used MS supplied with TDZ, (5 µM) + BAP (5 µM) proved to be most responsive in terms of % response (100 %) and maximum number of shoots/ explant (14.33 ± 0.14)	Hajong et al. (2013)
Ocimum basilicum	BA, 2-iP	Nodal segments	MS	MS + BA (10 µM) proved best among different concentrations of BA and 2-iP forming 5.7 ± 0.35 shoots/explant. This no. further enhanced to 13.4 ± 1.80 with the addition of glutamine (30.0 mg/L)	Shahzad et al. (2012)

(continued)

Table 1.1 (continued)

Plant	PGR	Explant	Medium	Observation	References
Cassia siamea	BA, Kn, TDZ	CN	MS	Among different PGRs used, plant responded best at BA (1.0 μM) with 80 % regeneration rate giving 8.20 ± 0.66 shoots/ explant. A combined effect of optimal concentration of BA with NAA (0.5 μM) enhanced multiplication further giving 12.20 ± 0.73 shoot/explant with 90 % regeneration frequency	Perveen et al. (2010)
Carlina acaulis	BA, Kn, Zea	ST, Hypocotyl	MS	Morphogenesis was best studied from ST explant cultured on MS + BA (4.4 μM) obtaining 7.9 ± 0.4 shoots/explant, but 100 % response was achieved on MS + BA (13.3 μM). Moreover with subculture passage no. of shoots reduced to 5.6 ± 0.4	Trejgell et al. (2009)

(continued)

Table 1.1 (continued)

Plant	PGR	Explant	Medium	Observation	References
Centaurium erythraea	BA, CPPU, 2-iP, Kn, TDZ, Zea	In vitro raised normal and hairy roots	½MS	Urea-derived PGRs like TDZ and CPPU were more effective than adenine-based PGRs in evoking morphogenesis between normal and hairy root explants. Normal roots at 3.0 μM CPPU were more effective in morphogenesis giving 25.61 ± 0.53 number of shoots	Subotic et al. (2008)

BA 6-benzyladenine, *Kn* kinetin, *CPPU* N-(2-chloro-4-pyridyl)-N′-phenylurea, *2-iP* 2-isopentenyl]-adenine, *TDZ* thidiazuron, *2,4-D* 2,4-dichlorophenoxyacetic acid, *mT* meta-topolin, *MemTR* meta-methoxy topolin, *MemTTHP* meta-methoxy topolin 9-tetrahydropyran-2-yl

procumbens. The protocol describes highest bud break and multiple shoot formation between July and September on MS + BA (1 mg/l), whereas explants inoculated during December were least responsive. Verma et al. (2011) also studied the seasonal effect on shoot proliferation through nodal segment of *Stevia rebaudiana*. Nodal segments cultured during June to August on MS + BA (0.5 mg/l) + Kn (0.5 mg/l) exhibited maximum bud break (80.5 %) and shoot multiplication (17.5 shoots/explant). While, in *Vitex negundo*, the nodes inoculated during March–May showed maximum bud break (95 %) with 7.29 ± 0.28 shoots/explant in MS medium fortified with 1 mg/l BA, but the activity declined to 26 % with only 2.20 ± 0.21 shoots/explant during September–November (Steephen et al. 2010). Seasonal effect of explant in *Glycyrrhiza glabra* has also been discussed by Yadav and Singh (2012). According to their study, nodal segments planted during May–August were more responsive with 86.6 % bud break and 3.0 ± 0.8 shoots/explant as compared to other months (Table 1.3).

1.3.1.4 Effect of Genotype

The effect of genotype has been an important aspect for plant tissue culture (PTC) mainly because an elite germplasm is sought for this purpose. A study was conducted on *Melissa officinalis* genotypes taken from different places by Mohebalipour et al. (2012). A maximum of 4.97 ± 0.20 shoots were obtained in Iranian landrace Hamadan 2 genotype, but the genotype Fars showed more shoot elongation, whereas

Table 1.2 Effect of explant type on regeneration

Plant	Explant type	Medium	Observation	References
Dianthus caryophyllus	ST, NS	MS	Highest number of shoots (4.30 shoots/explant) were achieved from nodal segments on MS + BA (2 mg/l)	Al-Mizory et al. (2014)
Curcuma caesia	Leaf, root, rhizome sections, mature bud of rhizome, sprouted bud of rhizome	MS	Sprouted buds of rhizome showed best response in a combination medium containing 4 mg/l BA and 100 mg/l adenine sulphate giving 3.8 ± 0.32 shoots/explant	Behar et al. (2014)
Bauhinia variegata	Cotyledons, hypocotyl, leaves	MS (liquid and solid media)	Direct organogenesis was best observed in liquid media supplemented with 2-iP (2 mg. dm-3) from cotyledons showing emergence of 212.2 ± 26.6 mean number of shoot buds	Banerjee (2013)
Saintpaulia ionantha	Leaf disc and petiole	MS	Leaf disc in the presence of MS + BA (0.5 mg/l) + IBA (0.5 mg/l) gave highest no. of shoot buds (80 shoots/explant)	Ghasemi et al. (2012)
Prunus microcarpa subsp. tortusa	Cotyledons, hypocotyl, root of seedling	Nasand Read mediun (2004)	Cotyledon explant exhibited maximum regeneration rate	Nas et al. (2010)
Spilanthes mauritiana DC.	ST, leaf explants	MS	A combination of BA and IAA was more efficient in inducing 18.8 ± 0.3 shoots per ST without undergoing any callus phase during the culture	Sharma et al. (2009)
Cinnamomum tamala	Petiole, apical shoot, shoot with internode, leaf	WPM	Indirect organogenesis was best achieved in petiole explant forming 4 shoots/explant in a combination of BA (2.5 μM) and IBA (5 μM)	Sharma and Nautiyal (2009)

MS Murashige and Skoog medium, *WPM* woody plant medium

genotypes Karaj and Qazvin 2 produced highest callus. Xing et al. (2010) used four genotypes of *Rosa rugosa* for regeneration studies. Genotype Purple Branch among Tang Red, Puce Dragon and Tang White was best in achieving maximum number of shoots (4.87 ± 0.51) on MS medium augmented with BA (2.2 μM) + NAA (0.054 μM) + GA_3 (0.4 μM) with glucose as the carbon source (Table 1.4).

Table 1.3 Effect of seasonal variation

Plant	Harvesting season	Medium	Observation	References
Pithecellobium dulce	Jan–March April–June July–Sept Oct–Dec	MS	Explants harvested during Oct–Dec were more responsive in giving max bud break and showed less pathogen contamination	Goyal et al. (2012)
Celastrus paniculatus	Dec–march April–July Aug–Nov	MS	90 % bud break was observed in explants taken during April–July which declined to 70 % during Aug–Nov	Yadav et al. (2011)
Tylophora indica	Dec–Feb March–May June–Aug Sept–Nov	MS	During Sep–Nov highest % bud break (95.74 ± 3.19) was observed giving 4.50 ± 0.20 no. of shoots/explant. In this case winter season (Dec–Feb) was least responsive	Rani and Rana (2010)
Lilium ledebourii	Spring Summer Winter	MS	Highest no. of bulbets/explant were observed during summer season but for the other parameters, viz. rooting, post-acclimatization survival, winter harvesting was suitable	Azadi and Khosh-Khui (2007)
Myrica esculenta	Jan–Dec	WPM	Winter season (Nov–Dec) marked maximum bud breaks and explant establishment. During spring explants died due to phenolics released from growing shoots	Bhatt and Dhar (2004)

MS Murashige and Skoog medium, *WPM* woody plant medium

1.3.1.5 Effect of Culture Room Conditions

The culture requires incubation under controlled condition which includes optimum temperature range, humidity, light quality as well as intensity and duration of photoperiod. An account of all the factors influencing culture condition has been described in Table 1.5.

1.3.2 Somatic Embryogenesis

Somatic embryogenesis (SE) is an extremely important aspect of induced regeneration, occurring in vitro, either indirectly from callus, suspension or protoplast culture or directly from the cell(s) of an organized structure such as leaf, cotyledon, stem segment or zygotic embryo. It is a complex developmental programme by which haploid or diploid competent somatic cells undergo differentiation into complete plants through various characteristic embryological stages without the

Table 1.4 Effect of genotype

Plant	Genotype	Medium	Observation	References
Arbutus unedo	AL2, AL3, AL4, AL6, AL7, IM1, IM2, IM4, IM6 AND JF3	FS basal medium (1974)	Genotype AL7 showed best morphogenic response among the other tested genotypes forming 1.90 ± 0.73 number of shoots per test tube	Gomes et al. (2010)
Buddleia cultivars	Black Knight, Royal Red, White Ball, Nanhoensis, *B. Lochinch*, Pink Delight, White Profusion, Empire Blue, Ile de France and Border Beauty	MS medium	*Buddleia* cultivars showed genotype-independent regeneration. The bisected internodes in four cultivars, viz. *Lochinch*, Border Beauty, Pink Delight and Ile de France, were more responsive in terms of number of adventitious shoot formation	Phelan et al. (2009)
Allium cepa	B-780 Hisar-2 N-2-4-1	MS medium	Among different genotypes B-780 was significantly superior in all explants studied (ST, RT seed) in inducing callus and multiple shoot formation	Khar et al. (2005)
Morus alba	Chinese white Kokuso- 27 Ichinose	MS basal medium (fortified with 0.1 mg/l TIBA)	Kokuso-27, among the three genotypes studied, was best in forming regenerative calli (90 %) and number of shoots/ callus (11.4)	Bhau and Wakhlu (2001)
Dianthus caryophyllus	Coral Jaguar Salome Sarinah	MS medium containing B_5 vitamins	Salome and Jaguar cultivars were intensively caulogenic but developed roots only. Coral and Sarinah genotypes were low caulogenic but evidenced intensive organogenic capacity developing both roots and shoots	Kallak et al. (1997)

MS Murashige and Skoog medium, *WPM* Woody plant medium

Table 1.5 Effect of culture room conditions

Plant	Factor (Light)	Medium	Explant	Observation	References
Lysionotus pauciflorus	WL, BL, OL, RL	MS with varied composition of nitrogen	Leaf	RL proved to be superior with 30.4 ± 7.5 shoots/explant and showing 100 % regeneration rate	Lu et al. (2013)
Alternanthera brasiliana	WL, RL, GL,BL	MS	Axenic nodes of germinated plantlet	BL was significant in terms of largest no. of leaf/ explant. RL resulted in formation of lower parameters	Macedo et al. (2011)
Cattleya hybrid	WL, BL, RL, FRL	MS	Shoots regenerated from protocorm-like bodies	Enhanced adventitious bud formation in RL and BL. RL promoted elongation of shoots and BL promoted rhizogenesis and elongation of aerial roots	Cybularz-Urban et al. (2007)
Alternanthera brasiliana	WL + UV-A	MS	Nodal segments	Regeneration frequency enhanced to 96 % with 100 % rooting and showed comparatively lesser value of chl a /chl b ratio	Silva et al. (2005)
	Temperature				
Mentha sp.	20 °C and 25 °C	MS	Apical and nodal explants	Nodal explants at 25 °C exhibited maximum no. of leaves	Islam et al. (2005)

intervention of a sexual fusion. Thus, the various developmental stages of somatic embryos correspond to that of zygotic embryos (Dodeman et al. 1997). Advantages of somatic embryogenesis over organogenesis include several practical means of propagation. The time-consuming subculture steps and in vitro root induction in recalcitrant plant species during organogenesis are not required during somatic embryogenesis (Thangjam and Maibam 2006). Somatic embryoids, being bipolar in organization, required a single step to get differentiated into an integrated root-shoot axis unlike the development of monopolar structures, either root or shoot through organogenesis. The origin and development of adventitious embryoids in culture was first reported by Steward et al. (1958) and Reinert (1959) in carrot cell suspension cultures. Carrot served as a model system for the detailed study of structural and developmental patterns of somatic embryogenesis, since most of the early work on somatic embryogenesis was concentrated on this plant (Wetherell and Halperin 1963; Kato 1968; Homes 1968). Since then the somatic embryogenesis has been successfully reported in many plants (Gharyal and Maheshwari 1981; Schuller et al. 1989; Martin 2004; Nowak et al. 2012) including many medicinally important plants (Murthy and Saxena 1998; Jayanthi and Mandal 2001; Kumar et al. 2002; Paramageetham et al. 2004; Ma et al. 2011). Secondary embryogenesis, i.e. phenomenon of induction of new somatic embryos in a cyclic manner from the preexisting one, is of common occurrence in many plant species. Secondary embryogenesis ensures high multiplication rate with greater uniformity of the emblings and is also independent on the explant availability (Shi et al. 2010). Also embryogenicity of an established culture could be maintained for long durations, i.e. up to many years through the process of cyclic or recurrent embryogenesis (Uzelac et al. 2007; Konan et al. 2010; Shi et al. 2010; Sahai et al. 2010; Saeed and Shahzad 2015). The responsive cells (also called as embryogenic cells) have the ability to activate embryo-responsive genes, thus leading to the initiation of the embryogenic pathway (Nomura and Komamine 1995; Quiroz-Figueroa et al. 2002). The explant changes its established gene expression programme to embryogenic gene expression as soon as the embryo responsive genes become activated (Quiroz-Figueroa et al. 2006). The key step in embryogenic induction is to determine specific factors that act as signalling molecules to change the somatic cells expression pattern towards embryogenic pathways. Internal and/or external cellular levels of plant growth regulators (PGRs), various stress factors such as osmotic shock, water stress, heavy metal ions, alterations of culture medium, pH, heat or cool shock treatments, hypoxia, antibiotics, ultraviolet radiation and mechanical or chemical treatments as well as reduced nitrogen are important inductive factors in generating signal transduction cascade leading to a series of cell division which may either give rise to unorganized embryogenic callus or polarized growth resulting into direct or indirect embryogenesis, respectively (Dudits et al. 1991; de Jong et al. 1993; Trigiano et al. 1992). Williams and Maheswaran (1986) suggested that the two pathways, direct and indirect somatic embryogenesis, proceed from different types of cells. Pre-embryogenic determined cells (PEDCs), which were already determined for embryogenic development prior to explanting, required only minimal reprograming of tissues for the expression of direct embryogenesis, while indirect embryogenesis proceeds from induced embryogenically determined cells (IEDCs)