Review of Literature

Chapter 2
REVIEW OF LITERATURE

Biodiversity loss, a threat to food security of the world is a major global concern, dire need for a revolution can only be achieved through a multidisciplinary approach. Increasing pollution, global warming and decreasing fertile lands put forth an imbroglio making the application of plant biotechnology inevitable, for conservation and sustainable utilization of the important plant species.

Plant tissue culture, is an important biotechnology tool useful to basic as well as applied plant studies, while also having commercial applications. It has become a popular method for vegetative propagation of plants and is possible because of the continuous efforts of many scientists.

2.1 Historical background of Plant Biotechnology

It all started with the invention of microscope in the 1500s, which gave us the ability to appreciate the smallness in the world. Microscopic studies and discovery of microorganisms by Robert Hooke and Antoni van Leeuwenhoek during 1665-83 (Gest 2004), started a revolution which lead to the proposal of cell theory by Schleiden (1838) and Schwann (1839), establishing the cell as the structural and functional unit of life. A century later the concept was experimentally tested by Haberlandt (1902) exploring the possibility of culturing plant cells and tissues under in vitro conditions. He used cells from different origin i.e. mesophyll cells (Lamium purpureum and Eichhornia crassipes), epidermal cells (Ornithogalum) and hair cells (Pulmonaria) and cultured them using Knop’s nutrient solution, sucrose, asparagine and peptone. Though, he couldn’t induce cell division in the mature plant cells in vitro, but from his observation he construed that if appropriate conditions are maintained one could successfully cultivate artificial embryos from vegetative cells, enabling investigations from a new experimental approach to solve important problems. His pioneering efforts deservedly earned him the title of the “Father of Plant Tissue Culture”

Cell division was successfully induced in plant cell cultures by White (1934) in isolated tomato roots on a medium containing sucrose, mineral salts and yeast extract and by Gautheret (1934) in cambial tissue of Acer pseudoplatanus, Ulmus campestre, Robinia pseudo-acacia and Salix capraea on agar-solidified medium of Knop’s
solution. The studies of White (1934) and Gautheret (1934) provided a platform for further studies. The finding of the importance of vitamin B and auxin (IAA) in cell division instigated significant advances in tissue culture investigations (Went 1926), and the concept was utilised by Gautheret (1939), White (1939) and Nobecourt (1939) concurrently for indefinite-cultures.

During the next three decades, new techniques were developed while those already present were significantly improved, use of coconut water (van Overbeek et al. 1941) and discovery of kinetin (Miller et al. 1955) drastically enhanced the number of species that could be cultured, thus increasing the applicability of tissue culture. However, perhaps the most important breakthrough was the discovery, establishing ratio of auxin and cytokinin as the factor determining the morphogenetic fate of the cells (Skoog and Miller 1957). Another big achievement was the formation of bipolar somatic embryos by Reinert (1959) and Steward et al. (1958). Unlike those early culture media formulations which were based on the nutrient requirement of whole plant, Murashige and Skoog (1962) formulated a culture media based on the ash of tobacco callus which suited to most of the plant species and till date it remains the most widely used nutrient media in plant tissue culture (Thorpe 2007). Another major event was when Guha and Maheshwari (1964) produced the haploid plants via anther culture in Datura innoxia, opening new vistas for plant improvement.

Plant tissue culture have been widely used for morphogenetic studies in forest trees as reviewed by Bonga (1982), Anis et al. (2012) and Kataria et al. (2013). While, the legumes have also received a fair attention, important studies on legume trees include those done on Acacia spp. (Girijashanker 2014; Javed et al. 2013), Albizia spp. (Rajeshwari and Paliwal 2008; Ghosh et al. 2010; Perveen et al. 2012), Bauhinia spp. (Naz et al. 2011, 2012), Pterocarpus spp. (Chand and Singh 2004; Vipranarayana et al. 2012), Cassia spp. (Anis et al. 2012), Pongamia spp. (Kesari et al. 2012).

However, physiological and biochemical studies on organogenesis have also been carried out to evaluate the response of plant tissues to various factors that influence plant growth and metabolism (Thorpe 1990; Rout et al. 1999; Ibrahim and Yousir 2009).
Micropropagation of forest trees *in vitro* open new vistas for genetic improvement and other molecular biology studies along with clonal propagation of elite genotype, conservation and development of integrated sustainable forest management system.

The advances made in the plant biotechnology is undoubtedly augmenting and strengthening progress in fundamental science, while also developing new and innovative state of the art techniques for better applicability of the scientific knowledge. This, remains our only hope for achieving sustainable growth.

2.2 Micropropagation

One of the most incredible discoveries for organogenesis was the role of auxin and cytokinin in determining the fate of plant cells (Skoog and Miller 1957). This pioneering work laid the foundation of the science of organogenesis and culminated into numerous tissue culture systems.

Micropropagation is a method for rapid vegetative propagation of plants using very small starting material (explants) under *in vitro* condition. It is one of the most widely used application of plant tissue culture. It can serve as a source for important plant species, independent of seasonal variations, with enhanced storage ability and convenient transportability using less energy and space as compared to the conventional ways. It can be accomplished either through the multiplication of shoots from axillary buds or by the formation of adventitious shoots/somatic embryos via direct or indirect organogenesis.

In general, micropropagation can be broadly divided into four stages as described by Murashige (1974). Another stage-zero was also described later by other authors (Deberg and Maene 1981).

STAGES OF MICROPROPAGATION
Stage 0: Donor plant selection and preparation
Stage I: Establishing an aseptic culture
Stage II: Proliferation and multiplication
Stage III: Rooting
Stage IV: Acclimatization and transfer to the natural environment
2.2.1 In vitro propagation using shoot meristem

Apical or the axillary meristems are already programmed to develop into shoots but are dormant till they receive proper cues. Their ability to form shoots can be augmented greatly by the application of hormonal conditions that promote shoot formation. Since re-differentiation/trans-differentiation is not required in this case, genetic stability is not affected. However, in some cases the number of shoots produced might be less as compared to those produced adventitiously. Therefore, this strategy is most effective when clonal propagation is intended. The efficiency of explants used for in vitro propagation depends mainly on the aim of the study and also to a certain extent on the species. Apical meristems (shoot tip) culture has been exploited for rapid clonal multiplication, virus elimination and germplasm preservation of both vegetative and seed propagated plants. While, nodal explants with pre-formed bud are preferred for vegetative propagation as it is easy to handle, more responsive and can tolerate the sterilization procedure better than shoot tip (Cassells et al. 1980).

Morel (1960) pioneered the axillary bud culture in orchid culture. Thereafter, numerous species of plant have been propagated using this explant and some of the more recent works by Rout et al. (2008) in Acacia chundra, Vipranarayana (2012) in Pterocarpus santalinus, and Naaz et al. (2014) in Syzygium cumini. It is increasingly being recommended by workers as they are least likely to induce somaclonal variation (Ahmad and Anis 2011; Anis et al. 2014; Javed and Anis 2014).

2.2.2 In vitro Propagation by adventitious shoot organogenesis

Adventitious shoots refer to the de novo generated shoots, it is a representation of the totipotency of the plant cells as the differentiated somatic cells changes their path to develop shoots. Molecular events preceding de novo shoot organogenesis have been reviewed critically by Duclercq et al. (2011) that have been revealed as a consequence of advancements made in molecular biology tools and techniques, but still the exact molecular mechanisms determining 'when' and 'where' remain elusive. However, as established, the process requires manipulations at the genetic level, thus, increasing the chances of mutations and genetic aberrations, more so in callus mediated indirect organogenesis than direct organogenesis. These variations are not always
unfavourable, and have been utilized as a tool for crop improvement (Larkin and Scowcroft 1981).

Therefore, where maintenance of genetic uniformity is not important or where we intend to develop variants for genetic improvement, *de novo* shoot organogenesis is an effective method for propagation. It has been extensively used, including many tree species such as *Dalbergia latifolia* (Lakshmisita et al. 1986), *Aegle marmelos* (Islam et al. 1993), *Liquidambar styraciflua* (Kim et al. 1997), *Prunus avium* (Hammatt and Grant 1998), *Dalbergia sissoo* (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), *Albizia lebbeck* (Perveen et al. 2011) and *Cassia angustifolia* (Siddique et al. 2014).

2.3 Factors affecting *in vitro* shoot regeneration and growth of plants

*In vitro* growth and differentiation is influenced by numerous factors, more pronounced being the genotype, type of explants, nutrient media, plant growth regulators and *in vitro* conditions before and after the regeneration process.

2.3.1 Explant type

The choice of the explant is largely governed by the objective of the study and has a major impact on the success of the protocol. The nature and condition of explant have a significant influence on multiplication rate (Mao et al. 1995). Therefore, it is a critical step and requires meticulous execution to develop an effective regeneration system.

Explants having meristems are preferred for axillary shoot multiplication (Elliott 1970; Davies 1980; Rout et al. 1990; Arya et al. 1995; Rout et al. 2000; Faisal and Anis 2003; Sivakumar and Krishnamurthy 2004; Avani et al. 2006; Prakash and Staden 2007). Nodal explants having axillary meristem have been extensively used for clonal propagation using axillary bud proliferation and have been reported to be effective in *Cephaelis pecacuanha* (Jha and Jha 1989), *Ocimum basilicum* (Sahoo et al. 1997), *Vitex negundo* (Sahoo and Chand 1998; Ahmad and Anis 2007), *Psoralea corylifolia* (Jeyakumar and Jayabal 2002), *Holostemma ada-kodien* (Martin 2002), *Leptadenia reticulata* (Arya et al. 2003), *Mucuna pruriens* (Faisal et al. 2006), *Ocimum basilicum* (Siddique and Anis 2009; Shahzad et al. 2012), *Erythrina variegata* (Javed and Anis 2014).

However, shoot tip have also been shown to be potent explants for the process, as reported by various workers (Karthi and Gamborg 1978; Mante et al. 1980; Kothari et al. 1991; Rahman et al. 1993; Johanson et al. 1997; Sudha et al. 1998; Soniya and Das 2002; Skala and Wysokinska 2004; Krogstrup et al. 2005; Husain and Anis 2006; Siddique and Anis 2007; Ahmed et al. 2011; Meena et al. 2013; Sherif et al. 2014; Chien et al. 2015).

While some of the species have been propagated in vitro using other explants such as cotyledonary node in *Melissa officinalis* (Tavares et al. 1996), *Psidium arguta* (Kodja et al. 1998), *Vicia faba* (Khalafalla and Hattori 1999), *Pterocarpus marsupium* (Chand and Singh 2004), *Capsicum annuum* (Siddique and Anis 2006), *Mucuna pruriens* (Faisal et al. 2006), *Trichosanthes anguina* (Ambetkar et al. 2012), *Cucumis
melo (Zhang et al. 2013a), Glycine max (Zhang et al. 2014), leaf segment in Hyoscyamus niger (Yamada and Hashimoto 1982), Centella asiatica (Banerjee et al. 1999), Artemisia annua (Gulati et al. 1996), Datura stramonium (Iranbakhsh et al. 2010), Populus spp (Wang et al. 2011), Muscari armeniacum (Lee et al. 2012), Tylophora indica (Haque and Ghosh 2013), Phoenix dactylifera (Kurup 2014) and Inflorescence in Ocimum sanctum (Singh and Sehgal 1999), Populus euphratica (Zhou et al. 2012), Vitis spp. (Sedighi-Dehkordi et al. 2014) and Miscanthus giganteus (Perera et al. 2015).

2.3.2 Media Type

The morphogenic response of the explant is greatly influenced by the nutrient composition of the culture media (Ahmad and Anis 2011; Perveen et al. 2011). Murashige and Skoog medium was developed for optimal growth of tobacco callus, the development involved a large number of dose response curve of the various essential minerals. It was a milestone in plant tissue culture, the nutrient composition supported growth of most of the plant and thus, became the most common nutrient media used in plant tissue culture studies. The medium has been found to be commonly used for the propagation and mass multiplication of various species (Ahmad and Anis 2011; Perveen et al. 2011; Khan et al. 2011; Javed et al. 2013; Javed and Anis 2014). However, some other media have also been proposed by various workers (Gamborg et al. 1968; Llyod and McCown 1981) differing in the concentration and composition of the nutrients, to meet the special requirements of different groups of plants. Many of the species have been found to grow well on these derived media (Bhatt and Dhar 2005; Khan et al. 2011). While, MS medium was found superior to other for culture of Pterocarpus santalinus (Rajeshwari and Paliwal 2008), Acacia nilotica (Abbas et al. 2010), Albizia lebbeck (Perveen et al. 2011), Vitex negundo (Ahmad and Anis 2011), Erythrina variegata (Javed and Anis 2014). Each plant species has its own characteristic elementary composition which may be different from the MS medium. Optimization of the media components can significantly enhance the growth of the cultures (Booman and Tiekstra 2005; Iain et al. 2009; Fatima et al. 2011).
2.3.3 Plant Growth regulators

Plant growth and development is an intricate process involving interactions between various essential and non-essential factors. Among all factors plant hormones are in a commanding positions as the relative concentration of these, decides the fate of the plant cell (Skoog and Miller 1957). Plant hormones are chemicals which are synthesized endogenously at extremely low concentration in a particular organ of plants, transported to targeted organ and induce chemical action/reaction. These interactions in the target tissue controls the biochemical, physiological and molecular events of plant developmental processes. In addition, plants also respond to some synthetic compounds and other biologically active compounds that acts like plant hormones and called as plant growth regulators (PGRs) (Davies 1995). Generally, plant physiologist have classified all phytohormones into five classical phytohormone groups (auxins, cytokinins, gibberellins, abscisic acid and ethylene). However, besides these five classical hormones researchers have found significant involvement of other compounds influencing growth and developmental processes, such as salicylic acid (SA), jasmonic acid (JA), polyamines (PAs), nitric oxide (NO) and brassinosteroids (BRs) have been considered as phytohormones but contradictory statements about these chemicals kept them under the category of PGRs (Gross and Parthier 1994).

Furthermore, new growth substances having potentiality to regulate morphogenesis and growth in tissue culture have been discovered such as phenyl urea derivatives (Thidiazuron), oligosaccharides, sterols, phosphorinositosides and systemins. The effects of phytohormones and PGRs are specific on growth and development. Responses of cells, tissues, and organs to phytohormones and PGRs in vitro may vary with cultural conditions, the type of explants, and the genotypic variation of plants. Besides these promoter i.e., phytohormones and PGRs there are some compounds known as growth inhibitors and growth retardants. The plant growth inhibitors are those compounds which retard physiological processes of the plant growth and development such as root and shoot elongation. It has been reported that growth inhibitors can be derived from natural and artificial auxins with bulky benzoyl group. This conjugation can prevents movement of the inhibitor out of the cell. Growth inhibitors include: abscisic acid, ancymidol, butralin, carbaryl, chlorphonium, chlorpropham, dikegulac, flumetralin, fluoridamid, fosamine, glyphosate, isopyrimol,
maleic hydrazide, mepiquat, piproctanyl, prohydrojasmon, propham, tiaojien, 2,3,5-
tri-iodobenzoic acid.

Cytokinins regulates several plant growth aspects and developmental processes,
including cell division, apical dominance, chloroplast biogenesis, nutrient
mobilization, leaf senescence, vascular differentiation, morphogenetic development,
shoot differentiation and anthocyanin production (Mok and Mok 2001; Davies,
2004). Type and concentration of the applied cytokinins significantly influences the
response of the tissue as the uptake, transport, metabolism and interaction with the
endogenous hormones is unique for each cytokinin (Van Staden et al. 2008).

Benzyadenine (BA) is the most frequently used cytokinin in plant tissue culture
systems and has been found effective in regeneration of different woody species
species (Vengadesan et al. 2002; Shirin et al. 2005; Faisal et al. 2012; Javed et al.
2013). BA favoured regeneration of multiple shoots from the nodal explants in several
tree species of legume family (Saamudritha et al. 2005; Chand and Singh 2004;
Rajeswari and Paliwal 2008; Perveen et al. 2012). While kinetin (Kin) was also found
effective for establishing in vitro regenerative protocol for many plant species such as
Eupatorium adenophorum (Borthakur et al. 2000), Swietenia macrophylla (Mona
2012), Ricinodendron heudelotii (Fotos et al. 2007) and Prunus persica (Obaid 2012)

Several other synthetic compounds have also been found effective for in vitro
regeneration of woody plant species, N-Phenyl-N′-(1,2,3-thiadiazol-5-yl) urea;
thidiazuron (TDZ) is a fine example, it is a plant bioregulator that induces axillary and
adventitious shoots induction (Huettteman and Preece 1993, Pradhan et al.1998). It act
through modulation of the endogenous plant growth regulators, modification in cell
membrane, energy levels, nutrient uptake or nutrient assimilation (Murthy et al.1998).
Effectiveness of TDZ in modulating organogenesis has been established in numerous
plant species (Pradhan et al. 1998; Shaik et al. 2009), and explants such as nodal
segments (Jahan and Anis 2009; Asghari 2013), cotyledons (Mulwa and Bhatta 2006;
Cseke et al. 2007), roots (Guo et al. 2012) and hypocotyls (Jahan et al 2014).

Auxins is another group of phytohormones known to cause cell elongation, cell
division and mediate the tropistic response of bending in response to gravity and light.
The auxin supply from the apical bud suppresses growth of lateral buds, delays leaf
senescence, inhibit or promote leaf and fruit abscission by influence of ethylene and
induce fruit setting. Auxins are also involved in assimilate movement toward auxin possibly by an effect on phloem transport, and stimulates the production of ethylene at high concentrations (Davies 1995; Mauseth 1991; Salisbury and Ross 1992). Indole-3-acetic acid (IAA) is one of the most important naturally occurring auxins, however, its use in plant tissue culture is limited owing to its heat and light labile nature. There are certain other compounds showing analogy with the naturally occurring auxins which are more stable, these analogues have different structures but similar biological properties. 2,4 dichlorophenoxy acetic acid (2, 4-D), α-naphthalene acetic acid (NAA) and indole-3-butyric acid (IBA) are some of the more commonly used analogues.

Cytokinin and auxin have long been recognized as crucial signalling molecules controlling plant growth and development (Skoog and Miller 1957). The mode of interaction between cytokinin and auxin often depends upon the plant species and organ being cultured. Hence, organogenesis relies on the inherent plasticity of plant tissues, and is regulated by altering the components of the medium. Interaction between various factors, in particular, auxin and cytokinin regulates the organogenic pathway of the regenerating tissue. Addition of low levels of auxins along with cytokinin is known to increase shoot number in many plant species like Gymnema sylvestre (Reddy et al. 1998), Tectona grandis (Shirin et al. 2005) and Vitea negundo (Ahmad and Anis 2011). BA in combination with NAA increases the shoot forming capacity of nodal and cotyledonary nodes manifolds in Mucuna pruriens as reported by Faisal et al. (2006 a, b). Similar observations were made by Rajeshwari and Paliwal (2008) in Albizia odoratissima and Girijashankar (2011) in Acacia auriculiformis. Whereas, BA-IAA combinations was efficient in Aegle marmelos (Nayak et al. 2007) and Chrysanthemum morifolium (Waseem et al. 2009). Also, Kin+NAA synergism and their triggering effect on shoot bud induction and multiplication has been established in Alpinia officinarum (Selvakkumar et al. 2007) and Gardenia jasminoides (Duhoky and Rasheed 2010).

2.3.4 Carbon source

Since an artificial carbon source is essential for tissue culture of plants when heterotrophic or mixotrophic systems are used. The type and concentration of carbon source has been shown to greatly influence the growth and development of cultures.
Sugar not only provides the required carbon, maintain osmotic potential, enzyme activities, metabolism, and development, but also regulates the expression of many genes responsible for photosynthesis and thus growth of the plant (Koch 1996). The role and control of sugar in the media on organogenesis can also be construed by reports of Calamar and Klerk (2002), Gibson (2000) and Smeekens (2000). A number of tissue culture studies have evaluated the effect of the carbon source on the in vitro morphogenesis (Neto and Otoni 2003). Sucrose has been the most preferred and have been used in most of the studies (Fuentes et al. 2000; Javed et al. 2013). However, some of the species favored other sources (George 1993; Norgaard 1997; Rout et al. 2000; Ramarosandrata et al. 2001). Some of the specie favored nutrient medium with partial or complete substitution of sucrose with other sugars (George 1993; Rout et al. 2000; Ramarosandrata et al. 2001).

2.3.5 Medium pH levels

The pH of the medium governs the availability of nutrients present in the basal media to the plant tissue. Substrate pH has been shown to affect the plant growth both in vitro and in vivo (Staniene and Stanyte 2007; George et al. 2008). As it not only affects the uptake of nutrients but also facilitates various enzyme mediated chemical reactions because the enzyme activity is pH dependent (Thorpe et al. 2008). The ionization of compounds at unregulated pH can change their function at the cellular level making them ineffective or deleterious (Sakano1990). For proper growth and development of cultures, pH needs to be standardized. Furthermore, in tissue culture generally basal media is gelled using agar, which do not bind properly under highly acidic conditions (pH < 5.0) and becomes very hard when pH of the medium exceeds 6.0 hampering the availability of nutrients (Bhatia and Ashwath 2005). Therefore, optimization of the pH level becomes essential for efficient shoot regeneration.

A pH range from 5.2 – 6.0 is generally recommended for in vitro culture however, this may vary significantly between species and explants. Influence of pH of media and various growth parameters have been studied by various workers, showing its significant effect (Nair and Seenii 2003; Ostrolucka et al. 2004; Staniene and Stanyte 2007). In general pH greater than 6.0 has been found more detrimental, reducing seed germination, number of shoots and dry weight of the cultures (Finn et al. 1991).
Highly acidic pH has also been found to reduce the micropropagation yield of the cultures (Ostrolucka et al. 2004).

Many of workers screened different pH levels for establishing optimum conditions and have found 5.8 to be optimal in most of the cases (Nair and Seeni 2003; Karim et al. 2007; Siddique and Anis 2007; Perveen et al. 2011). However, some species may have a different requirement.

2.4 Rooting

One the most essential step in vegetative propagation of plants is adventitious root formation, as it is required, especially in case of micropropagation for the successful establishment of the plant in the field, fatalities at this point results in huge economic losses (De Klerk 2002). Some of the plant species are easy to root like Salix, where only wounding can induce rooting (Carlson 1950), while other species might require complex treatments for rhizogenesis. Adventitious root formation is an intricate process that involves interaction between various endogenous and exogenous factor. Though the exact molecular mechanism remains to be revealed, recent advances in the field of biotechnology have increased our knowledge greatly. Various workers have evaluated the effect of different factors such as plant growth regulators, light, carbon source, nutritional status, responses to stresses such as wounding and genetic characteristics (Gronroos and Arnold 1985; Dumas and Monteuuis 1995; Fogaca and Fett-Neto 2005; Sorin et al. 2005; Aloni et al. 2006; Yasodha et al. 2008; Abarca and Díaz-Sala 2009; Li et al. 2009; Thwe et al. 2013). The intricacies of adventitious root development have been reviewed in detail by Haissig (1974), George and Sherrington (1984), Gaspar et al. (1994) and Rout et al. (2000).

Strength of the culture media has also been shown to exercise a strong influence on in vitro rooting response of the micropropagated shoots (Javed and Anis 2014; Naz et al. 2011; Perveen et al. 2011; Khan et al. 2011). Full strength MS medium was found suitable for root induction in a number of plant species viz., Acacia ehrenbergiana (Javed et al. 2013), Anemopaegma arvense (Percira et al. 2003), Pongamia pinnata (Sugia et al. 2007), Cassia angustifolia (Siddique et al. 2010). However, in Erythrina variegata (Javed and Anis 2014), Bauhinia tomentosa (Naz et al. 2011), Albizia lebbeck (Perveen et al. 2011), Nyctanthes arbor-tristis (Jahan et al. 2011) and Salix tetrasperma (Khan et al. 2011), reduction in strength of the basal media was required
to initiate rooting. Application of auxins for initiation and elongation of adventitious roots is a routine process (De Klerk et al. 1999; Li et al. 2009). However, effectiveness of different auxins may vary for different species, NAA has been found most suitable for initiating root formation in regenerated shoots (Ahmad and Anis 2005; Ahmad et al. 2006; Burdyn et al. 2006).

Alternative methods such as ex vitro rooting have also been used in some species. This technique combines the rooting and acclimatization procedure, reducing time, labour and cost substantially (Yan et al. 2010) as the in vitro rooting has been estimated to be approximately 35-75% of the total cost of micropropagation (Debergh and Maene 1981). Success of the procedure has been well documented in *Vitex negundo* (Ahmad and Anis 2007), *Malus zumi* (Xu et al. 2008), *Terminalia bellirica* (Phulwaria et al. 2012), and *Albizia lebbeck* (Perveen et al. 2013).

2.5 Synthetic seed technology

The synthetic or artificial seed was conceptualized by Murashige (1977) as a mechanism to develop seed like structures that may serve as a clonal propagule and germinated just like a natural seed. Initially, somatic embryos were encapsulated in various water soluble gelling agents such as polyoxyethylene glycol (Kitto and Janick 1982) or alginate hydrogel (Redenbaugh et al. 1984). Later, to increase its applicability, the process was made more liberal and other explants too were used for encapsulation such as nodal segments (Chand and Singh 2004), cotyledonary nodes (Naik and Chand 2006) and shoot tips (Nunes et al. 2003). Potential advantages of the encapsulation technology includes ease in handling, genetic uniformity of plants and direct delivery to the field. Synthetic seeds can be made available throughout the year where most of tree species produce seeds only in certain months of the year (Bapat and Mhatre 2005). The most important use of synseeds for trees 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). Several authors have comprehensively reviewed the advances in the technology (Rai et al. 2009; Sharma et al. 2013). The technology have successfully utilised in several tree species including *Decalepis hamiltonii* (Sharma and Shahzad 2012), *Rubus idaeus* (Alvarez et al. 2003), *Quercus* spp (Tsvetkov and Hausman 2005), *Morus* spp (Kavyashree et al. 2006),
Psidium guajava (Rai et al. 2008), Vitex negundo (Ahmad and Anis 2010), Citrus sinensis × Poncirus trifoliata (Germana et al. 2011).

2.6 Acclimatization

The success of a micropropagation protocol is often judged by the number of plantlets established under natural conditions. All the efforts made in establishing a protocol can be lost if the regenerated plantlets do not adapt to the field conditions. Acclimatization process, thus is consequential to the success of the procedure (Pospisilova et al. 1999). Under developed water regulation system, susceptibility to photoinhibition and mixotrophic behaviour due to reduced photosynthetic ability are some of the major issues reported by various authors (Precece and Sutter 1991; Kozai 1991; Ziv 1991; Argita et al. 2002; Chen et al. 2006; Kornova and Popov 2007).

High exogenous sucrose degrades the photosynthetic ability of cultured plants by suppressing the gene expression, reducing chlorophyll content, Calvin cycle enzyme’s content and their activity affecting photosynthetic rate (Argita et al. 2002; Fuentes et al. 2005). To sum up, growth under high humidity, low irradiance, limited CO2, high sugar levels and presence of exogenous growth regulators may lead to development of modified morphology, anatomy and physiology in the culture grown plants (Pospisilova et al. 1999; Hazarica 2006; Pinto et al. 2011), making it difficult to adapt to the natural conditions instantly. A gradual exposure to the field conditions, thus, is helpful in acclimatization as it provides the crucial time required by the regenerated plantlets to develop the required physiological and morphological competence to cope with the variations of the natural environment.

2.7 Heavy metals

Metal toxicity has emerged as one of the most challenging threat to the agricultural system worldwide. Phytotoxicity induced by heavy metals may be the result of replacement of enzyme cofactors and transcription factors, cellular redox imbalance, ionic transport imbalance, DNA damage, protein oxidation, and inhibition of photosynthetic pigments and processes (Syta et al. 2013). However, some of these metals are essential for the growth and development of plants, while some are beneficial and augment the growth, but these are required in very small amounts. There is a threshold limit beyond which toxic effects become apparent. Exposure at
higher concentration of heavy metals can increase the production of ROS and change antioxidant responses (Gratao et al. 2005).

Copper is an essential micronutrient for plants as it is a constituent of protein components of several enzymes, mainly of those participating in electron flow, catalyzing the redox reaction in mitochondria and chloroplasts in the cytoplasm of plant cells (Ouzounidou et al. 1995). It works as a cofactor for photosynthetic electron transport in chloroplast and is also an integral component of plastocyanin (Raven et al. 1999). Copper is also a cofactor of Cu/Zn SOD enzyme (Bowler et al. 1994).

Essentiality of cobalt for animal kingdom is well established. However its role in the plant kingdom is not quite as articulate. Requirement of the metal by lower plant is well accepted while it is frequently cited as a beneficial micronutrient for the higher plants (Reeves and Baker 2000). Many of the earlier workers have reported the influence of cobalt on morphogenic processes in higher plants (Miller 1952; Miller 1954; Thiman 1956; Grover and Purves 1976; Lua and Yang 1976). Cobalt seems to be having a more intricate role in the nitrogen metabolism of the higher plant, many authors have reported enhanced nitrogen content in plants exposed to higher cobalt concentrations (Gad 2012). Evaluating the effect of cobalt on nodule free plants by some workers lead them to the conclusion that there is a requirement for the metal to the plants (Hallsworth et al. 1965; Wilson and Nicholas 1967). Also studies on excised plant tissue showed enhanced growth in presence of cobalt (Bollard 1983). Although cobalt has not been ascribed to any specific role in the plant metabolism of higher plants, it is still an integral part of most of the culture media proposed for tissue culture of plants.

*In vitro* techniques such as in plant tissue culture apart from micropropagation and plant regeneration also provides a good model to evaluate the effect of a particular factor as it is free from environmental contingencies. Moreover, the time required for the study can also greatly reduced through the use of these biotechnological tools.

*In vitro* techniques have been used as an excellent tool for the trial and selection of metal tolerant plant lines (Rout et al. 1999; Rai et al. 2011). Vera-estrella et al. (2009) reported *Arabidopsis halleri* as a model Zn hyper-accumulating plant by performing *in vitro* studies. Several authors have used the technique to evaluate the effects of metals on various plant species, namely *Populus* (Spirochova et al. 2003), *Salvinia natans* (Mohan and Hosetti 2006), *Alianthus altissima* (Gatti 2008), *Arachis hypogea*
(Dinaker et al. 2008), *Cicer arietinum* (Tantrey and Agnihotri 2010) and *Albizia lebbeck* (Perveen et al. 2012).

### 2.8 Genetic uniformity

Clonal propagation of elite genotypes is one of most important application of plant tissue culture. However, micropropagation may be severely restrained by incidences of somaclonal variations (Larkin and Scowcroft 1981). In woody plant Chaturvedi and Mitra (1975) reported it first time in *Citrus*. Somaclonal variation mostly occurs in response to stress imposed on the plant in culture conditions and is manifested in the form DNA methylations (Phillips et al. 1994), consequently giving rise to genetically variable plants, which is a potential drawback for the propagation of an elite plant species. Isozyme and allozymes have been used to detect variation in the regenerants (Bindiya and Kanwar 2003; Rout et al. 1998). However, their use is limited as these are developmentally regulated and difficult to compare when the age of the plants are different. DNA markers are more attractive means for examining genetic variations and are not affected by age or other environmental factors, various PCR-based molecular techniques including RAPD, RFLP, AFLP and ISSR have proved to be powerful tool used for a quick assessment of the genetic stability of micropropagated plants. Application of these techniques to evaluate genetic integrity has been applied successfully in several plants species (Archak et al. 2003; Molinari et al. 2003; Martin et al. 2004; Ray et al. 2006; Ahmad et al. 2013; Fatima et al. 2013; Perveen and Anis 2014).