

Abstract

Gottlieb Haberlandt (Math Naturwiss 111:69–92), a German plant physiologist, for the first time initiated the work on tissue culture. His work arose as a research tool and attempts were made to culture the isolated, fully differentiated cells in nutrient medium in vitro as early as 1898. The theoretical basis of tissue culture lies in the cell theory given by Schleiden and Schwann (1838–1839). Practically, this technique stands on the concept of “totipotency,” i.e., each cell has the ability to regenerate into a new plant. The field finds a wide range of applications starting from mass clonal propagation to plant improvement, molecular biology, bio-processing as well as a basic research tool. It has advanced the production in forestry and agriculture to many folds. There are a number of reviews published on tissue culture of woody and tree species which provide the wide-ranging micropropagation reports of various plant species. In this way, this chapter highlights the current review in tree tissue culture in vitro and micropropagation of other valuable plants, their significance, and the wide scope existing for investigations on mass multiplication and conservation of these plants.

2.1 Introduction

Plant biotechnology is founded on the demonstrated totipotency of plant cells, combined with the delivery, stable integration, and expression of transformed plants, and the Mendelian transmission of transgenes to the progeny. The concept of totipotency itself is inherent in the cell theory of Schleiden (1838) and Schwann (1839), which forms the basis of modern biology by recognizing the cell as the primary unit of all living organisms. The cell theory received much impetus from the famous aphorism of Virchow (1858), “Omnis cellula a cellula” (all cells arise from cells), and by the very persistent observation of

Vochting (1878) that the whole plant body can be built up from ever so small fragments of plant organs. However, no sustained attempts were made to test the validity of these observations, until the beginning of the twentieth century because the required technologies did not exist and the nutritional requirements of cultured cells were not fully understood (Gautheret 1985).

Haberlandt (1902) was the first to conduct experiments designed to demonstrate the totipotency of plant cells by culturing chloroplast-containing differentiated cells from leaves of *Lamium purpureum*, cells from the petioles of *Eichhornia crassipes*, glandular hairs of *Pulmonaria* and *Urtica*, and stamen hairs of *Tradescantia* in

Knop's (1865) salt solution in hanging drop cultures. The cells grew in size, but did not undergo any cell divisions. He failed largely because of his unfortunate choice of experimental material and because of the inadequacy of nutrition provided by Knop's salt solution.

Even now, more than a century later, the culture of isolated leaf cells, and other materials used by Haberlandt, is either extremely difficult or impossible in most species. Nevertheless, based on his experiences, Haberlandt made some bold predictions. He advocated the use of embryo sac fluids (coenocytic liquid endosperm, such as coconut milk that was later widely and successfully used in tissue culture studies) for inducing cell divisions in vegetative cells and pointed to the possibility of successfully cultivating artificial embryos (i.e., somatic embryos, which are now the predominant means of plant regeneration in a wide range of species (Thorpe 1995; Vasil 1999)) from vegetative cells in nutrient solutions. It is for these reasons that Haberlandt is rightfully credited with being the founder of the science of plant cell culture.

Went's discovery of auxin (Went 1928) and its subsequent identification as indole-3-acetic acid (IAA) in 1934 (Went and Thimann 1937; Haagen-Smit 1951) opened the road to early successes in tissue culture (Gautheret 1983, 1985). Lyophilized leaf extract from leaves of dodder host plants (a preparation which probably contained auxin) and pure IAA were incorporated in culture media with mixed results starting about a decade after the discovery of this hormone (Fiedler 1936; Geiger-Huber and Burlet 1936; Gautheret 1935, 1937; Loo 1945a).

Skoog and Tsui (1951) reported the continued cell division and bud formation in the cultured pith tissues of tobacco on nutrient media containing adenine and high levels of phosphate. However, Jablonski and Skoog (1954) observed the occurrence of cell divisions when the explants containing vascular tissue were cultured only on nutrient medium. A variety of plant extracts, including coconut milk, were added to the nutrient medium in an attempt to replace vascular tissues and to identify the factors responsible for their beneficial effect. Among these, yeast extract was found to be the most effective and its active com-

ponent was shown to have purine-like properties. This finding led to the addition of DNA to the medium which greatly enhanced cell division activity (Vasil 1959). These investigations resulted in the isolation of kinetin (6-furfurylaminopurine, Kn) from old samples of herring sperm DNA (Miller et al. 1955) and the understanding of the hormonal (auxin–cytokinin) regulation of shoot morphogenesis in plants (Skoog and Miller 1957).

Later experiments led to the isolation of naturally occurring as well as many synthetic cytokinins, the elucidation of their role in cell division and bud development, and their extensive use in the micropropagation industry related to their suppression of apical dominance resulting in the development of many axillary shoots.

The development of improved nutrient solutions, informed choice of plant material, and appreciation of the importance of aseptic cultures led to long-term or indefinite cultures of excised tomato roots and cambial tissues of tobacco and carrot, by White (1934a and b, 1939) in the USA and Gautheret (1934, 1939) and Nobecourt (1939) in France.

White (1943) and others believed that the nutrient solutions based on Knop's (1865) and other formulations neither provided optimal growth nor were stable or satisfactory over a wide range of pH values. These concerns led to the development of White's (1943) medium, which was widely used until the mid-1960s. During this period, a systematic study of mineral and other requirements of plant tissues grown in culture was carried out (Hildebrandt et al. 1946; Heller 1953), demonstrating the need for a greatly increased level of mineral salts in the medium (Ozias-Akins and Vasil 1985). In a similar study, designed to optimize the growth of cultured tobacco pith tissue, a marked increase in growth obtained by the addition of aqueous extracts or ash of tobacco leaves to White's medium was found to be caused largely by the inorganic constituents of the extracts, leading to the development of the first chemically defined and most widely used nutrient solution for plant tissue cultures (Murashige and Skoog 1962). The principal novel features of the Murashige and Skoog (MS) medium were the very high levels of inorganic constituents, chelated iron in order to make it

more stable and available during the life of cultures, and a mixture of four vitamins and myo-inositol.

The discovery of the protoplast culture by Cocking in 1960, haploid plants from anther culture (Guha and Maheshwari 1966), mericlone (Morel 1964), and stages of tissue culture by Murashige (1974) further added aspects of the plant cell culture.

Though hardwood trees were among the first plants cultured in vitro (Gautheret 1940), the first complete plant from tissue culture of free living angiosperm tree species was reported several years later by Winton (1968) from leaf explants of black cotton wood (*Populus trichocarpa*) and by Wolter (1968) for *Populus tremuloides*. The tissue culture of perennial and woody species, being difficult to yield quick results because of their inherent slow-growing nature besides intractable regeneration potential, in addition to some other factors, naturally prompted less effort. But of late, the accent has shifted to a good extent to regenerate trees which used to pose insurmountable challenges in conventional practices of propagation.

Previous work with other woody species, including members of the *Pinus* and *Populus* genera, has provided robust knowledge about the need for a biotechnology-based breeding program in order to develop domesticated, and preferably clonal, elite varieties able to sustain the consistent and homogeneous production of high-quality plant material (Park et al. 1998; Giri et al. 2004; Boerjan 2005). Clonal forestry has enabled rapid genetic gain, thus opening up the possibility for domestication and massive proliferation of elite individuals within a relatively short time (Campbell et al. 2003; Merkle and Nairn 2005). Micropropagation has been used in breeding programs of the most important tropical forest genera, such as *Eucalyptus* (Pinto et al. 2002; Ruiz et al. 2005), *Tectona* (Hausen and Pal 2003), *Cedrela* (Nunes et al. 2002; Pena-Ramirez et al. 2010), *Acacia* (Vengadesan et al. 2002), *Gmelina* (Naik et al. 2003), *Azadirachta* (Quraishi et al. 2004; Morimoto et al. 2006), *Buchanania* (Sharma et al. 2005), *Simmondsia* (Bashir et al. 2007), *Pterocarpus* (Husain et al. 2010), and *Tabebuia*

donnell-smithii rose (Gonzalez-Rodriguez et al. 2010).

2.2 Micropropagation

Schaeffer (1990) defined micropropagation as the in vitro clonal propagation of plants from shoot tips or nodal explants, usually with an accelerated proliferation of shoots during subcultures. Micropropagation is usually described as having the following four distinct stages (a stage “0” is added by some authors):

1. Stage “0”: prepreparation of in situ donor material (fungicide and/or plant growth regulator (PGR) treatments, hedging, etiolation, etc.)
2. Stage “I”: initiation (including surface sterilization) of explants
3. Stage “II”: shoot multiplication (optimization of proliferation media)
4. Stage “III”: root induction on microcuttings (in vitro or ex vitro)
5. Stage “IV”: acclimatization of rooted shoots (or unrooted microcuttings, to ex vitro conditions)

Shoot culture remains the most widely used form of clonal tissue culture and with care can provide a ready source of disease-free and contaminant-free material (George 1993). Induction of shoots into culture (provided the initiation of viable sterile cultures is possible) involves manipulation of the growing medium components, chiefly PGRs to achieve optimal shoot multiplication (stage II) and root induction (stage III) prior to acclimatizing and transferring rooted shoots into the extra vitrum environment (stage IV).

Various degrees of difficulty can be encountered in stages I–IV between species and even between genotypes within a species (Naik et al. 2003). Inability to achieve sterile explants, poor explant performance due to oxidation, phenolic leakage, and the premature death of explants can be encountered in stage I (Lynch 1999). Lack of response to cytokinins, slow growth, abnormal growth, e.g., hyperhydric transformation, shoot miniaturization, or stunting, prolonged phenolic exudation, shoot necrosis, or excessive callusing

may impede optimization of shoot multiplication media in stage II, especially with woody species (Benson 2000). Lack of response (poor or no root induction) to auxin(s), excessive callusing, or deterioration in overall shoot quality can be encountered in stage III, especially with woody plants (Lynch 1999).

In stage IV, transfer to soil, plants need physiological adjustment to the *ex vitro* environment. This entails physiological adjustment to reduced mineral nutrient loading, more variable temperatures, higher lighting levels, reduced humidity, reinstatement of waxy leaf coatings to prevent desiccation and resumption of stomatal function, and regaining root function to allow mass flow transpiration (Preece and Sutter 1991). In addition, plants must successfully survive the transition from primary dependence on medium sugar as the carbon source (photoheterotrophic or photomixotrophic) and become photoautotrophic again (Pospisilova et al. 1999a and b). All these changes need to occur relatively quickly for the plant to regain physiological competence and avoid a prolonged transition period in stage IV with subsequent risk of senescence resulting from oxidative trauma (Batkova et al. 2008) and pathogen infection (Williamson et al. 1998).

2.3 Various Approaches for Micro-propagation

The methods that are available for propagation of plants *in vitro* are described in the following sections of the chapter. They are essentially as follows:

- By the multiplication of shoots from axillary buds

By the formation of adventitious shoots, and/or adventitious somatic embryos, either (a) directly on pieces of tissue organs (explants) removed from the mother plant or (b) indirectly from unorganized cells (in suspension cultures) or tissues (in callus cultures) established by the proliferation of cells within explants, or on semiorganized callus tissues or propagation bodies (such as protocorms or pseudobulbils) that can be obtained from explants (particularly those from certain specialized whole plant organs)

2.3.1 The Propagation of Plants from Axillary Buds or Shoots

The production of plants from axillary buds or shoots has proved to be the most generally applicable and reliable method of true-to-type *in vitro* propagation. The two methods commonly used are as follows:

- Shoot culture
- Single, or multiple, node culture

Both depend on stimulating precocious axillary shoot growth by overcoming the dominance of shoot apical meristems.

Robbins (1922) seems to have been the first person to have successfully cultured excised shoot tips on a medium containing sugar, but a significant shoot growth from vegetative shoot tip explants was first achieved by Loo (1945a, b, 1946a, b) in *Asparagus* and dodder plants. He has made several significant observations in *Asparagus* cultures showing that:

- Growth depended on sucrose concentration, higher levels being necessary in the dark than in the light.
- Explants, instead of being supported, could apparently be continued indefinitely (35 transfers were made over 22 months).
- Shoot tip culture afforded a way to propagate plant material (clones were established from several excised shoot apices).

This work failed to progress further because no roots were formed on the *Asparagus* shoots in culture. Honors for establishing the principles of modern shoot culture must therefore be shared between Loo and Ball.

Ball (1946) was the first person to produce rooted shoots from cultured shoot apices. His explants consisted of an apical meristem and two to three leaf primordia. There was no shoot multiplication but plantlets of *Tropaeolum majus* and *Lupinus alba* were transferred to soil and grown successfully.

Haramaki (1971) described the rapid multiplication of *Gloxinia* by shoot culture, and, by 1972, several reports of successful micropropagation by this method had appeared (Adams 1972; Haramaki and Murashige 1972). Since then, the number of papers on shoot culture published an-

nually has increased dramatically and the method has been utilized increasingly for commercial plant propagation (George and Debergh 2008).

Currently, node culture is of value for propagating species that produce elongated shoots in culture, especially if stimulation of lateral bud break is difficult to bring about with available cytokinins. Nowadays, the technique becomes more and more popular in commercial micropropagation. The main reason is that it gives more guarantee for clonal stability. Indeed, although the rate of multiplication is generally less than that which can be brought about through shoot culture, there is less likelihood of associated callus development and the formation of adventitious shoots, so that stage II subculture carries very little risk of induced genetic irregularity (George and Debergh 2008). For this reason, node culture has been increasingly recommended by research workers as the micropropagation method least likely to induce somaclonal variation (Prakash and Van Staden 2008; Ahmad and Anis 2011; Asthana et al. 2011).

2.3.1.1 Multiple Shoots from Seeds

During the early 1980s, it was discovered that it was possible to initiate multiple shoot clusters directly from seeds. Seeds are sterilized and then placed onto a basal medium containing a cytokinin. As germination occurs, clusters of axillary and/or adventitious shoots ("multiple shoots") grow out and may be split up and serially subcultured on the same medium. High rates of shoot multiplication are possible. For instance, Hisajima (1982) estimated that ten million shoots of almond could be derived theoretically from one seed per year. Similarly, Malik and Saxena (1992a, b) found that 5–20-fold more shoots were induced when intact seeds of pea, chickpea, and lentil were exposed to a medium with high concentrations of cytokinins such as benzyladenine (BA) or thidiazuron (N-fenil-N'-1,2,3-thidiazol-5-il-urea, TDZ), compared to isolated explants such as cotyledonary nodes. Malik and Saxena (1992b) hypothesized that the high regeneration potential of seeds is caused by the physiological structural integrity of the explants.

It is likely that multiple shoots can be initiated from the seeds of many species, particularly dicotyledons. The technique is effective in both herbaceous and woody species: soybean (Hisajima and Church 1982), sugar beet (Powling and Hussey 1981), almond (Hisajima 1981), walnut (Rodriguez 1982), chickpea, lentil (Malik and Saxena 1992a), *Pisum sativum* (Malik and Saxena 1992b; Zhihui et al. 2009), *Murraya koenigii* (Bhuyan et al. 1997), *Litchi chinensis* (Das et al. 1999), and *Sterculia urens* (Hussain et al. 2008).

2.3.2 Propagation by Adventitious Shoot Organogenesis

During adventitious organogenesis, new organs (shoots, roots) can develop on explants of different plant tissues such as leaves, stems, and roots. Leaves and hypocotyls, for instance, do not have any apparent preexisting meristems, and therefore most plant cells could be considered totipotent.

The huge amount of variability seen in the frequency of organogenesis between varieties and species suggests that it is the proportion of cells that are receptive to in vitro culture conditions that vary. Organogenesis in vitro consists of several factors, such as PGR perception and transduction, redifferentiation after dedifferentiation of differentiated cells, organization for specific organ primordial and meristems, etc. The process depends on external and internal factors, such as exogenously applied PGRs, and the ability of plant tissue to perceive these PGRs.

According to the requirement for a specific PGR balance, three phases of organogenesis are distinguishable (Sugiyama 1999):

1. Competence (dedifferentiation): Cells become able to respond to hormonal signals of organ induction (Howell et al. 2003). Dedifferentiation means the acquisition of organogenic competence. Wounding usually triggers dedifferentiation in tissue culture (Sugiyama 1999).
2. Determination: The competent cells in explants are determined for specific organ formation and this process is influenced by a specific PGR balance. From competent cells,

adventitious shoot formation could be induced by cytokinins (Gahan and George 2008).

3. Morphogenesis: This proceeds independently of exogenously added PGRs (Sugiyama 1999). However, Yancheva et al. (2003) found that the type of auxin and the length and timing of its application are critical for both activation and progression of the plant cell developmental program.

There are two distinct pathways of adventitious meristem formation: direct and indirect. During the direct pathway, the formation of a meristem proceeds without intermediate proliferation of undifferentiated callus tissue. However, meristems can be formed indirectly from unspecialized and dedifferentiated cells of callus or suspension culture (Yancheva et al. 2003; Gahan and George 2008).

In certain species, adventitious shoots which arise directly from the tissues of the explants (and not within previously formed callus) can provide a reliable method for micropropagation. The main advantages of micropropagation by direct adventitious shoot regeneration are as follows:

- Initiation of stage I cultures and stage II shoot multiplication are more easily achieved than by shoot culture.
- Rates of propagation can be high, particularly if numerous small shoots arise rapidly from each explant.

However, the induction of direct shoot regeneration depends on the nature of the plant organ from which the explants were derived and is highly dependent on plant genotype.

Regeneration via adventitious shoots in woody species has been reported in *Dalbergia latifolia* (Lakshmisita et al. 1986), *Aegle marmelos* (Islam et al. 1993), *Sesbania grandiflora* (Detrez et al. 1994), *Liquidambar styraciflua* (Kim et al. 1997), *Prunus avium* (Hammatt and Grant 1998), *D. sissoo* (Pradhan et al. 1998; Pattnaik et al. 2000; Chand et al. 2002), *Azadirachta indica* (Eswara et al. 1998; Sharma et al. 2002), *Salix nigra* (Lyyra et al. 2006), *Paulownia tomentosa* (Corredoira et al. 2008), *Platanus occidentalis* (Sun et al. 2009), *Jatropha curcas* (Kumar et al. 2010), *Cassia angustifolia* (Siddique et al.

2010), *Tabebuia donnell-smithii* rose (Gonzalez-Rodriguez et al. 2010), *Pterocarpus marsupium* (Husain et al. 2010), and *Albizia lebbeck* (Perveen et al. 2011).

2.3.2.1 Regeneration from Roots

Among the possible initial explants, roots have proven to be highly regenerative explants for in vitro regeneration in different species, including forest ones (George 1993). According to Morton and Browse (1991), root explants are advantageous over other explants in terms of their easy manipulation, higher regeneration, and excellent susceptibility for *Agrobacterium* transformation. Roots have also received considerable attention as a potential production system for stable metabolite production (Zobayed and Saxena 2003). Besides being useful for micropropagation, the root culture could be successfully applied for germplasm preservation (Chaturvedi et al. 2004).

Since the pioneering work on the establishment of root culture of tomato (White 1934a), roots of many plants are capable of independent growth to form secondary roots in culture (Butcher and Street 1964) and regenerate shoots. These shoots arise de novo from pericycle, as demonstrated in *Convolvulus* (Torrey 1958). Initiation of shoots from root explants has been described in several plant species, indicating a possibility of developing regenerative excised root culture for mass multiplication and their germplasm preservation, viz., *Citrus mitis* (Sim et al. 1989), *Citrus aurantifolia* (Bhat et al. 1992), *Averrhoa carambola* (Kanthrajah et al. 1992), *Lonicera japonica* (Georges et al. 1993), *Acacia albida* (Ahee and Duhoux 1994), *Albizia julibrissin* (Sankhla et al. 1994), *Aeschynomene sensitiva* (Nef-Campa et al. 1996), *Azadirachta indica* (Sharma et al. 2002; Shahin-un-zamam et al. 2008; Arora et al. 2010), *Shorea robusta* (Chaturvedi et al. 2004), *Melia azedarach* (Vila et al. 2005), *Populus alba* (Tsvetkov et al. 2007), *Cleome rosea* (Simoes et al. 2009), *Swertia chirata* (Pant et al. 2010), *Passiflora edulis* (Viana da Silva et al. 2011), and *Albizia lebbeck* (Perveen et al. 2011).

2.4 Factors Affecting In Vitro Shoot Regeneration and Growth of Plants

Several factors, such as genotype, type of explants, PGRs, type of media, and in vitro conditions before and after the regeneration process, can influence the success of in vitro shoot regeneration.

2.4.1 Explant Type

The tissue which is obtained from the plant to culture is called an explant. Based on work with certain model systems, particularly tobacco, it has often been claimed that a totipotent explant can be grown from any part of the plant. In many species, explants of various organs vary in their rates of growth and regeneration, while some do not grow at all. Also, the risk of microbial contamination is increased with an inappropriate explant. Thus, it is very important that an appropriate choice of explant be made prior to tissue culture.

The specific differences in the regeneration potential of different organs and explants have various explanations. The significant factors include differences in the stage of the cells in the cell cycle, the availability of or ability to transport endogenous growth regulators, and the metabolic capabilities of the cells. The most commonly used tissue explants are the meristematic ends of the plants such as the stem tip, axillary bud tip, and root tip. These tissues have high rates of cell division and either concentrate or produce the required growth-regulating substances including auxins and cytokinins (Akin-Iidowu et al. 2009).

Some studies have shown that explant characteristics such as type, source, genotype, and history affect the success and commercial viability of tissue culture systems (Bhau and Waklu 2001; Chan and Chang 2002; Hoy et al. 2003). The effect of explant type on successful tissue culture of various plants has been reported (Gubis et al. 2003; Blinstrubiene et al. 2004; Tsay et al. 2006; Gitonga et al. 2010).

Moreover, juvenile plants are an excellent explant source to achieve successful in vitro propagation of tropical forest trees. Nodal segments have been widely used for in vitro shoot proliferation of woody plants such as *Syzygium cumini* (Jain and Babbar 2000), *Terminalia chebula* (Shyamkumar et al. 2003), *Oroxylum indicum* (Dalal and Rai 2004), *Holarrhena antidysenterica* (Kumar et al. 2005), *Boswellia ovalifoliolata* (Chandrasekhar et al. 2005), *Tectona grandis* (Shirin et al. 2005), *Pterocarpus santalinus* (Rajeswari and Paliwal 2006), miracle berry (Ogunsola and Ilori 2007), *Stereospermum personatum* (Shukla et al. 2009), *Vitex negundo* (Ahmad and Anis 2011), and *Sapindus trifoliatus* (Asthana et al. 2011). This is probably due to the readily available axillary buds in nodal segments that only require a trigger for bud break in contrast to root, leaf, and cotyledonary tissue that would otherwise require initiation of adventitious buds (Lombardi et al. 2007, Sivanesan et al. 2007) and somatic embryos (Naz et al. 2008, Husain et al. 2010) before any shoot regeneration is achieved.

The different explants such as nodal segments, cotyledonary nodes, hypocotyls, roots, and cotyledons selected also could influence the rate of shoot regeneration in many trees including different species of *Acacia*, teak, *Eucalyptus*, *Salix tetrasperma*, *V. negundo*, *P. marsupium*, and *Albizia lebbek* (Vengadesan et al. 2002; Yasodha et al. 2004; Anis et al. 2012).

Different age of explants may have different levels of endogenous hormones and, therefore, the age of explants would have a critical impact on the regeneration success; these results have been reported in many plants, including *Prunus* (Mante et al. 1989), *Lachenalia* (Niederwieser and Van Staden 1990), *Cydonia oblonga* (Baker and Bhatia 1993), *Aegle marmelos* (Islam et al. 1993), *Malus* (Famiani et al. 1994), *Cercis canadensis* (Distabanjong and Geneve 1997), *Morus alba* (Thomas 2003), and *Sapindus trifoliatus* (Asthana et al. 2011).

The genotype, in addition to the type and age of the explants, is a key criterion in determining the material suitable for micropropagation (Kunze 1994; Tang and Guo 2001; Tereso et al. 2006; Cortizo et al. 2009).

2.4.2 Plant Growth Regulators

Some chemicals occurring naturally within plant tissues (i.e., endogenously) have a regulatory rather than a nutritional role in growth and development. These compounds, which are generally active at very low concentrations, are known as plant hormones (or plant growth substances). Synthetic chemicals with physiological activities similar to plant growth substances or compounds having an ability to modify plant growth by some other means are usually termed PGRs.

There are several recognized classes of plant growth substance. Until relatively recently, only five groups were recognized, namely:

- Auxins
- Cytokinins
- Gibberellins
- Ethylene
- Absciscic acid (ABA)

Auxins and cytokinins are by far the most important plant growth substances for regulating growth and morphogenesis in plant tissue and organ cultures; in these classes, synthetic regulators have been discovered with a biological activity, which equals or exceeds that of the equivalent natural growth substances.

No chemical alternatives to the natural gibberellins or ABA are available, but some natural gibberellins are extracted from cultured fungi and are available for use as exogenous regulators.

2.4.2.1 Auxins

Auxins are very widely used in plant tissue culture and usually form an integral part of the nutrient media. Auxins promote, mainly in combination with cytokinins, the growth of calli, cell suspensions, and organs and also regulate the direction of morphogenesis.

At the cellular level, auxins control basic processes such as cell division and cell elongation. Since they are capable of initiating cell division, they are involved in the formation of meristems giving rise to either unorganized tissue or defined organs. The choice of auxins and the concentration administered depend on:

- The type of growth and/or development required

- The rate of uptake and of transport of the applied auxin to the target tissue
- The inactivation (oxidation and/or conjugation) of auxin in the medium and within the explants
- The sensitivity of the plant tissue to auxin (and other hormones as well)
- The interaction, if any, between applied auxins and the natural endogenous substances

The most commonly detected natural auxin is IAA which may be used in plant tissue culture media, but it tends to be oxidized in culture media and is rapidly metabolized within plant tissues. However, for many purposes, it is necessary or desirable to use one of the many synthetic analogues of IAA. These analogues have different structures but similar biological properties and are also called auxins. The synthetic auxins 2,4-dichlorophenoxyacetic acid (2,4-D), α -naphthalene acetic acid (NAA), and indole-3-butyric acid (IBA) are commonly used in the tissue cultures.

Indeed, all active auxins are weak organic acids. The relative degree of activity of individual auxins in different growth processes is very variable. It differs not only from plant to plant but also from organ to organ, tissue to tissue, cell to cell, and also with the age and physiological state of the plant (tissue; Davies 2004).

In tissue culture, depending on other hormones present in the medium, changes in auxin concentrations may change the type of growth, e.g., stimulation of root formation may switch to callus induction. In this respect, each tissue culture system is unique, and the effects of different concentrations of auxins and other hormones must be tested for each case individually, and only to some extent, the results can be transferred to other cultures.

Plants—like other higher organisms—have to possess intra-organismal communication system(s) working over relatively long distances. As no nervous system is present, the main signaling systems are hormone dependent (Libbenga and Mennes 1995). Auxins are a component of such systems. Auxins and cytokinins impact at several levels in many different processes of plant development.

The ability of auxins (together with cytokinins) to manage key events in plant morphogenesis was documented, among others, by Skoog and Miller's (1957) discovery of the regulation of organogenesis in vitro by means of the auxin to cytokinin ratio in culture media. It has been further supported by recent investigations on the relationships between auxin and cytokinin levels and the morphogenetic response of various plants (Li et al. 1994; Leyser et al. 1996; Centeno et al. 1996).

A range of auxins in combination with cytokinins played a vital role in multiple shoot regeneration in many tree species (Vengadesan et al. 2002; Giri et al. 2004; Anis et al. 2012). Addition of low levels of auxins along with cytokinin is known to increase shoot numbers in many plant species like *Wrightia tinctoria* (Purohit and Kukda 1994), *Rauwolfia micrantha* (Sudha and Seeni 1996), *Sapium sebiferum* (Siril and Dhar 1997), *Gmelina arborea* (Tiwari et al. 1997), *Gymnema sylvestre* (Reddy et al. 1998), and *Tectona grandis* (Tiwari et al. 2002; Shirin et al. 2005). Moreover, the role of auxins in root development is well established and the effect of various auxins on rooting of the excised microshoots in vitro and ex vitro has been discussed in the section 2.5 "Rooting" of this chapter.

2.4.2.2 Cytokinins

Among PGRs, cytokinins have proven to be the most important factor affecting shoot regeneration, and their significant effects may be related to the histological changes in induced tissues (Magyar-Tabori et al. 2010).

Cytokinins are N⁶-substituted adenines with growth regulatory activity in plants that promote cell division and may play a role in cell differentiation (McGaw and Burch 1995). Cytokinins added to the medium are very important during tissue culture of plants because they induce division and organogenesis (Howell et al. 2003) and affect other physiological and developmental processes (Heyl and Schumling 2003; Ferreira and Kieber 2005; Van Staden et al. 2008).

The success of a culture is affected by the type and concentration of applied cytokinins, because their uptake, transport, and metabolism differ be-

tween varieties and they can interact with endogenous cytokinins of an explant (Werbrouck et al. 1996; Strnad et al. 1997; Van Staden et al. 2008).

There are two main classes of cytokinins according to the chemical structure of the side chain: isoprenoid and aromatic cytokinins, which differ in their biochemistry, receptors, biological activity, and their metabolism (Strnad et al. 1997; Werbrouck et al. 1996; Van Staden et al. 2008). Considering natural cytokinins, BA or sometimes Kn (Barciszewski et al. 1999) is most frequently used in tissue culture systems. Vengadesan et al. (2002) reported that BA singly was common in most of the in vitro micropropagation systems of different species of *Acacia* irrespective of the explants and media type. Similarly, the positive effect of BA on shoot multiplication of teak has been reported by a number of researchers (Gupta et al. 1980; Devi et al. 1994; Shirin et al. 2005).

Likewise, the superiority of BA over other cytokinins on multiple shoot bud differentiation has been demonstrated in a number of cases (Jeong et al. 2001; Loc et al. 2005; Phulwaria et al. 2012). Bhattacharya and Bhattacharya (1997) developed an in vitro culture protocol for *Jasminum officinale* using BA at an elevated level (17.76 μ M) in MS medium. Similarly, Purkayastha et al. (2008) obtained maximum number of shoots at 10 μ M BA. A considerable higher range of BA (22.2 μ M) was used by Koroch et al. (1997) for the micropropagation of *Hedeoma multiflorum*. However, lower concentrations of BA (2.22 μ M) were applied with full-strength MS medium by Elangomathvan et al. (2003) to obtain multiple shoots in *Orthosiphon spiralis*. Nayak et al. (2007) investigated the effect of BA at low concentrations on shoot proliferation in *Aegle marmelos* and found optimal response in MS medium supplemented with 6.6 μ M BA. Gokhale and Bansal (2009) obtained multiple shoots directly from apical and axillary buds of *O. indicum* in MS medium amended with 4.43 μ M BA.

Above and beyond the utilization of BA in tissue culture, Kn has also found to be very effective in establishing in vitro regenerative protocol for many plant species such as *Picrorhiza kurroa* (Lal et al. 1988), *Hemidesmus indicus* (Pattnaik

and Debata 1996), *Limonium cavanillesii* (Amo-Marco and Ibanez 1998), *Eclipta alba*, and *Eupatorium adenophorum* (Borthakur et al. 2000). In addition, the application of this growth regulator for multiple shoot production has been successful in a number of medicinally important plant species including *Alpinia galanga* (Borthakur et al. 1999), *Eurycoma longifolia* (Hussein et al. 2005), *Tinospora cordifolia* (Gururaj et al. 2007), *Ricinus communis* (Chaudhary and Sood 2008), *Rauvolfia tetraphylla* (Harisaranraj et al. 2009), *Thymus vulgaris* and *T. longicaulis* (Ozudogru et al. 2011).

Moreover, 2-isopentenyladenine (2-iP) is reported to be the best cytokinin for shoot multiplication in blueberry by Cohen (1980) and in garlic by Bhojwani (1980). Chattopadhyay et al. (1995) achieved rapid micropropagation protocol for *Mucuna pruriens* using 2-iP. Mills et al. (1997) mentioned that 2-iP at a higher concentration of 30.5 mg dm⁻³ was optimum for differentiating maximum number of shoots in *Simmondsia chinensis*. However, Taha et al. (2001) reported that the shoot bud proliferation ability of date palm shoot tips was strongly enhanced by low concentration of 2-iP (3 mg dm⁻³). Similarly, Jakola et al. (2001) obtained best results in *Vaccinium myrtillus* and *V. vitis-idaea* utilizing higher levels of 2-iP (49.2 and 24.6 µM) on modified MS medium, while lower concentrations (12.3 or 24.6 µM) were recommended by Pereira (2006) for other species of *Vaccinium cylindraceum* micropropagation on Zimmermann and Broome medium. In case of *Rhododendrons*, Vejsadova (2008) found the highest shoot multiplication rate on MS medium amended with 2-iP. Similarly, Singh and Gurung (2009) proved 2-iP to be the most effective cytokinin in comparison with BA or Kn for multiple shoot induction in *Rhododendron maddenii*.

There are also some important synthetic cytokinins, such as TDZ. The activity of TDZ varies widely depending on its concentration, exposure time, the cultured explant, and the species tested (Murthy et al. 1988). Like other synthetic cytokinins, it appears less susceptible to enzymatic degradation in vivo than other naturally occurring amino purine cytokinins and has been

found to be effective at very low concentrations (0.0091–3.99 µM) for micropropagation of several species (Lu 1993). However, it has been used at higher concentrations (2.27–145.41 µM) for propagation of some species including *Zanthoxylum rhetsa* (Augustine and D'Souza 1997), a mature forest tree species. It has been shown to induce high bud regeneration rates in comparison to purine-based cytokinins and also has the capability of fulfilling both the cytokinin and auxin requirements of regeneration responses in a number of woody plants (Mok et al. 2005; Jones et al. 2007). However, it can also cause undesirable side effects, such as inhibited shoot elongation and rooting, fasciated shoots, and hyperhydricity.

In woody plants, TDZ has been shown to be suitable for micropropagation and regeneration of recalcitrant species or genotypes (Huetteman and Preece 1993; reviewed in Durkovic and Misalova 2008). A perusal of literature reveals that it has successfully been used to induce axillary or adventitious shoot proliferation in a number of plant species including herbaceous, perennials, and tree species such as *Cassia angustifolia* (Siddique and Anis 2007a and b; Parveen and Shahzad 2011), *P. marsupium* (Husain et al. 2007), *Pongamia pinnata* (Sujatha and Hazra 2007), *Cardiospermum halicacabum* (Jahan and Anis 2009), and *Bacopa monnieri* (Ceaser et al. 2010).

• Cytokinins and auxins in synergy

Since the action of cytokinin and auxin has been linked from early studies, they are known to interact in several physiological and developmental processes, including apical dominance, control of cell cycle, lateral root initiation, regulation of senescence, and vasculature development (Coenen and Lomax 1997; Swarup et al. 2002).

Exogenous applications of cytokinins and auxins have been known to be important for shoot induction and elongation of many plant species in vitro (George 1993). Various successful combinations have been reported, such as BA+IAA for *Aegle marmelos* (Islam et al. 1993), *Tecomella undulata* (Nandwani et al. 1995), *Paulownia* species (Rao et al. 1996; Bergmann and Moon 1997), *Tectona grandis* (Tiwari et al. 2002), *O. indicum* (Dalal and Rai 2004), *Malus zumi* (Xu

et al. 2008), *Terminalia bellirica* (Phulwaria et al. 2012); BA + IBA for *G. arborea* (Sukartiningsih et al. 1999), *J. curcas* (Shrivastava and Banerjee 2008), *L. styraciflua* (Durkovic and Lux 2010); BA+NAA for *Syzygium alternifolium* (Sha Valli Khan et al. 1997), *Acacia catechu* (Hossain et al. 2001), *Zyziphus jujuba* (Hossain et al. 2003), *Tectona grandis* (Shirin et al. 2005), *Cornus mas* (Durkovic 2008), *Teucrium fruticans* (Frabetti et al. 2009), *Celastrus paniculatus* (Martin et al. 2006), and *Acacia auriculiformis* (Girijashankar 2011).

Also, elevated level of shoot multiplication and proliferation rate was achieved in *Myrica esculenta* using Kn in concurrence with NAA by Bhatt and Dhar (2004). Complete plantlets of *R. communis* were successfully raised by Chaudhary and Sood (2008) while applying Kn in combination with NAA in MS medium. Kn–NAA synergism and their triggering effect on shoot bud induction and multiplication have established rightly in *Cephaelis ipecacuanha* (Jha and Jha 1989), *Gossypium hirsutum* (Rauf et al. 2004), *Cordia verbenacea* (Lameira and Pinto 2006), *Alpinia officinarum* (Selvakkumar et al. 2007), and *Gardenia jasminoides* (Duhoky and Rasheed 2010). In addition, Iapichino and Airo (2008) reported that the addition of 2-iP with IAA is found to be the suitable PGR regime for propagation in *Metrosideros excelsa*.

2.4.2.3 Gibberellins

Plant tissue cultures can generally be induced to grow and differentiate without gibberellins, although gibberellic acid (GA_3) may become an essential ingredient of media for culturing cells at low densities (Stuart and Street 1971). GA_3 is known to break the dormancy of several types of seeds at a critical concentration. It stimulates seed germination via the synthesis of α -amylase and other hydrolases (Shepley et al. 1972). Thus, in recent papers, GA_3 has been used to break dormancy and morphogenesis (Chaturvedi et al. 2004; Shahzad et al. 2007; Parveen et al. 2010; Balaraju et al. 2011).

GA_3 is added to the medium, together with auxin and cytokinin, in stages I and II of shoot cultures of certain plants. At stage I, its presence

can improve establishment; for example, De Fossard and De Fossard (1988) found that the addition of GA_3 to the medium was useful to initiate growth in cultures from adult parts of trees of the family Myrtaceae. Additions of GA_3 with BA caused high-frequency bud break and shoot multiplication in apical shoot buds and nodal explants of *Morus cathayana* (Pattnaik and Chand 1997). Moreover, the benefits of using GA_3 singly or in combination with other PGRs in the culture medium for shoot multiplication have been well documented in a number of plant species (Kotsias and Roussos 2001, Farhatullah and Abbas 2007, Moshkov et al. 2008).

2.4.3 Medium pH Levels

The relative acidity or alkalinity of a solution is assessed by its pH. This is a measure of the hydrogen ion concentration; the greater the concentration of H^+ ions (actually H_3O^+ ions), the more acidic the solution. As pH is defined as the negative logarithm of hydrogen ion concentration, acidic solutions have low pH values (0–7) and alkaline solutions have high values (7–14). To judge the effect of medium pH, it is essential to discriminate between the various sites where the pH might have an effect: (1) in the explants, (2) in the medium, and (3) at the interface between explants and medium (Thorpe et al. 2008).

According to Thorpe et al. (2008), the pH of a culture medium must be such that it does not disrupt the plant tissue. Within the acceptable limits, the pH also:

- Governs whether salts will remain in a soluble form
- Influences the uptake of medium ingredients and plant regulator additives
- Has an effect on chemical reactions (especially those catalyzed by enzymes)
- Affects the gelling efficiency of agar

This means that the effective range of pH for media is restricted. As will be explained, medium pH is altered during culture, but as a rule of thumb, the initial pH is set at 5.5–6.0. In culture media, detrimental effects of an adverse pH are

generally related to ion availability and nutrient uptake rather than cell damage.

The pH of the medium has an effect on the availability of many minerals (Scholten and Pierik 1998). In general, the uptake of negatively charged ions (anions) is favored at acidic pH, while that of cations (positively charged) is best when the pH is increased. As mentioned earlier, the relative uptake of nutrient cations and anions will alter the pH of the medium. The release of hydroxyl ions from the plant in exchange for nitrate ions results in media becoming more alkaline; when ammonium ions are taken up in exchange for protons, the media become more acidic.

One of the chief advantages of having both NO_3^- and NH_4^+ ions in the medium is that uptake of one provides a better pH environment for the uptake of the other. The pH of the medium is thereby stabilized. Uptake of nitrate ions by plant cells leads to a drift towards an alkaline pH, while NH_4^+ uptake results in a more rapid shift towards acidity (George 1993). In media containing both NO_3^- and NH_4^+ with an initial pH of 5–6, the preferential uptake of NH_4^+ causes the pH to drop during the early growth of the culture. This results in increased NO_3^- utilization (Martin and Rose 1976). The final pH of the medium depends on the relative proportions of NO_3^- and NH_4^+ (Gamborg et al. 1968).

It has been reported that medium pH influences developmental processes in tissue culture, among other regenerative processes: xylogenesis in *Citrus* and *Zinnia elegans* (Khan et al. 1986; Roberts and Haigler 1994), androgenesis in winter triticale and wheat (Karsai et al. 1994), adventitious bud regeneration in tobacco (Pasqua et al. 2002), and adventitious root formation in apple (Harbage et al. 1998).

Changes in the pH of a medium do, however, vary from one kind of plant to another. In a random sample of papers on micropropagation, the average initial pH adopted for several different media was found to be 5.6 (mode 5.7) but adjustments to as low as 3.5 and as high as 7.1 had been made. Kartha (1981) found that pH 5.6–5.8 supported the growth of most meristem tips in culture and that cassava meristems did not grow for a prolonged period on a medium adjusted to

pH 4.8. Shoot proliferation in *Camellia sasanqua* shoot cultures was best when the pH of a medium with MS salts was adjusted to 5–5.5. Many plant cells and tissues in vitro will tolerate pH in the range of about 4.0–7.2; those inoculated into media adjusted to pH 2.5–3.0 or 8.0 will probably die (Butenko et al. 1984). Bhatia and Ashwath (2005) reported that a high pH above 6.0 produces a very hard medium and a pH lower than 5.0 does not sufficiently solidify the medium.

Parlman et al. (1982) tested the effect of different medium pH values (3.5, 4.5, 5.5, and 6.5) in *Dionea muscipula* and found that the optimum pH for shoot proliferation and elongation was 5.5, which was severely inhibited in more acidic medium. In chickpea, pH 6.5 was proved to be the optimum for embryo formation, which was adversely affected by pH above 7.0 and below 4.0 (Barn and Wakhlu 1993). Wang et al. (2005) showed that pH level of 5.8–6.6 was broadly effective for shoot regeneration for *Camptotheca acuminata*. The best result of shoot regeneration was found for the medium at pH 5.8 with 90% regeneration frequency. On a medium with pH 7.0 or below 5.4, the regenerated shoot number was low. Moreover, on the medium with pH value below 5.4, the regenerated shoots showed serious vitrification.

A similar study was conducted by Faisal et al. (2006a and b) where a wide range of pH was tested for shoot induction and it reported maximum multiplication rate at a pH of 5.8. Similar findings were reported by Siddique and Anis (2007a) in *Cassia angustifolia* and *B. monnieri* (Naik et al. 2010) and Perveen et al. (2011) in *Albizia lebbeck*. These studies indicated that the effect of pH value on plant regeneration depends on plant species.

2.4.4 Basal Media

The basic components of plant tissue culture media are the mineral nutrients. How rapidly a tissue grows and the extent and quality of morphogenetic responses are strongly influenced by the type and concentration of nutrients supplied.

Early research by Gautheret (1939); Heller (1953); White (1942); Hildebrandt et al. (1946); Nitsch and Nitsch (1956) culminated in the development of MS medium by Murashige and Skoog (1962). The organic and mineral compositions of the culture medium are particularly important to improve differentiation and to optimize explant's growth. It is well known that the amount of nutrients present in the culture medium must be sufficient to foster growth throughout the entire culture period.

The potential benefits of optimizing the nutrient component of culture media for a particular response are well documented across a wide range of species and applications. For example, the concentration of NH_4^{4+} and NO_3^{-} affects numerous in vitro responses including the development of somatic embryos (Meijer and Brown 1987; Poddar et al. 1997; Elkonin and Pakhomova 2000; Leljok-Levanic et al. 2004), the plating efficiency of protoplasts (Attree et al. 1989), the efficiency of plant recovery after ovule culture (McCoy and Smith 1986), shoot regeneration (Leblay et al. 1991), regulation of growth and biomass of bioreactor-grown plantlets (Sivakumar et al. 2005), and controlling the rate of root initiation on shoot cultures (Hyndman et al. 1982).

Because there are 13 mineral elements essential for plant growth (Epstein and Bloom 2005), the experimental determination of optimal nutrient levels is complex. This complexity illustrates why the "revised medium" developed by Murashige and Skoog (1962) was an important development. Although MS medium is not optimal for many tissues, many tissues will grow on it to some degree; hence, MS medium represents a starting point to begin the process of improving a response. The significant and distinguishing feature of MS (1962) medium is its high nitrate, ammonium, and potassium contents.

The composition, type, and strength of basal medium also played an important role in shoot multiplication. Full strength of MS medium was found favorable for multiple shoot production in *H. antidysentrica* (Mallikarjuna and Rajendrudu 2007), *Actinidia deliciosa* (Akbas et al. 2007), *P. santalinus* (Rajeswari and Paliwal 2008), *Acacia nilotica* (Abbas et al. 2010), *Albizia lebbbeck*

(Perveen et al. 2011), and *V. negundo* (Ahmad and Anis 2011). Modification in the MS medium such as MS salts reduced to one half, one third, one fifth, or three fourth has been found effective in *Acacia senegal* (Badji et al. 1993), *A. mearnsii* (Huang et al. 1994), *Anacardium occidentale* (Das et al. 1996).

While many studies have concentrated on the influence of PGRs, the influence of the nutrient medium has received less attention. Most studies have used MS medium (Murashige and Skoog 1962) without modification, but a few have reduced the overall ion concentration or modified the nitrogen concentration or nitrogen sources (Chevreau et al. 1992; Bell and Reed 2002). Only a few of the studies made comparison among several nutrient media for axillary, adventitious shoot regeneration, as well as for somatic embryogenesis. Nedelcheva (1986) found that shoot proliferation of "Bartlett" was the greatest on a medium devised by Quoirin and Lepoivre (1977; QL), in comparison to MS medium. In contrast, Baviera et al. (1989) obtained better shoot proliferation of "Conference" on MS than on QL. Wang (1991) observed a higher degree of multiple shoot formation of the *Pyrus communis* L. rootstock BP10030 on woody plant medium (WPM; Lloyd and McCown 1981) and QL than on MS in a double-phase culture system consisting of a liquid medium overlaid on a semisolid medium. Yeo and Reed (1995) found that the nutrient medium of Cheng (1979) was better for shoot multiplication than WPM for a genetically diverse group of root stocks, such as "OH × F 230" (*P. communis* L.), "OPR 260" (*Pyrus betulifolia* Bunge), and "OPR 157" (*Pyrus calleryana* Decne), whereas Thakur and Kanwar (2008) reported that WPM resulted in enhanced axillary shoot proliferation of *Pyrus pyrifolia* when compared to MS and various modifications, and Gonzalez-Rodriguez et al. (2010) found that it was true for adventitious shoot regeneration from stem explants of *Tabebuia donnell-smithii* rose.

Recently, Khan et al. (2011) demonstrated the best shoot induction response in nodal explants of *Salix tetrasperma* in WPM medium supplemented with different PGRs. Bhatt and Dhar (2004) established a higher efficiency of WPM over other types of medium like B₅ (Gamborg et al. 1968)

and MS and their different strengths were tried for shoot proliferation in *Myrica esculenta*. They observed that neither MS nor B₅ gave satisfactory response even when the salt concentration is reduced to half, and shoot response was severely inhibited.

Earlier, Gharyal and Maheshwari (1990) reported 36% direct shoot regeneration from petioles of different leguminous plant species on a B5 medium supplemented with 0.5 mg l⁻¹ IAA and 1 mg l⁻¹ BA. Lakshmisita et al. (1992) and Anuradha and Pulliah (1999) used B₅ medium for *P. marsupium* and *P. santalinus*. Similarly, Kaneda et al. (1997) found that B₅ medium was more superior over L₂ (Philips and Collins 1979) and MS media in obtaining maximum number of multiple shoots in *Glycine max*. Likewise, Wang et al. (2005) showed that B₅ and WPM media were the optimal basal media for shoot regeneration from axillary bud in *Camptotheca acuminata*, while Douglas and McNamara (2000) obtained adventitious shoot regeneration in *Acacia mangium* using Driver and Kuniyuki (1984) and McGranahan et al. (1987) (DKW) medium.

Bosela and Michler (2008) tested different media types in *Juglans nigra* where both MS and DKW media were suitable for long-term culture maintenance but the hyperhydricity frequencies were unacceptably high (70–100%) for the WPM and ½×DKW nutrient formulations. The major differences in macronutrients among these media are in ammonium and nitrate ion concentrations and total ion concentration. Full-strength MS is high in ammonium (20.6 mM) and nitrate (39.4 mM) ions, while QL is a low-ammonium medium (5 mM). WPM contains low concentrations of both ammonium (5 mM) and nitrate (9.7 mM) ions. In addition, QL uses calcium nitrate as a nitrogen source. A medium originally developed for walnut (Driver and Kuniyuki 1984), designated DKW, also has lower ammonium ion content (17.7 mM) than MS and contains calcium nitrate instead of potassium nitrate.

2.4.5 Carbohydrate Source

Plants growing under tissue culture conditions are semiautotrophic (Hazarika 2003) and leaves

formed during in vitro growth may never attain photosynthetic competence (Van Huylenbroeck and Debergh 1996). Moreover, plantlets growing under in vitro conditions have limited accessibility to CO₂ inside the vessel (Hazarika 2003). Therefore, sugar is supplemented as a carbon source to maintain an adequate supply of carbon source for in vitro multiplication and growth of plant cell, tissue, and organs or whole plantlets.

Addition of sugar to the culture media also helps in the maintenance of osmotic potential of cells and conservation of water (Hazarika 2003). The conservation of water is essentially important for ex vitro settlement of plants, because in vitro-grown plants lack a well-developed cuticle and epicuticular wax (water housekeeping system; Van Huylenbroeck et al. 2000). Moreover, exogenous supply of sugar increases starch and sucrose reserves in micropropagated plants and could favor ex vitro acclimatization and speed up physiological adaptations (Pospisilova et al. 1999a). Nonetheless, addition of sugar to the culture media has been shown to be negatively correlated with growth (Kwa et al. 1995), photosynthesis (Serret et al. 1997; Hazarika 2003), and expression of enzymes of the carbon assimilatory pathway (Kilb et al. 1996).

George and Sherrington (1984) had drawn the conclusion that for optimal growth and multiplication, 2–4% sucrose was found to be optimum whereas Capellades et al. (1991) found that the size and number of starch granules of *Rosa* cultivated in vitro increase with the sucrose level in the culture medium.

Hazarika et al. (2000) have demonstrated that in vitro preconditioning of *Citrus* microshoots with 3% sucrose concentration is advantageous for ex vitro survival and acclimatization. There was a linear increase in biochemical constituents, viz. reducing sugar, starch, and total chlorophyll on addition of sucrose to the medium. Likewise, high-frequency in vitro shoot multiplication of *Plumbago indica* was possible in a medium containing 3% sucrose (Chetia and Handique 2000).

Mehta et al. (2000) reported that an increase in sucrose concentration from 2 to 4% in the medium increases caulogenic response in tamarind plantlets from 34 to 48% in explants. On the contrary,

studying acclimatization of Asiatic hybrid lily under stress conditions after propagation through tissue culture, Mishra and Dutta (2001) reported that liquid medium having 9% sucrose and other phytohormones was found suitable for the growth of bulblets in the isolated unrooted shoots. Due to the high concentration of sucrose, the size of the bulblets increased from 0.5 cm in diameter to approximately 1–1.5 cm within 2 months of inoculation. Further, the increase in sucrose to 6% induced browning of media which was detrimental for the growth of the shoots whereas the induction of multiple shoots using shoot tips of *Gerbera* was accomplished on MS medium supplemented with 3% sucrose and other phytohormones, and almost 100% survival rate was obtained after transfer (Ashwath and Choudhury 2002).

Akbas et al. (2007) tested the effects of different carbon sources (sucrose, maltose, and dextrose at 3% concentration) in *Actinidia deliciosa* and the best results were obtained on MS medium using 3% sucrose.

Recently, Jo et al. (2009) observed the effect of sucrose concentrations (0–9%) on the multiplication and growth of the plantlets of *Alocasia amazonica* under in vitro condition and their subsequent acclimatization under ex vitro condition. An absence of sucrose in the growth medium induced generation of leaves; however, it decreased multiplication.

Sucrose supply of 6 or 9% increased multiplication, corm size, fresh weight, dry weight, and root number; however, it decreased photoautotrophic growth (leaves). A similar reduction in the growth of plantlets was observed by Serret et al. (1997), when they incorporated sugar into the medium. It has previously been shown that decrease in sucrose concentration in the medium enhanced the photosynthetic ability of plantlets (Desjardins et al. 1995). Plantlets growing on the sucrose-supplemented media exhibit reduced photosynthesis, probably, due to the presence of sufficient energy source (sugars) for other metabolic activities (Rolland et al. 2002; Amiard et al. 2005). When in vitro-grown plantlets were transferred to ex vitro condition, the best growth was observed in those plantlets which have been micropropagated with 3% sucrose.

2.4.6 Subculture Passages

Subculturing often becomes imperative when the density of cells, tissues, or organs becomes excessive and when there is a need to increase the volume of a culture or to increase the number of organs (e.g., shoots or somatic embryos) for micropropagation.

Rapid rates of plant propagation depend on the ability to subculture shoots from proliferating shoot or node cultures, from cultures giving direct shoot regeneration, or from callus or suspensions capable of reliable shoot or embryo regeneration. The reason for transfer or subculture is that the growth of plant material in a closed vessel eventually leads to the accumulation of toxic metabolites and the exhaustion of the medium, or to its drying out. Thus, even to maintain the culture, all or part of it must be transferred onto fresh medium.

Shoot cultures are subcultured by segmenting individual shoots or shoot clusters. Bajaj et al. (1988) obtained around 2,200 plantlets of *Thymus vulgaris* from a single shoot grown in vitro in 5 months (four passages). Ajithkumar and Seeni (1998) reported that repeated subculturing of nodes and leaf from shoot cultures of *Aegle marmelos* helped to achieve a continuous production of callus-free healthy shoots at least upto five subculture cycles. Borthakur et al. (1999) established a mass multiplication protocol for *Alpinia galanga* by subculturing the regenerated explants to Kn-supplemented medium for more than 1 year. They obtained an average of 1,000 plantlets with four to five successive subculture cycles, i.e., within 40–45 days. Likewise, Raghu et al. (2007) observed that the micropropagated shoots of *Aegle marmelos* could be subcultured up to 20 cycles without loss of vigor to produce shoots free from morphological and growth abnormalities. Similarly, in *Simmondsia chinensis*, around 10–15 shoots were produced by repeated subculturing up to three successive subcultures (Singh et al. 2008).

The increase in shoot number may be due to the suppression of apical dominance during subculture that induced basal dormant meristematic cells to form new shoots (Shukla et al. 2009).

Hence, by adopting this procedure of shoot excision and reculturing of the mother explants in the fresh medium, a large number of shoots could be obtained per explants within few months (Asthana et al. 2011). This approach of increasing the yield of shoots at an enhanced pace was adopted earlier for other woody taxa (Kaveriappa et al. 1997; Jain and Babbar 2000; Hiregoudar et al. 2005; Prakash et al. 2006; Anis et al. 2010; Tripathi and Kumar 2010; Shekhawat and Shekhawat 2011).

2.5 Rooting of In Vitro-Regenerated Shoots

Rooting of in vitro-regenerated shoots and transplantation of the plantlets to the field is the most important, crucial, and essential step, but a difficult task in tissue culture of woody trees (Murashige 1974). Generally, rooting in micropropagated shoots can be achieved by two different methods, i.e., in vitro and ex vitro methods. Root induction and elongation are complex processes that are influenced by a large number of factors, such as genotype, type, and concentration of PGRs, and culture conditions (Bennett et al. 1994; Mylona and Dolan 2002).

The intricacies involved in adventitious rooting were reviewed by Haissig (1974), George and Sherrington (1984), Gaspar et al. (1994), and Rout et al. (2000). The in vitro-regenerated shoots of various medicinal plants rooted readily on growth regulator-free MS basal medium (Cristina et al. 1990; Binh et al. 1990; Faisal and Anis 2003; Shan et al. 2005; Mallikarjuna and Rajendrudu 2007; Jahan and Anis 2009). The ease with which microshoots root in vitro in the absence of exogenously supplied hormones was supported by the fact that there may occur high endogenous auxin in these in vitro-regenerated shoots. A full-strength MS medium was found to be satisfactory for root induction in a number of plant species but as the concentration of salts were reduced to half or to much lower levels (1/3 or 1/4), a striking increase in the rooting efficiency was observed. In woody trees, Rai et al. (2010) and Tripathi and Kumar (2010) have sup-

ported the fact that relatively low salt concentration in the medium is known to enhance rooting efficiency of microshoots. Recently, Phulwaria et al. (2012) have reported that half-strength MS medium supplemented with auxin was the most effective for rooting of shoots in *Terminalia belirica*.

Exogenous auxins are often used in a number of plant species to promote in vitro rooting of in vitro-produced microshoots and their efficacy depends on several factors such as the affinity for the auxin receptor protein involved in rooting, the concentration of free auxin that reaches target competent cells, the amount of endogenous auxins, and the metabolic stability (De Klerk et al. 1999; Gaba 2005).

Auxins most frequently incorporated in the medium to induce rooting are IAA, IBA, and NAA. In particular, higher auxin concentrations are generally required during the induction phase, whereas during the formation phase growth regulators become inhibitory. This effect is particularly evident when microcuttings are cultured continuously on media with auxins, as demonstrated by De Klerk et al. (1997) in apple microcuttings previously exposed for 3 weeks to IAA and which rooted ex vitro more efficiently than those exposed to IBA or NAA. The different response was attributed to the higher stability and consequent supraoptimal concentrations of IBA and NAA for further rooting formation as compared to IAA, which, being unstable and easily degradable, would, instead, favor root growth and elongation. Nair and Seenii (2001) reported that MS medium supplemented with IAA, IBA, and NAA, for which IAA under initial dark conditions, gave the maximum rooting within a period of 5 weeks in *Celastrus paniculatus*.

Generally, IBA has been observed to induce strong rooting response over IAA or NAA and has been extensively used to promote rooting in a wide range of tree species such as *Myrtus communis* (Ruffoni et al. 1994; Scarpa et al. 2000), *Pistacia vera* (Onay 2000), *Terminalia arjuna* (Pandey and Jaiswal 2002; Pandey et al. 2006), *Terminalia chebula* (Shyamkumar 2003), *Kigelia pinnata* (Thomas and Puthur 2004), *Stereospermum personatum* (Shukla et al. 2009), *Semecar-*

pus anacardium (Panda and Hazra 2010), and *Sapindus trifoliatus* (Asthana et al. 2011). Moreover, this group of plant hormone is readily and easily available commercially worldwide (Epstein and Ludwig-Miller 1993). Ludwig-Muller (2000) has documented that the stimulatory effects of IBA on the root development may be due to several factors such as its preferential uptake, transport, and stability over other auxins and subsequent gene activation.

Many researchers emphasized on ex vitro rooting because plants developed after ex vitro rooting have a better root system than the plants raised after in vitro rooting (Borkowska 2001). In addition, the ex vitro technique is comparatively less time consuming and cost-effective and requires less labor, chemicals, and equipment than in vitro rooting because plantlets rooted ex vitro do not need any additional acclimatization prior to transplanting in the field conditions (Yan et al. 2010). The main advantages of ex vitro rooting are that the chance of root damage is less, rooting rates are good, and root quality is better (Bellamine et al. 1998).

Ex vitro rooting of in vitro-multiplied shoots has been reported for several species, including *G. jasminoides* (Economou and Spanoudaki 1985), apple (Zimmerman and Fordham 1985; Stimart and Harbage 1993), *Actinidia deliciosa* (Pedroso et al. 1992), *Cornus nuttallii* (Edson et al. 1994), blueberry (Isutsa et al. 1994), hazelnut (Nas and Read 2004), *Nyctanthes arbor-tristis* (Siddique et al. 2006), *V. negundo* (Ahmad and Anis 2007), *Malus zumi* (Xu et al. 2008), *Metrosideros excelsa* (Iapichino and Airo 2008), and *Terminalia bellirica* (Phulwaria et al. 2012). The system is now preferred in woody plant micropropagation to rapidly produce high-quality plantlets and to avoid the potential of off-types (Suttle 2000).

2.6 Acclimatization and Hardening of Plantlets

The ultimate success of in vitro propagation in a reforestation program depends on a reliable acclimatization protocol, ensuring low cost and

high survival rates. In vitro protocols provide minimal stress and optimum conditions for shoot/plant multiplication (Hazarika 2006). As a consequence of these special conditions (e.g., high air humidity, low irradiance, low CO₂ during photoperiod, high levels of sugars as carbon source and growth regulators), in vitro-grown plantlets usually exhibit abnormal morphology, anatomy, and/or physiology (Pospisilova et al. 1999b; Premkumar et al. 2001; Hazarika 2006). Under these conditions, in vitro plantlets can develop specific features (e.g., nonfunctional roots and/or stomata) that are inconsistent with the development under greenhouse or field conditions. Also, the mixo-heterotrophic mode of nutrition and poor mechanism to control water loss render micropropagated plants vulnerable to the transplantation shocks when directly placed in a greenhouse or field.

Understanding the physiological characteristics of micropropagated plants and the changes they undergo during the hardening process should facilitate the development of efficient transplantation protocols and will help to make decisions on, if necessary, adjusting environmental conditions (Hazarika 2006). For example, water/osmotic stress is often the cause of micropropagated plant's mortality and its monitorization is particularly important when acclimatization occurs in a degraded land as is the case reported by Brito et al. (2003).

The improvement of the photosynthetic competence during acclimation is a common characteristic in various plant species grown in vitro (Yue et al. 1993; Van Huylenbroeck et al. 1998a; Pospisilova et al. 1999b). Grout (1988) suggested that based on the behavior of in vitro-formed leaves, plants can be classified into two groups. In the first group, the in vitro leaves are photosynthetically competent and function as normally formed leaves; in the second group, these leaves act as storage organs and never become fully autotrophic. Moreover, the correlation between their performances and in vitro culture condition was also reported (Van Huylenbroeck et al. 1996, 1998a).

The presence of sugars in the medium may promote mixotrophy, leading to a downregulation of photosynthesis due to feedback inhibi-

tion of the Calvin cycle (Amancio et al. 1999; Premkumar et al. 2001; Van Huylenbroeck et al. 2000). Among photosynthetic enzymes, Rubisco deserves much attention since it performs a dual role as a catalyst in the carboxylation of CO₂ and as a major storage protein being 40–80% of the total soluble leaf proteins (Premkumar et al. 2001). Both roles could be important in overcoming the critical acclimatization phase, when the mixo-heterotrophic behavior of the in vitro plants is shifted to an autotrophic functioning.

In vitro plantlets grow generally under low level of light, with plenty of sugar and nutrients to favor heterotrophic growth and in an atmosphere with high percentage of relative humidity (Hazarika 2003). Due to these factors, in vitro plants have low rates of photosynthesis and an incipient photosynthetic apparatus. After transfer to ex vitro conditions, most micropropagated plants develop a functional photosynthetic apparatus, although the increase in light intensity is not linearly translated to an increase in photosynthesis (Amancio et al. 1999). The results on the enhancement in the contents of the photosynthetic pigments in micropropagated plantlets during the acclimatization have been reported by Kadlecěk et al. (1998) and Pospisilova et al. (1999b). The significant increase in chlorophyll (a and b) contents with exposure to high light levels suggested that chlorophyll synthesis enzymes vital for chlorophyll biosynthesis were induced. Since, both chlorophyll a and b are associated with light-harvesting complexes of photosystem II (PS II), an increase in the levels of pigments during the exposure to high light intensity did not impair the core complexes of PS II (Von Willert et al. 1995). Also, carotenoids play a key function in protecting chlorophyll pigments under stress conditions (Kenneth et al. 2000).

There are reports available where an initial abrupt decrease in chlorophyll contents during the starting days followed by a continuous and subsequent increase was noticed as in *Ocimum basilicum* (Siddique and Anis 2008) towards the final days of acclimatization. A similar pattern of photosynthetic efficiency in micropropagated plants of neem was detected by Lavanya et al. (2009). Faisal and Anis (2010) compared the

chlorophyll contents of ex vitro-formed leaves of *Tylophora indica* with that of in vitro ones during the acclimation period and found significant higher levels of pigments in the fully hardened plantlets at the 28th day of acclimation.

Amancio et al. (1999) indicated that high light regime during acclimatization has a direct influence on the transition to in vitro characteristics and on the final yield, without symptoms of light stress. Also, it has been documented in the majority of the reports that the acclimatized plants had normal leaf development and lacked detectable morphological variation and showed apparently uniform growth and true-to-type morphology.

2.7 Advancement in Plant Tissue Culture: Synthetic Seed Technology

Synthetic seed technology in the last decade has emerged as one of the major branches of plant biotechnology and has opened up unprecedented opportunities in many areas of basic and applied biological researches. Tissue cultures of several plant species produce somatic embryos which proceed through similar developmental stages like that of seed zygotic embryos to form a plant. Synthetic seed refers to the encapsulation of somatic embryos or encapsulated buds, bulbs, or any form of meristem which can develop into plantlets.

Encapsulation is usually done in a suitable gel (sodium alginate) matrix to produce a “synthetic seed coat” and the resulting encapsulated propagules can be treated like natural seeds. The facility to incorporate nutrients, biofertilizers, antibiotics, or other essential additives to the matrix and the easy handling, storing, shipping, and planting are the major attractions to imply the synthetic seed as a unit of delivery of tissue-cultured plants.

The uniform and simultaneous production of somatic embryos in culture followed by uniform germination of encapsulated embryos could possibly remove many disadvantages associated with natural seeds. Many trees produce seeds in certain periods of the year whereas synthetic seeds would be available throughout the year.

The first synseeds of desiccated carrot and celery somatic embryos (Kitto and Janick 1982) were obtained on encapsulation in “Polyox,” polyoxyethylene, which is soluble in water, non-toxic to embryos, inert to microbial growth, and dries to form a thin film.

Synseeds of alfalfa were obtained on encapsulation of somatic embryos in 2% solution of calcium alginate (Redenbaugh et al. 1991). Since then, this has been the most practiced method of synseed production. An embryo and sodium alginate mixture was dropped in 100 mM solution of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. In an ion exchange reaction, Na^+ ions were replaced by Ca^{2+} ions forming calcium alginate gel. For the production of synseed of carrot, 1% Na-alginate, 50 mM Ca^{2+} , and 20–30 min of complexing were satisfactory (Molle et al. 1993).

The technique of synseed has been widely studied and works with various plant species including fruits, cereals, medicinal plants, vegetables, ornamentals, forest trees, and orchids (Ballester et al. 1997; Gonzalez-Benito et al. 1997; Standardi and Piccioni 1998; Pattnaik and Chand 2000; Ara et al. 2000; Nyende et al. 2003; Chand and Singh 2004; Tsvetkov and Hausman 2005; Malabadi and Staden 2005; Naik and Chand 2006; Faisal et al. 2006; Faisal and Anis 2007; Antonietta et al. 2007; Micheli et al. 2007; Pintos et al. 2008; Rai et al. 2008; Singh et al. 2009; Sundararaj et al. 2010; Singh et al. 2010; Germana et al. 2011; Mishra et al. 2011). In addition, during cold storage, encapsulated nodal segments require no transfer to fresh medium, thus reducing the cost of maintaining germplasm cultures (West et al. 2006).

In this context, the most important application of synthetic seeds for these plants could be in exchange of elite and axenic plant material between laboratories due to small bead size and relative ease of handling these structures (Rai et al. 2008).

Conservation is an important aspect of encapsulation technology. For short- and medium-term storage, the aim is to increase the interval between subcultures by reducing growth. This is achieved by modifying the environmental conditions and/or the culture medium. Various approaches have been applied for slow-growth maintenance of cultures such as maintenance under reduced tem-

perature and/or reduced light intensity, use of growth retardants such as ABA, use of minimal growth medium (restrict the growth of cultures by alteration of mineral content and/or sucrose in medium), use of osmoticum, reduction in oxygen concentration, and combination of more than one treatment (Gupta and Mandal 2003).

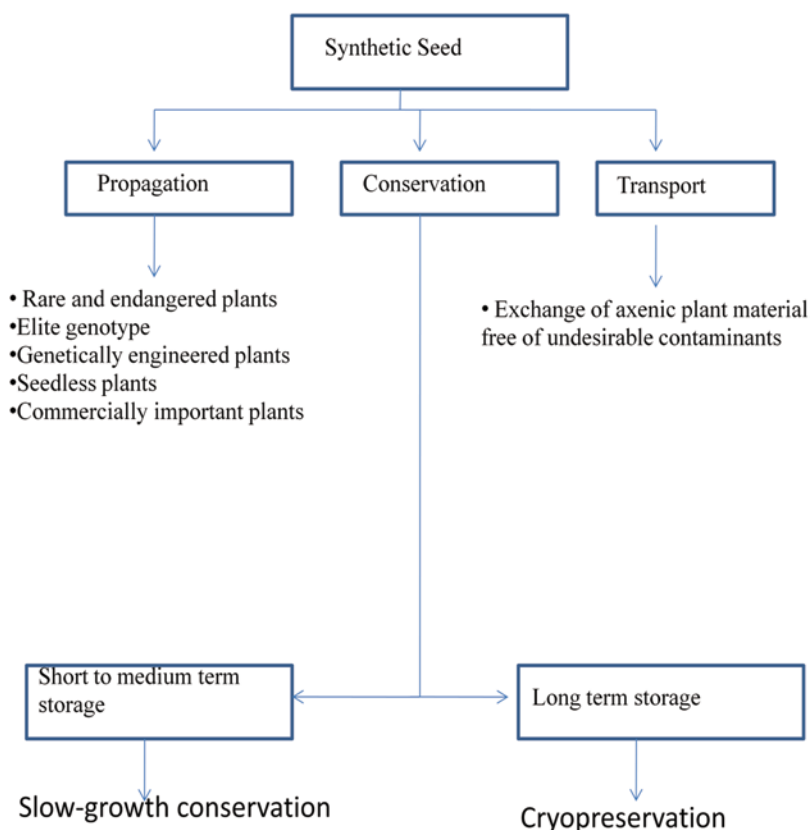
Long-term storage of synthetic seeds can be achieved through storage at ultralow temperature, termed as cryopreservation, and is usually carried out by using liquid nitrogen (-196°C). At this temperature, all cellular division and metabolic processes are suspended, and hence plant material can be stored for an unlimited period (Engelmann 1997). In recent years, several new cryopreservation techniques like encapsulation-dehydration and encapsulation-vitrification methods based on technology have been developed for the production of synseeds (Fig. 2.1).

2.8 Clonal Fidelity of Micropropagated Plants

Clonal fidelity is a major consideration in commercial micropropagation using in vitro tissue culture methods. In vitro-regenerated plants are usually susceptible to genetic changes due to culture stress and might exhibit somaclonal variation (Larkin and Scowcroft 1981). Somaclonal variation was first reported for woody plants in *Citrus grandis* (Chaturvedi and Mitra 1975). Therefore, it should be a common practice to assure the true-ness of tissue culture plants after regeneration.

However, somaclonal variation is of immense importance for the isolation of improved clones of forest trees (Ahuja 1993). In addition to somaclonal variation, mislabeling and mixing of clones in germplasm collections have also been reported (Keil and Griffin, 1994). Materials from germplasm banks are frequently used in breeding and tree improvement programs. The economic implications of such inadvertent variations and mixings of accessions could be serious as considerable time and money are spent before the mistakes are detected. This necessitates the development of suitable strategies for assessing genetic uniformity and for identifying the variations.

Fig. 2.1 Scope of synthetic seeds. (Source: Rai et al. 2009)



The need to test the genetic fidelity of tissue culture plants in tree species is important because they are harvested on long rotations and the *in vitro* cultures are maintained through many subcultures. Molecular techniques are at present powerful and valuable tools in the analysis of genetic fidelity of *in vitro*-propagated plants. Evaluation of genetic diversity using molecular techniques could provide useful baseline information for breeding programs.

Molecular markers are not influenced by environmental factors and can be estimated using DNA from any growth stage and, therefore, are very useful in assessing genetic diversity of plant species. Among the various molecular markers, polymerase chain reaction (PCR)-based markers, such as random amplified polymorphic DNA (RAPD; Williams et al. 1990), inter-simple sequence repeat (ISSR; Zietkiewicz et al. 1994), sequence-related amplified fragment length polymorphism (Li and Qulros

2001), and amplified fragment length polymorphism (AFLP; Vos et al. 1995), have become popular as their application does not require any prior sequence information.

2.8.1 PCR-Based DNA Markers

The development of PCR for amplifying DNA sequences led to the revolution in the applicability of molecular methods, and a range of new technologies were developed which could overcome the technical limitations of hybridization-based methods. In a PCR, arbitrary or known sequence primers are used to amplify one or discrete DNA segments that can be resolved in agarose or polyacrylamide gels. Each product is derived from a region of the genome containing two DNA sites with sequences complementary to the primer(s) on the opposite strand and sufficiently close for the amplification to work.

2.8.1.1 RAPD-PCR Markers

Welsh and McClelland (1991) developed a new PCR-based genetic assay, namely RAPD. This procedure detects nucleotide sequence polymorphisms in DNA by using primer of arbitrary nucleotide sequence. In this reaction, a single species of primer anneals to the genomic DNA at two different sites on the complementary strands of the DNA template. If these primary sites are within an amplifiable range of each other, a discrete DNA product is formed through thermocyclic amplification. On an average, each primer directs amplification of several discrete loci in the genome, making the assay useful for efficient screening of nucleotide sequence polymorphism among individuals.

Polymorphisms in RAPD result from different types of changes in the genomic DNA: base-pair substitution, insertions, and deletions, which modify or eliminate the primer-annealing sites; insertions in the genomic sequence that change the intervening length of DNA between the primer sites; and insertions which separate the primer sites to a distance that will not permit amplifications (Williams et al. 1990). Each of these results in the presence or absence of a particular RAPD fragment. This procedure usually amplifies 1–15 DNA fragments from a single primer reaction (Reiter et al. 1992). The primers are usually 10 bp in length with GC content of at least 50% and have a low annealing temperature (36–40°C). However, due to the stochastic nature of DNA amplification with random sequence primers, it is important to optimize and maintain consistent reaction conditions for reproducible DNA amplification. RAPD is the method generally employed for the detection of genetic diversity because it has the advantage of being technically simple, quick to perform, and requires only small amounts of DNA (Ceasar et al. 2010).

Many investigators have reported genetic stability in *Picea mariana* (Isabel et al. 1993), *Pinus thunbergii* (Goto et al. 1998), *Lilium* (Varshney et al. 2001), chestnut rootstock hybrid (Carvalho et al. 2004), *Prunus dulcis* (Martins et al. 2004), banana (Venkatachalam et al. 2007), *V. negundo* (Ahmad and Anis 2011), *Sapindus trifoliatus* (Asthana et al. 2011), *P. santalinus* (Balaraju

et al. 2011), *Simmondsia chinensis* (Kumar et al. 2011), etc. using RAPD.

2.8.1.2 ISSR-PCR Markers

In this technique, primers based on microsatellites are utilized to amplify ISSR DNA sequences. Here, various microsatellites anchored at the 3' or 5' end are used for amplifying genomic DNA which increases their specificity. This technique is more reproducible and generates three to five times the variation of RAPD (bands/marker; Nagaoka and Ogihara 1997). ISSR markers have been shown to be more reliable and conform closely to dominant Mendelian inheritance which makes them useful for genotype analysis and genome mapping (Tsumara et al. 1996; Nagaoka and Ogihara 1997). An unlimited number of primers can be synthesized for various combinations of di-, tri-, tetra-, penta-nucleotides $[(4)^3=64, (4)^4=256]$, etc. with an anchor made up of a few bases and can be exploited for a broad range of applications in plant species.

ISSR markers have been successfully applied in the analysis of genetic fidelity within lines of cauliflower (Leroy et al. 2000), almond (Martins et al. 2004), banana (Venkatachalam et al. 2007), *Swertia chirayita* (Joshi and Dhawan 2007), *Dicotspermum ovalifolium* (Chandrika et al. 2008), *Platanus acerifolia* (Huang et al. 2009), and *Nothapodytes foetida* (Chandrika and Rai 2010). Moreover, ISSR markers offer other advantages in the detection of somaclonal variation, notably a high degree of sensitivity, reproducibility, and the dominant representation of polymorphic genetic alleles.

2.9 Antioxidant Enzymes

It is known that reactive oxygen species (ROS) production increases during abiotic and biotic stresses and that ROS and some resultant metabolites are important signaling molecules (Moller et al. 2007). According to these authors, the production of ROS, such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (HO^\cdot), and singlet oxygen (1O_2), is a consequence of aerobic metabolism, both as products of mainstream

enzymatic reactions such as photorespiration or as an unavoidable accident (e.g., the O_2^- produced by the mitochondrial electron transport chain).

The chloroplasts, peroxisomes, and mitochondria are the main centers for ROS production in green plants, producing several types of ROS that have different properties and specific cellular roles (Moller et al. 2007). 1O_2 is produced in PS II and O_2^- at both photosystem I (PS I) and mitochondria, while the peroxisomes produce O_2^- and H_2O_2 in several key metabolic reactions (Moller et al. 2007). H_2O_2 is relatively stable and can be removed by normal cellular antioxidant systems (Yannarelli et al. 2006).

The other ROS are very unstable and are present at much lower concentrations. Several authors have reported oxidative damage caused by ROS and resultant products (Clijsters et al. 1999; Mittler 2002; Moller et al. 2007; Smeets et al. 2005; Ammar et al. 2008), including: (1) membrane damage caused by changes in the lipid composition of cellular membranes and accumulation of lipid peroxidation products, e.g., aldehydes such as malondialdehyde (MDA) and complex mixtures of lipid hydroperoxides; (2) oxidative damage of proteins leading to denaturation of functional and structural proteins, with inhibition of some enzymatic systems; (3) oxidative damage of nucleic acids; (4) oxidation of other components of the antioxidative system such as glutathione (GSH) and ascorbate; and (5) oxidation of free carbohydrates like sugars and polyols by reaction with HO^- .

According to Mittler (2002), plant cells require both the control of low levels of ROS for signaling purposes and the stress-induced detoxification of excess ROS. To help in the detoxification of excess ROS, plants have an efficient antioxidant defense system composed of enzymatic and nonenzymatic mechanisms (Gratao et al. 2005; Yannarelli et al. 2006) located in distinct cell organelles (peroxisomes, chloroplasts, and mitochondria). These enzymatic mechanisms include enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR).

SOD catalyzes the dismutation of superoxide into H_2O_2 and O_2 . SOD is found in almost all cel-

lular compartments and is one of the main ROS-scavenging pathways of plants, participating in the water–water and the ascorbate–glutathione cycles in chloroplasts, cytosol, mitochondria, peroxisomes, and apoplast (Mittler 2002).

In plants, CAT is one of the main H_2O_2 -scavenging enzymes that converts H_2O_2 into H_2O and O_2 in peroxisomes and is involved in the decomposition of H_2O_2 formed during photorespiration, without the need of an additional substrate (Pereira et al. 2002). The elimination of H_2O_2 in other cell compartments depends on distinct peroxidases, such as APX and glutathione peroxidase (GPX), which catalyze the breakdown of H_2O_2 using different reducing substrates (Pereira et al. 2002). APX isozymes are localized in chloroplasts, cytosol, mitochondria, peroxisomes, and apoplast (Jimenez et al. 1997; Mittler 2002). GPX is located in the cytosol (Mittler 2002) and also metabolizes H_2O_2 , although at rates that are small compared with the large rates of H_2O_2 generation in plants (Noctor et al. 2002). Other peroxidases such as guaiacol peroxidases are enzymes that metabolize H_2O_2 to water, using the oxidation of a wide variety of substrates, mainly phenols. These enzymes are also involved in numerous physiological roles in plant tissues, including lignin biosynthesis and pathogen defense, among others (Yannarelli et al. 2006).

The ascorbate–glutathione cycle is also an important defense mechanism against oxidative stress caused by metals (Cuyppers et al. 2002; Mittler 2002; Smeets et al. 2005). The different affinities of APX (μM range) and CAT (mM range) for H_2O_2 suggest that they belong to two different classes of H_2O_2 -scavenging enzymes with CAT participating in the removal of excess ROS during stress (Mittler 2002).

The ubiquitous tripeptide GSH, which occurs mostly as a low molecular weight thiol compound in almost all cells, acts as a disulphide reductant to protect the thiol groups of enzymes, regenerate ascorbate, and react with 1O_2 and OH^- . GSH detoxifies herbicides by conjugation, either spontaneously or by the activity of a glutathione-S-transferase (GST), and regulates gene expression in response to environmental stress and pathogen attack. GSH also participates in

the regeneration of ascorbate from docosa-hexaenoic acid (DHA) via the enzyme dehydroascorbate reductase (DHAR; Noctor and Foyer 1998). GR catalyses the NADPH-dependent formation of a disulphide bond in glutathione disulphide (GSSG) and is thus important for maintaining the reduced pool of GSH. The role of GSH and GR in H_2O_2 scavenging has been well established in the Halliwell–Asada pathway (Noctor and Foyer 1998; Asada 2000). GR catalyzes the rate-limiting last step of the Halliwell–Asada pathway. An increase in GR activity in plants results in the accumulation of GSH and ultimately confers stress tolerance in plants. Expression of GR is unregulated under stresses such as high light, mechanical wounding, high temperature, chilling, and exposure to heavy metals and herbicides (Apel and Hirt 2004; Karuppanapandian et al. 2011).

Van Huylbroeck et al. (2000) reported that micropropagated plants develop antioxidant mechanism during acclimation. Under high irradiance, significant changes in the activity of the antioxidant enzymatic system were observed in micropropagated plants of *Calathea* (Van Huylbroeck 2000), *Phalaenopsis* (Ali et al. 2005), *Zingiber officinale* (Guan et al. 2008), *Rauwolfia tetraphylla* (Faisal and Anis 2009), *Tylophora indica* (Faisal and Anis 2010), *Ocimum basilicum* (Siddique and Anis 2009b), *Tecomella undulata* (Varshney and Anis 2011), and *Ulmus minor* (Dias et al. 2011).

In addition, Batkova et al. (2008) recognized that ex vitro transfer is often stressful for in vitro-grown plantlets. Water stress and photoinhibition, often accompanying the acclimatization of in vitro-grown plantlets to ex vitro conditions, are probably the main factors promoting the production of ROS and, in consequence, oxidative stress. The extent of the damaging effects of ROS depends on the effectiveness of the antioxidative systems which include low molecular mass antioxidants (ascorbate, glutathione, tocopherols, carotenoids, phenols) and antioxidative enzymes (SOD, APX, CAT, GR, monodehydroascorbate reductase, DHAR). Authors have focused on ROS production and development of antioxidative system during in vitro growth and their further changes during ex vitro transfer.

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