Chapter 1
Historical Perspective and Basic Principles of Plant Tissue Culture

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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.

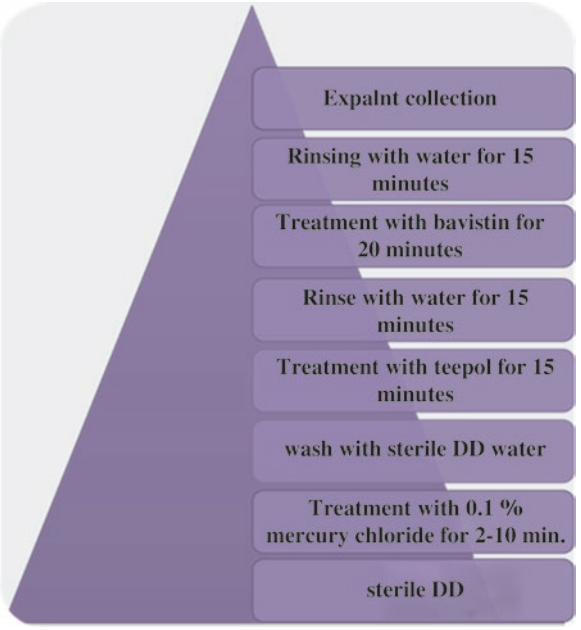

Fig. 1.2 Schematic representation of protocol for surface sterilization

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)

that require major reprogramming to get proliferated calli with embryogenic ability before embryo formation. Another point of discussion is a single- or multiple-cell origin of somatic embryoids. Induction of somatic embryo from a superficial cell possibly indicates its unicellular origin (Haccius 1978) or from subepidermal cells, representing a multicellular origin (Tisserat et al. 1978). The various events occurring during somatic embryogenesis have been schematically represented in Fig. 1.3.

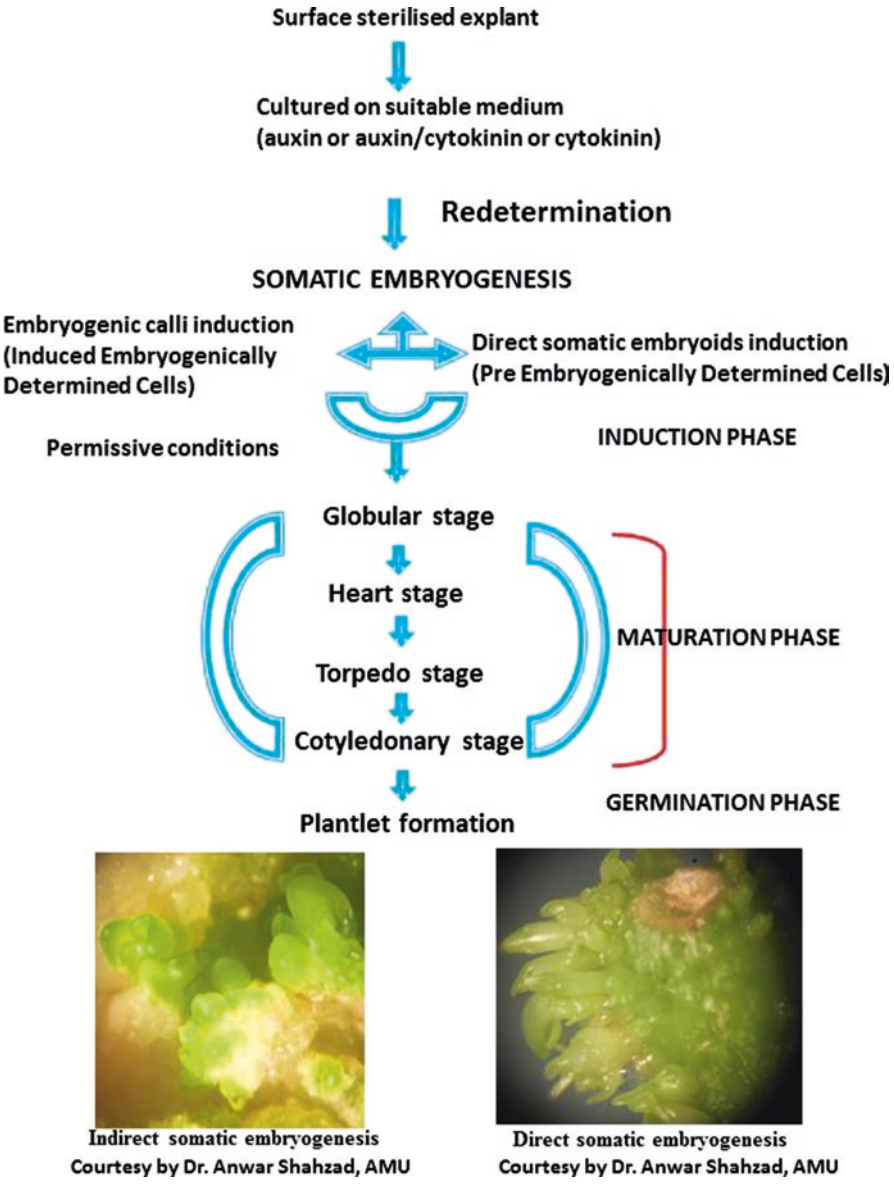

Fig. 1.3 Agents used for surface sterilization

1.3.2.1 Effect of Media

The different types of media (MS, WPM, B5, N6, SH, LS) too have a significant impact on somatic embryogenesis; MS is the most commonly used nutritional medium for the induction of somatic embryogenesis (Martin 2003; Husain et al. 2010; Silja et al. 2014). Differences in the concentrations or combinations of nutrients have a very influential role in the optimal expression of embryogenic potential. Among all the inorganic nutrients, nitrogen is a major nutrient component that influences in vitro morphogenesis of the species or the plant organ considered (Samson et al. 2006) (Table 1.6).

1.3.2.2 Effect of PGRs

PGRs are the key players in the induction of somatic embryogenesis. Auxin is considered to be the most important inducer of somatic embryogenesis in vitro (FehÈr 2008). It has been reported that 2,4-dichlorophenoxyacetic acid (2,4-D) is the

Table 1.6 Effect of media on SE of some selected medicinal plants

Plant	Explant used	Media used	Response	References
Paris polyphylla			*% SE*	
	Immature zygotic embryos	B5; ½ B5	20.7; 29.6	Raomai et al. (2014)
		MS; ½ MS	17.0; 32.6	
		N6; ½ N6	18.5; 26.6	
		SH; ½ SH	24.4; 28.9	
Murayya koengi			*% Callus induction*	
	Zygotic embryos	MS + 2.675 µM NAA + 4.44 µM BA	90 (embryogenic callus)	Paul et al. (2011)
		WPM + 2.675 µM NAA + 4.44 µM BA	63.33 (non-embryogenic callus)	
Eucalyptus globulus			*% SE*	
	Mature zygotic embryos	MS; ½ MS	30; 20	Pinto et al. (2008)
		B5	20	
		DKW	8	
		WPM	4	
		JADS	12	
Sesbania sesban			*% SE*	
	Cotyledonary explants	LS	10	Shahana and Gupta (2002)
		MT	11	
		N	9	
		MS	2	
		B5	0	
		N6	0	
		SH	0	
		W	0	

classic auxin for the induction of somatic embryos (SEs) in many species (Pasternak et al. 2002). Higher contents of endogenous auxins in embryogenic cultures than their non-embryogenic counterparts have been reported in *Medicago falcata* (Ivanova et al. 1994) and wheat (Jimenez and Bangerth 2001). Further development of the somatic embryos generally occurs through the reduction or removal of 2,4-D from the culture medium. Cytokinins are also the key determinants of embryogenic response in several plant species. BA was found suitable for SE in *Hygrophila spinosa* (Varshney et al. 2009), *Sapindus mukorossi* (Singh et al. 2015), *Albizia lebbeck* (Saeed and Shahzad 2015). Thidiazuron (TDZ), a phenylurea-derived cytokinin, has proved its potential in high frequency direct induction and development of somatic embryoids even from the mature tissues (Panaia et al. 2004; Zhang et al. 2005). The effect of GAs on SE is highly variable from one to another species or tissues, for example, GA inhibited SE in carrot (Tokuji and Kuriyama 2003), whereas it stimulated embryogenesis in petiole-derived tissue cultures of *Medicago sativa* L. (Ruduś et al. 2002). ABA was found to induce somatic embryos directly from the epidermal cells of seedlings in carrot, and the concentration of this hormone determines the number of induced embryos (Nishiwaki et al. 2000). The positive role of ABA includes normal development of plantlets from somatic embryoids, as it inhibits the precocious germination and stimulates their maturation (Kuklin et al. 1994) (Table 1.7).

1.3.2.3 Effect of Explant Types

Age, developmental stage and the physiological state of the donor plants play vital role in the induction of somatic embryos in cultured tissues. Almost any part of the plant can be used as explant to initiate embryogenic cultures such as in carrot, which is very responsive plant (Jiménez et al. 2005), whereas in other recalcitrant plants such as in cereals and conifers, very specific, usually juvenile explants are responsive for the induction of somatic embryogenesis (Bhaskaran and Smith 1990; Stasolla et al. 2002). Various tissue explants such as immature and mature zygotic embryos (Raomai et al. 2014; Rai and McComb 2002), cotyledons (Kumar et al. 2002; Parveen and Shahzad 2014b), hypocotyl (Choi et al. 1999; Kumar et al. 2002), leaf (Jayanthi and Mandal 2001; Sahai et al. 2010), petiole (Choffe et al. 2000), root (Franklin and Dias 2006), shoot (Dhandapani et al. 2008), nodal segments (Devendra et al. 2011), internode (Martin and Pradeep 2003), etc. have been exploited so far for the induction of somatic embryogenesis. Hypocotyl explants of *Catharanthus roseus* produce embryogenic callus on 1.0 mg/L 2,4-D-supplemented MS medium, while the calli induced from root, leaf and stem explants on the same medium proved to be non-embryogenic (Dipti and Mujib 2014). In another study on the same plant, direct somatic embryogenesis with maximum embryoid induction (48.7 %) was observed from mature embryos on 7.5 μM TDZ-augmented MS medium, while indirect somatic embryogenesis was induced from petiole, shoot tip as well as stem node on the same medium. Hypocotyl and cotyledon explants did not produce somatic embryoids at all. Akula et al. (2003) reported direct

Table 1.7 Effect of PGRs on SE of some selected medicinal plants

Plant	Explant	MS (1962) supplemented with different PGRs	Response	References
Cassia angustifolia	Immature cotyledons		*% SE*	
		10 µM 2,4-D	83.90	Parveen and Shahzad (2014a, b)
		10 µM 2,4-T	10.26	
		10 µM IAA	05.86	
		10 µM IBA	00.00	
		10 µM NAA	30.30	
Petiveria alliacea	In vitro raised shoot-derived leaf segments		*% Response*	
		2.2 µM TDZ	Necrosis	Cantelmo et al. (2013)
		22.8 µM TDZ	Friable callus (33)	
		2.0 µM PIC	SE/compact callus (100)	
		20.0 µM PIC	SE/friable callus (100)	
		2.2 µM 2,4-D	SE/friable callus (100 %)	
		22.6 µM 2,4-D	SE/friable callus (100)	
Catharanthus roseus			*% SE*	
	Mature seeds	2.5 µM TDZ	18.0	Dhandapani et al. (2008)
		2.2 µM BA	25.6	
		2.4 µM IBA	75.6	
		2.5 µM BA + 5.3 µM NAA	82.2	
Corydalis yanhusuo			*Mean no. of SE per callus*	
	Tuber	0.5 mg/l BA	10.9	Sagare et al. (2000)
		0.5 mg/l Kn	07.5	
		0.5 mg/l Zea	09.0	

BA, Kn, Zea, NAA, TDZ, PIC, 2,4-D, IBA, MS medium

recorded on the percentage of plant regeneration from somatic embryos, i.e. the response varies from 74.44 to 85.56 %; the maximum percentage was observed in PCR cultivar. Akula et al. (2003) observed significant variation in the frequency of somatic embryogenesis from root segments in seven different genotypes of *Azadirachta indica*. Only four out of seven genotypes tested showed somatic embryogenesis that too with different frequencies. The highest response with 68 % frequency was observed in clone 5.6, while the lowest frequency (23 %) of somatic embryogenesis was exhibited by clone 20. Clones 10 and 11 did not exhibit somatic embryogenesis, but slight callus development was observed. No response (neither callus nor somatic embryos) was observed for clone 16. In clone 20, more than 50 % of explants showed callus induction, while induction of somatic embryos was observed in 23 % of explants. In alfalfa, among the three genotypes, A70–34 was found to be highly

Table 1.8 Effect of explant types on somatic embryogenesis of some medicinal plants

Plant and explant type	Medium	Type of embryogenesis	Response	References
Catharanthus roseus			*Mean no. of SE/culture*	
Hypocotyl	MS + 1 mg/L 2,4-D (ECIM); MS + 1 mg/L NAA + 1.5 mg/L BAP	Indirect	92.6	Dipti and Mujib (2014)
Root			00.0	
Leaf			00.0	
Stem			00.0	
Ochna integerrima			*Mean no. of shoots and SE*	
Shoot explant	MS + 15.0 μM TDZ	Indirect	48.2	Guohua Ma et al. (2011)
Leaf explant		Indirect	15.9	
Catharanthus roseus			*% SE*	
Mature embryo	MS + 7.5 μM TDZ	Direct	48.7	Dhandapani et al. (2008)
Hypocotyl		No response	00.0	
Cotyledon		No response	00.0	
Petiole		Indirect	06.0	
Shoot tip		Indirect	04.0	
Stem node		Indirect	04.0	
Piper nigrum			% SE	
Intact seeds	PGR-free SH medium	Direct	16.0	Nair and Gupta (2005)
In vitro germinated seeds		Direct	24.0	
In vitro abortively germinated seeds		Direct	32.0	
Unripened green fruits (zygotic embryo removed)		Direct	05.0	
Zygotic embryos		Direct	00.0	
Azadirachta indica			% SE	
Root	MS I (half-strength macrosalts + full-strength microsalts of MS medium + 1 g/l CH + 100 mg/l myo-inositol + 100 mg/l AdS + 100 mg /l glutamine)	Direct	72.0	Akula et al. (2003)
Nodal segment			66.2	
Leaf	MS I + 2.3 μM TDZ + 0.5 μM 2,4-D	Indirect	35.2	
Eleutherococcus sessiliflorus			% SE	
Hypocotyl	MS + 4.5 μM 2,4-D	Direct	45.0	Choi et al. (2002)
Cotyledon		Direct	33.0	
Root		Direct	08.0	

MS, 2, 4-D, TDZ, CH, AdS, SH, NAA, BAP

embryogenesis on MS I from root and nodal explants, wherein root exhibited better somatic embryogenesis (72 %) than nodal segments (66.2 %), while indirect embryogenesis (35.2 %) was observed from leaf explants of the same plant on TDZ and 2,4-D containing MS I medium (Table 1.8).

1.3.2.4 Effect of Liquid Culture System

Among other factors, liquid culture systems also have the profound effect on the induction and maintenance of somatic embryoids in many plant species. Suspension cultures have proven to be embryogenically uniform and can be maintained for a very long period. Also, embryogenic suspension cultures are an important source for the identification and examination of certain events in somatic embryo development. Liquid media were used from the beginning of research to study the developmental pathways of cells leading to somatic embryoid formation (Steward et al. 1958; Reinert 1958). Somatic embryo production through suspension cultures has also been used in medicinal plant species such as *Catharanthus roseus* (Kim et al. 2004) and *Plumbago roseus* (Silja et al. 2014). The use of bioreactors for the maintenance of embryogenic cell suspensions at large scale has been reported by various workers in different plant species (Bapat et al. 1990), sweet potato (Bienick et al. 1995), *Picea sitchensis* (Ingram and Mavituna 2000), etc. (Table 1.9).

1.3.2.5 Effect of Genotype

Genotype has a profound effect on the induction of somatic embryogenesis in various plant species. Although genotypic specificity regarding somatic embryogenesis has been reported in various plant species like wheat (Maës et al. 1996), melon (Yadav et al. 1996), maize (Close and Ludeman 1987), soybean (Parrott et al. 1989), cotton (Sakhanokho et al. 2001; Rao et al. 2006) and coffee (Molina et al. 2002), there is scarcity of literature on the genotype-dependent somatic embryogenesis and plant regeneration on medicinally important plants. Nair and Gupta (2005) reported that out of 15 genotypes (Jeerakamundi, Kalluvally, Karimunda, Kutching, Kuthiravally, Narayakodi, Neelamundi, Neyyattinkaramunda, Panniyur-1, Perambramunda, Sreekara, Subhakara, Thevanmundi, Thommenkodi and Vadakkan) of black pepper tested, 14 showed the embryogenic response while Malaysian cultivar ‘Kutching’ did not exhibit any somatic embryogenic response. Among the responded genotypes, ‘Karimunda’ exhibited the highest frequency of embryogenesis (28.0 %) with the formation of about 7.0 somatic embryos per explant.

Genotypic effect of three *Catharanthus roseus* cultivars Pacifica cherry red (PCR), Heatwave mix colour (HWMC) and Mediterranean Rose Red (MRR) on somatic embryogenesis through hypocotyl explants was elaborated by Yuan et al. (2011). They reported that similar responses of primary callus and embryogenic callus formation were observed in all the three cultivars, but a significant difference was

Table 1.9 Effect of liquid culture system on somatic embryogenesis of some medicinal plants

Plant	Initiation of embryogenic calli		Establishment of suspension culture	Result	References
	Explant	Medium			
Plumbago rosea	Leaf	MS + 2 mg/l 2,4-D + 1 mg/l NAA + 2.5 mg/l BA	1 g callus to liquid MS + 2 mg/l 2,4-D + 1 mg/l NAA + 2.5 mg/l BA	No. of SE (data not given	Silja et al. (2014)
Echinacea purpurea	In vitro grown leaf disc	MS + 1 μM TDZ	0.3 g slurry in 50 ml MS liquid media + 1.0 μM TDZ	No. of SE approx. 800	Jones et al. (2007)
Catharanthus roseus	Immature zygotic embryos	MS basal medium	1 g embryogenic callus in 20 ml of liquid MS + 4.52 μM 2,4-D medium; 5 ml of this suspension into 50 ml liquid MS + 4.52 μM 2,4-D	56.7 % conversion of SE to plantlet on solid MS basal medium	Kim et al. (2004)
Hylomecon vernalis	Petiole	B5 + 13.6 μM 2,4-D	1 g callus in liquid B5 medium + 4.52 μM 2,4-D; 5 ml of this suspension into50 ml B5 + 4.52 μM 2,4-D	70 % SE	Kim et al. (2003)
Acanthopanax koreanum Nakai	Internode	MS + 4.5 μM 2,4-D	Filtered cells (200 μl) in 30 ml MS liquid medium + 0.45 μM 2,4-D	≈350 SE	Choi et al. (1997)

B5, MS, 2,4-D, SE, NAA, BA, TDZ

Table 1.10 Effect of genotype on somatic embryogenesis (SE) of some potentially important medicinal plants

Plant and genotype	Explants used	Medium	Response	References
Catharanthus roseus			% Plant regenerated from SE	
cv. PCR	Hypocotyl	MSCP (MS 1962 + 150 mg/L CH + 250 mg/ L proline, + 30 g/ L sucrose + 3 g/L gelrite.)	85	Yuan et al. (2011)
cv. HWMC			78	
cv. MRR			74	
Piper nigrum			% SE	
cv. Jeerakamundi	Germinating seeds	PGR-free SH medium	11.0	Nair and Gupta (2005)
cv. Karimunda			28.0	
cv. Kutching			0.0	
cv. Sreekara			23.0	
Azadirachta indica			% SE	
A. indica 5.6	Root	MS 1 (half-strength macrosalts and full-strength microsalts of MS medium + 1 g/L CH + 100 mg /L myo-inositol + 100 mg /L AdS + 100 mg/L L-glutamine)	68.2	Akula et al. (2003)
A. indica 10			00.0	
A. indica 20			23.1	
A. indica 5.2			50	
Medicago sativa			Mean no. of SE	
cv. RA3	Ovary and petiole tissue	SH	50	Skokut et al. (1985)
cv. RA3 × falcata regen.			20	
cv. RA3 × falcata non-regen.			0	

embryogenic, R3 produced callus but not somatic embryoids and MK did not show any response (Hernandez-Fernandez and Christie 1989). In contrast to above studies, Franklin and Dias (2006) found that different genotypes (Helos, Topas, Elixir and Numi) of *Hypericum perforatum* responded similarly with no significant differences in somatic embryogenesis and plantlet production (Table 1.10).

1.3.2.6 Effect of Culture Room Conditions

Somatic embryogenesis is also regulated by culture room conditions. Environmental factors such as light intensity, temperatures, humidity, etc. are important determinants for the acquisition of embryogenic competence by the somatic cells (FehÈr 2008). Yang et al. (2013) reported that relatively high temperature was effective for induction of secondary SEs from hypocotyls of germinated primary somatic embryos of *Hovenia dulcis*. The maximum number (97.2) of secondary SEs was formed at 30 °C, whereas the lowest (5.7) was induced at 20 °C. Therefore, high

temperature stress can turn somatic cells into embryogenic cells. Although many species can form embryo in light as well as in darkness (Gingas and Lineberger 1989; Mikuła and Rybczyński 2001), promotion and inhibition of embryo by light are also well documented. Gingas and Lineberger (1989) reported greatest number of somatic embryos from explants incubated in light, whereas high irradiance inhibited embryogenesis in cotyledon cultures of soybean (Lazzeri et al. 1987). In *Gentiana tibetica* cultures maintained in light formed the first embryogenic centres in the fifth week, whereas embryogenesis was delayed for further 2 weeks in cultures maintained in the dark (Mikuła and Rybczyński 2001). The effect of alternating exposures to dark and light incubation conditions has also been examined in olive, wherein somatic embryogenesis only occurred from zygotic embryos that were first incubated in dark for 3 weeks and thereafter in light. Incubation only in light completely inhibited embryogenesis (Rugini 1988) (Table 1.11).

1.3.3 Root Induction in Microshoots

Rooting in regenerated microshoots is an important step in micropropagation, which is essential for the development of complete plantlets. It involves three distinct phases, namely, induction, initiation and expression (Kevers et al. 1997; De Klerk et al. 1999). In the absence of proper root system, plantlets will not be able to survive under external or ex vitro conditions, and losses at this stage have vast economic consequences (De Klerk 2002). Rooting can be induced via in vitro or ex vitro methods.

1.3.3.1 In Vitro Rooting

Strength of the MS medium played an important role in rooting of microshoots. It was observed that in *Cassia angustifolia*, full-strength MS medium without any auxin failed to induce rooting, while reducing the strength of MS medium to half proved to be beneficial (Parveen et al. 2012). Rooting on auxin-free MS basal medium has been reported by Reddy et al. (1998) in *Gymnema sylvestre* and Pyati et al. (2002) in *Dendrobium macrostachyum*, while superiority of half-strength MS in rooting over full-strength MS medium has also been well documented in the literature (Nabi et al. 2002; Parveen et al. 2010; Shahzad et al. 2012; Sharma et al. 2014). IBA proved to be more efficient for rooting than other auxins in number of plants such as *Cunila galoides* (Fracaro and Echeverrigaray 2001), *Embelia ribes* (Raghu et al. 2006), *Clitoria ternatea* (Shahzad et al. 2007), *Cassia siamea* (Parveen et al. 2010) and *C. sophera* (Parveen et al. 2010). Another reason for being more potent auxin is that IBA is comparatively lesser degraded by autoclaving than IAA and is generally considered to be more stable in the light than IAA, which is rapidly photo-oxidized (Nissen and Sutter 1990; Epstein and Müller 1993; De Klerk et al. 1999).

Table 1.11 Effect of culture room conditions on SE induction of some selected medicinal plants

Plant name	Explant/medium used	Culture room conditions	Response	References
Hovenia dulcis		*Temperature (°C)*	*No. of secondary SEs/explant*	
	Mature seeds/ MS agar medium	20	5.7	Yang et al. (2013)
		25	65.3	
		30	97.2	
Eleutherococcus senticosus		*Temperature (°C)*	*Growth ratio of embryos ({harvested dry weight (g) – inoculated dry weight (g)}/ inoculated dry weight (g) of the inocula)*	
	Young leaves/ MS + 1 mg/L 2,4-D	12	8.62	Shohael et al. (2006a)
		18	12.34	
		24	16.61	
		30	7.81	
Eleutherococcus senticosus		*Light quality*	*Growth ratio of embryos ({harvested dry weight (g) – inoculated dry weight (g)}/ inoculated dry weight (g) of the inoculums)*	
	Young leaves/ MS + 1 mg/L 2,4-D	Dark	19.05	Shohael et al. (2006b)
		Fluorescent	19.81	
		Blue	17.77	
		Red	15.22	
		Blue + far red (1:1)	19.11	
Gentiana tibetica	Cotyledons/ MS + 0.5 mg/L 2,4-D + 1.0 mg/L Kn	Dark/light	Callus proliferation: 60–70 %/40 %	Mikula and Rybczynski (2001)
G. pannonica		Dark/light	Callus proliferation: 60–70 %/40 %	
G. cruciata		Dark/light	Callus proliferation: 40 %/30 %	

Phloroglucinol (PG), a phenolic compound, is responsible for the suppression of peroxidase activity in the culture and thus protects the endogenous auxin from peroxidase-catalyzed oxidation which facilitates healthy root formation (De Klerk et al. 1999; Parveen et al. 2012). The promotive effect of PG on rooting was identified in several plant species including *Prunus avium* (Hammatt and Grant 1996), *Malus pumila* (Zanol et al. 1998), *Decalepis hamiltonii* (Giridhar et al. 2005) and *Pterocarpus marsupium* (Husain et al. 2007, 2008).

The gelling substance used in rooting medium also had a great impact on in vitro rooting (Parveen and Shahzad 2014a, b). The superiority of liquid medium in rooting has been observed in different plant species (Gangopadhyay et al. 2002, 2004). The filter paper bridge/support provided in liquid medium gave better anchorage owing to its porosity that facilitated better absorption throughout its surface area. Faisal et al. (2006) reported that rooting medium solidified by agar was more suitable than phytagel or gelrite and provided more branched and thicker roots in *Mucuna pruriens*.

1.3.3.2 *Ex Vitro* Rooting

In vitro rooting in *Cassia angustifolia* involves several problems like necrosis of shoot tips and yellowing or abscission of leaves on transferring to rooting media. The formation of callus at cut end of the microshoots also prevented the development of roots in auxin-supplemented medium (Parveen and Shahzad 2011). Thus, to rectify the problems of in vitro rooting, an alternative method (ex vitro rooting) was adopted to induce rooting in *C. angustifolia*. Through ex vitro rooting technique, necrosis and leaf abscission have been minimized considerably, and healthy developmental pattern was observed. Pandeya et al. (2010) in *Clitoria ternatea* also reported *ex vitro* rooting through pulse treatment with 250 mg/l IBA for half an hour.

Bozena (2001) suggested that the plantlets of strawberry developed after ex vitro rooting have better root system than the ones raised through in vitro rooting. Rooting in the external environment is an aid for simultaneous hardening and acclimatization of plantlets and decreases the micropropagation cost as well as the time from laboratory to field conditions (Pruski et al. 2000). Ex vitro rooting proved to be more advantageous over in vitro rooting, as the latter requires utmost care during planting and also more labour and time. This is in corroboration with the earlier studies in several other plant species such as *Lagerstroemia parviflora* (Tiwari et al. 2002), *Prunus fruticosa* (Kris et al. 2005), *Celastrus paniculatus* (Martin et al. 2006), *Aegle marmelos* (Raghu et al. 2007), *Holarrhena antidysenterica* (Mallikarjuna and Rajendrudu 2007) and *Siraitia grosvenorii* (Yan et al. 2010) *Tecomella undulata* (Shaheen and Shahzad 2015).

1.3.4 Acclimatization of Plantlets in Natural Environment

The success of any micropropagation protocol depends on the acclimatization of regenerated plantlets in the external environment at low cost and with high survival rate. During this period of transition, from in vitro to ex vitro conditions, plants have to overcome many adverse conditions as they were cultured under aseptic conditions, with low light intensity on medium containing ample sugar and nutrients to allow heterotrophic growth in the atmosphere of high relative humidity. These conditions result in the plantlets with altered morphology, anatomy and physiology (Kozai et al. 1991; Pospíšilová et al. 2007). Therefore, after ex vitro transplantation, plantlets usually need few weeks of acclimatization and gradually overcome these inadequacies to adapt in the external environment. The survival of plantlets during acclimatization also depends on the use of suitable planting substrate.

After successful acclimatization, in vitro raised plantlets are transferred to earthen pots containing sterilized soil and manure (1:1) and kept under greenhouse for 2 weeks and then finally transferred to the field. These plants do not show any detectable variation in morphological or growth characteristics when compared to the control plants.

1.4 Summary and Future Prospects

Success of plant biotechnology is dependent on regeneration of intact plants following genetic modification, and it has been achieved using plant tissue culture technology. Plant tissue culture helps regenerating a whole plant from a small tissue or a cell, in a suitable culture medium under controlled environmental conditions, and has become an integral part of plant breeding also. It has been effectively used for mass production of elite clones of crop plants where other viable forms of propagation are not available. It has also been successfully used in agriculture-related business. Various types of fruits, flowers, medicinal plants and even trees have been successfully propagated through plant tissue culture.

Thus, plant tissue culture represents the most promising areas of application at present time and gives an outlook into the future. Quality control, however, is also very essential to assure high-quality plant production. The selection of explant source, disease-free material and authenticity of variety are some of the critical parameters which should be evaluated to ensure the quality of the plants produced through this technology.

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