CHAPTER 2

Review of Literature

REVIEW OF LITERATURE

The present literature, reviews the important aspects on germination,*in vitro* regeneration and synthetic seeds of mostly tree species.

Forests are our heritage but at the current rate of deforestation, mass extinction of species and considerable amount of genetic diversity is likely to be lost. Deforestation and fragmentation of habitats adversely affect the fate of rare species. Studies have shown that many plant species are in danger of extinction, while some have already become extinct. Thus, priorities need to be determined for conservation strategies in order to build a relatively small amount of relevant work on rare and threatened species. (Phartyal *et al,* 2002).In forestry, because of the long regeneration time required by trees, *in situ* conservation is the preferred approach for sustainable forest management (Simberloff, 1988). *Ex situ* conservation of forest genetic resources has become a common practice due to the alarming rate of deforestation, and the loss of species and genetic diversity (Phartyal *et al,* 2002).The medicinal plants have been an important source for different medicine but of them collected from the wild and exploitation coupled with increasing urbanization has lead to a steady erosion and loss of diversity from the natural habitats of these plants(Parveen and Shahzad, 2010). To satisfy growing market demands, new strategies are developled for better yield and quality which has opened new vistas in the forest biotechnology. Propagation can be achieved through tissue culture which has helped in conserving many valuable tree species (Dubey *et al,* 2004).

2.1 Tissue culture of trees

Plant tissue culture is a technique of vegetatively propagating important species and has a great potential commercially at present (Biondii and Thorpe, 1981).Large scale propagation of plants by tissue culture is now widely spread and the number of plants species, which can be multiplied by this technique has been steadily increasing (Bhojwani and Razdan, 1983).Propagating woody trees through tissue culture has many advantages over conventional propagation as helps in fast multiplication of the important genotypes, production of disease-free plants, season-independent production of plants, germplasm conservation and facilitating their easy exchange (Asthana *et al,* 2011).

Mascarenhas and Muralidharan (1989) had reviewed the tissue culture studies carried out on forest trees in India. Some of the important plants are : *Acacia nilotica, Albizia lebbeck, A. procera, Azadirachta indica, Bauhinia purpurea, Butea monosperma, Dalbergia* sp., *Dendrocalmus strictus, Eucalyptus* sp. *Ficus religiosa, Morus* sp., *Populus* sp., *Shorea robusta, Tectona grandis (all angiosperms), Biota oriental's, Cedrus deodara, Cryptomena japonica, Picea smithiana, Pinus* sp. (all gymnosperms).

This technique offers unparalleled opportunity for forest tree improvement. Tissue culture methods have been successfully employed for large scale multiplication of a number of woody plants some of them are listed below (Table 1).

Table 1. Tissue culture studies in some of the tree species

Tree species	References
Azadirachta indica	Arya *et al*, 1995
Acacia catechu	Kaur and Kant, 2000
Cinnamomum camphora	Nirmal Babu *et al*, 2003
Pterocarpus marsupium	Chand and Singh, 2004
Holarrhoena antidysentrica	Kumar *et al*, 2005
Tectona grandis	Shirin *et al*, 2005
Pterocarpus santalinus	Ekambaranellore *et al*, 2006
Aegle marmelos	Raghu *et al*, 2007
Sterculia urens	Hussain *et al*, 2007
Saraca asoca	Subbu *et al*, 2008
Terminalia bellerica	Rathore *et al*, 2008
Stereospermum personatum	Shukla *et al*, 2009
Wrightia tomentosa	Joshi *et al*, 2009
Parkia biglobosa	Sambe *et al*, 2010
Salix tetrasperma	Khan *et al*, 2011
Butea monosperma	Ratnaprabha *et al*, 2017
Coffea arabica	Gebremarium, 2017
Simarouba glauca	Bramhanapalli *et al*, 2017

One of the tissue culture technique is micrpropagation which offers a rapid means to produce clonal planting stock for afforestation woody biomass production and conservation of elite germaplasm of trees (Rout *et al,* 2008).

Toshivo Murashige of the University of California has defined three steps or stages(I-III) of micropropagtion (Murashige, 1974) which are widely adopted by research and commercial tissue culture laboratories. Debergh and Maene (1981) added a stage 0 in the micropropagation for preparation of stock plants(George *et al,* 2008).The advantages of micropropagation over conventional methods of vegetative propagation are: it has potentially high production rate, propagation of plants where conventional propagation is difficult, uneconomical, or impossible (e.g. plants that produce low quantities of seeds or produce seeds that are not viable or cannot be stored) and also protects from external pests, diseases, and environmental hazards(Mizuri *et al,* 2011). *In vitro* clonal propagation through adventitious or axillary bud has the capacity to produce large numbers of plants within limited space and time irrespective of the season, under controlled conditions of temperature, light intensity, and photo period. It offers an alternative cloning procedure for forest trees that do not respond well to conventional methods of propagation, either sexual or vegetative. (Bajaj, 1986;Bonga, 1987;Sharma *et al,* 2000)

Kaur and Kant (2000) pointed out that rapid and progressive deforestation is endangering several plant species therefore micropropagation systems have the potential for rapidly multiplying economically important genotypes for reforestation, which help to increase forest productivity (Bonga and Durzan, 1987; Gupta *et al*, 1993).

In vitro protocols producing quality planting stock on a large scale have been developed for several plants such as *Rauvolfia tetraphylla* (Faisal and Anis, 2002);*Pterocarpus marsupium* (Husain *et al,* 2006);*Acacia sinuata* (Shahzad *et al,* 2006);*Cannabis sativa*(Lata *et al*,2009) and *Veronica anagallis-aquatica* (Shahzad *et al,* 2010) through various strategies of micropropagation.

The tissue culture studies in some of the Bignoniaceae members have been reported as follows:

- In *Tecomella undulata* (sm.) *in vitro* regeneration has been carried out utilising internodal explants in MS medium fortified with BAP,Kn,2,4-D and NAA with different concentrations (Danya *et al,* 2012).
- *In vitro* propagation of *Oroxylum indicum* has been carried out by several researchers which includes:Dalal and Rai (2004) who reported the plant species

is naturally propagated by seeds, which have a short viability period and carried out *in vitro* propagation of *Oroxylum indicum* using cotyledonary node explants in MS medium with BA (2.22–17.74µM), kinetin (2.32–18.58µM), or thidiazuron TDZ (2.27– 9.08µM) of which the best medium for proliferation was Murashige-Skoog (MS) medium with BA (8.87µM) and IAA (2.85µM).

- Gokhale and Bansal (2009) used apical buds and axillary buds explant dissected from 15-20 days old seedling(8cm)and placed on MS medium supplemented with BAP(0.44-44.43 µM) and Kinetin (0.46-46.4 µM) tested individually and in combination. Gokhale and Bansal (2010) studied somaclonal variation in the same plant. Shoot regeneration from embryonic axis explant (both direct regeneration as well as through callus) has been studied previously in *O. indicum* (Gokhale and Bansal, 2005; Gokhale and Bansal, 2008).
- Whereas Tiwari *et al,* (2007) used six-week old *in vitro* seedlings of *Oroxylum indicum* and callus proliferation was observed from seedling stem sections sections on all the initial culture media supplemented with growth regulators BA (3.0 mg/l) and NAA(0.5 mg/l). They observed better root initiation (68%) and development on ½ MS with IBA (0.5 mg/l) as compared to medium with IBA(0.1 mg/l).
- *In Stereospermum suaveolens* untill now few references are documented one of the report by Shukla *et al,* (2012) who utilised nodal segments from field grown seedlings and terminal twigs of one and two year old *S.suaveolens* tree were used as explants and inoculated in MS,WPM and SH medium and obtained best result in MS medium fortified with BAP (4.44 µM) with maximum number of shoots and nodes.
- Micropropagation of *Handroanthus hetaphyllus* from seedling explants were carried out in MS medium fortified with BAP(22.2µM) (Duarte *et al*, 2016).
- Effect of different plant growth regulators on callus induction in *Catalapa bungei* has been studied by Juan *et al* (2010) whereas Thiadiazuron induced high frequency shoot organogenesis in callus from *Kigelia pinnata* has also been reported (Thomas and Puthur, 2004).
- Studies on micropropagation and *in vitro* conservation has been done in *Jacaranda decurrens* utilising nodal segments (Malosso *et al,* 2012).

2.2 Seed germination studies

AOSA (1970) defines germination as the emergence and development from the seed of those essential structures which are indicative of the ability to produce a normal plant under favourable conditions. Seed germination is regarded as a series of steps which occur prior to the emergence of radical from the seed coat (Mayerand and Shain, 1974; Rahman *et al,* 2007).Under natural conditions, the seeds of some tree species have low percent of germination and are difficult to grow through conventional methods. Because of that their large scale plantations becomes limited in agroforestry social forestry and home gardens (Azad *et al,* 2006a,2006b).Such seeds must be pretreated to hasten germination and seedling establishment (Maku *et al,* 2014).

There are several reports which mention methods for enhancing the seed germination as well as growth of seedlings (Kandya, 1990; Khan *et al,* 2001; Annapurna *et al,* 2005; Azad *et al,* 2011;Trivedi and Joshi, 2012; 2014; Maku *et al,* 2014) in tree species. According to Chalam *et al* (1967) the important requirements for normal seed germination are substrate, moisture, temperature and light.

2.2.1 Planting material

Since 1985 various forest industries and research institutions have tested different types of containers for growing seedlings. Root trainers were introduced in the forestry sector and presently this technology is being widely used for growing planting stock of selected forestry species. This technology has made a tremendous impact on forest nursery seedling production as it improves the quality of seedlings allows also less shock to seedling resulting from transportation. A root trainer, root-training pot or root pruning container is an aid to the cultivation of young plants and trees in nurseries which require less amount, of porting mixture and are easy to fill. They are well aerated and also do not cause root coiling (Mohanan and Sharma, 2005).

2.2.2 Substrates

The type of planting substrate is the another factor that influences germination and can be species-specific; therefore in nurseries importance is given to substrate for raising seedlings (Jaiswal and Chaudhary, 2005). An ideal planting substrate plays an important role in seed germination, it should be non toxic, free from moulds and microorganisms, cost effective, easy to handle (Agrawal, 1997) ,sufficiently porous and able to retain

moisture, (Richard *et al*, 1964; Jaiswal and Chaudhary, 2005). There are several reports which states the effect of substrates on germination on plants like *Jatropha curcas* (Gairola *et al,* 2011), *Gonystylus bancanus* (Utami *et al,* 2006) and *Calendula officinalis* (Ming *et al,* 1999).

Cocopeat is a commonly used substrate in horticultural practices for growing seedlings (Yau and Murphy, 2000). This substrate is beneficial for germinating seeds, as it is 100% organic with high water holding capacity, good aeration, has nutrient absorption qualities and is non toxic. Improved growth of seedlings using cocopeat has been reported in a number of species like *Pterocarpus macrocarpus* (Kijkar, 1991), *Eucalyptus tereticornis* (Kumar and Marimuthu, 1997) and *Swietenia macrophylla* (Woods *et al,* 1998). Utami *et al* (2006) reported highest percent of seed germination in cocopeat and least in soil for *Gonystylus bancanus*.

The other substrates like filter paper substrate can hold sufficient amount of water and dissipate water evenly all over the surface quickly and thus has effect on germination of seeds (Jaiswal and Chaudhary, 2005).MS and WPM medium have different mineral salt concentrations which greatly affects *in vitro* germination. It has been successful for seeds of *Senna macranthera* (Faria *et al,* 2012) while MS medium for *Pterocarpus marsupium* (Mishra *et al,* 2013) and *Salvia sclarea* (Ghanbari *et al,* 2012).Germination of *Simarouba glauca* has also been carried out in MS and WPM medium(Bramhanapalli *et al,* 2017).

Also the seed treatment can ensure success in seed germination by ensuring speed and guarantee germination procedures to be quick (Azad *et al,* 2011a; 2011b). The effects of pre sowing treatments on seed germination of several tropical forest tree species has also been reported (Matin and Rashid, 1992; Koirala *et al,* 2000; Khan *et al,* 2001; Matin *et al,* 2006).

2.3 Regeneration studies

The major factors responsible for *in vitro* regeneration are explant, culture medium and plant growth regulators (Nowak *et al,* 2004). Kataria *et al* (2013) also explained that *in vitro* techniques are dependent upon the strong and intricate interactions between the explant, plant growth regulators, culture conditions, and genotype.

2.3.1 Role of explants in shoot regeneration

Explant type has been shown to effect multiple shoot induction in a number of trees like *Dalbergia sisso* (Pradhan *et al,* 1998), *Pterocarpus marsupium* (Anis *et al,* 2005), *Albizzia lebbeck* (Mamun *et al,* 2004) and *Albizia odaratisssima* (Rajeshwari and Paliwal, 2006).Many tree species have been regenerated utilising various explants like hypocotyl, cotyledons (from aseptically germinated seedlings) or nodal explants (from mature plants) for large scale multiplication (Purohit *et al,* 1996; 1998; Hussain *et al,* 2008 ; Rathore *et al,* 2008). The differential response of explants had been observed in *Carthamus tinctorious* (Mahadevappa *et al,* 2014) and *Solanum melongena* (Sharma and Rajam, 1995). Eastern redbud (Distabanjong and Geneve, 1997), *Glycine max* (Kim *et al,* 1990), *Terminalia Bellerica* (Rathore *et al,* 2008) and in Guava (Shah *et al,* 2008) where the explants were also isolated from seedlings. Micropropagation of *Sterculia urens* an endangered tree species was carried out using intact seedlings which were placed in MS medium (Hussain *et al,* 2008). Micropropagation of woody trees from explants derived from mature plants has also been carried out (Giri *et al,* 2004; Pandey *et al,* 2006; Phulwaria *et al,* 2011).

2.3.1.1 Hypocotyl and cotyledonary leaf explants

The juvenile explants like hypocotyl and cotyledonary leaf have been utilised for *in vitro* regeneration by many researchers some of them are discussed as below.

The high frequency of plant regeneration from hypocotyl explants have been observed in documented in *Eucommia ulmoides* (Chen *et al,* 2008), *Dalbergia latifolia* (Rai and Chandra, 1989),*Camellia oleifera* (Li and Tan, 2016),*Bixa orellana* (de Paiva Neto *et al,* 2003).Shoot formation was obtained from cotyledon and hypocotyls explants excised from aseptically grown seedlings in *Eucalyptus tereticornis* (Prakash and Gurumurthi, 2005).In *Tectona grandis* hypocotyl and mature cotyledons have been utilised for indirect organogenesis (Tambarussi *et al,* 2017).Whereas in Safflower(*Carthamus tinctorious*) ten day old cotyledonary leaves were used as explants and *in vitro* growth response and regeneration potentials of the shoot tip, hypocotyls, and cotyledons of safflower were evaluated(Mahadevappa *et al,* 2014).

2.3.1.2 Cotyledonary Node explants

Another seedling explants which is widely used for establishing shoot cultures is cotyledonary node explant.

There are many reports documenting successful establishment of cultures utilizing cotyledonary nodes in tree species like *Sterculia urens* (Purohit and Dave, 1996), *Murraya koenigii* (Bhuyan *et al,* 1997), *Dalbergia sissoo* (Pradhan *et al,* 1998), *Quercus floribunda* (Purohit *et al,* 2002), *Pterocarpus marsupium* (Anis *et al,* 2005), *Gmelina arborea* (Behera *et al,* 2008),*Holarrhena antidysentrica* (Mallikarjun and Rajendrudu, 2009) and *Cassia sophera* (Parveen and Shahzad, 2010).

Devi *et al* (2011) studied multiple shoot induction and regeneration of whole plants from cotyledonary node explants which differentiated highest number of shoots in *Sterculia urens* as compared to nodal explants. According to them the explants isolated from younger plants exhibit a high regeneration capacity than explants excised from adult trees. Other reports where *in vitro* plant regeneration from cotyledonary nodes has been carried out includes *Tecomella undulata* (Varshney and Anis, 2012), *Soymida febrifuga* (Chiruvella *et al,* 2013) *Salvadora persica* (Mathur and Batra, 2014), *Sapindus emarginatus* (Srinivas *et al,* 2015).

2.3.1.3 Nodal explants

The other widely used explants are nodal segments for clonal propagation of trees owing to their high multiplication rates and genetic stability (Cassells and Curry, 2001). Besides genotype effects the responses of nodal segments over successive subcultures has been studied since subculturing is known to bring about changes in the rates of shoot growth and multiplication as well in the rooting ability of micropropagated plants (Norton and Norton, 1986; Grant and Hammatt, 1999).

Mulwa and Bhalla (2000) have reported on successful tissue culture of *M. tetraphylla* using nodal segments. It has been used for *in-vitro* shoot proliferation of number of woody plants such as *Azadirachta indica* (Saha *et al,* 1999), *Zizyphus mauntiana* (Wali and Siddiqui, 2001), *Tecomella undulata* (Teli *et al,* 2001), *Holarrhoena antidysentrica* (Raha and Roy, 2001), *Tectona grandis* (Tiwari *et al,* 2002),*Citrus limon* (Rathore *et al,* 2004), Rough lemon (Ali and Mizra, 2006)and miracle berry (Ogunsola and Ilori, 2008)*Capparis decidua* (Zhu *et al,* 2010), *Acacia mangium* (Annapurna and Rathore, 2010), *Jatropha*

curcas (Nayak *et al,* 2013),*Gardenia Latifolia* (Reddy and Saritha, 2013),*Psidium guajava* (Mousa *et al,* 2017).

2.3.2 Importance of medium in establishment of cultures

In vitro growth of plants is largely determined by the composition of the culture medium. The main components of most plant tissue culture media are mineral salts and sugar as carbon source and water. Other components may include organic supplements, growth regulators, a gelling agent, (Gamborg *et al,* 1968; Gamborg and Phillips, 1995). Medium components such as amino acids and vitamins are known to exert a profund effect on tissue culture systems of certain species. (Benson, 2000).The media compositions have been formulated for the specific plants and tissues (Nitsch and Nitsch, 1969; Conger, 1981) and tissue growth and the quality of morphogenetic responses are strongly influenced by the type and concentrations of nutrients present in the culture media (Niedz and Evens, 2007).Certain tissues respond much better on solid media while others on liquid media.

During the past decades, many types of media have been developed for *in vitro* plant culture (Street and Shillito, 1977; Pierik, 1989; Torres, 1989) and more than 60 different media have been used for tissues of various woody species (Skirvin, 1980).

Bonga (1980) has stated that commonly used media is that of Murashige and Skoog (1962) for plant regeneration.Similarly according to Cohen (1995) the most widely used culture medium is MS medium, because most plants respond to it favorably. It is classified as a high salt medium in comparison to many other formulations, with high levels of nitrogen, potassium and some of the micronutrients, particularly boron and manganese. *In vitro* regeneration has been done in MS medium in species like *Prunus avium* (Ruzic and Vujovic, 2008), *Psidium gujava* (Shah *et al*, 2008), *Eucalyptus citridora* (Jasrai *et al,* 2009), *Azadirachta indica* (Arora *et al,* 2009), *Andrographis neesiana* (Karuppuswamy and Kalimuthu, 2010),*Cassia sophera* (Parveen and Shahzad, 2010),*Cordyline* sp. (Chinnu *et al,* 2012),*Gardenia latifolia* (Reddy and Saritha, 2013),*Salix viminalis* (Joanna *et al,* 2017).

Some woody species are sensitive to high salts therefore Lloyd and McCown (1980) developed the woody plant medium in which the tissues of woody species grew better in medium as it was with a lower salt content. McCown and Sellmer (1987) reported that

some species give analogous response in all media while others show preference for a specific medium for explant establishment and growth.

WPM medium is also equally effective in shoot regeneration and many trees have achieved multiple shoot formation in trees like *Calophyllum inophyllum* (Thengane *et al,* 2006), *Syzigium cumini* (Remashree *et al,* 2007), *Terminalia bellerica* (Rathore *et al,* 2008),*Salix tetrasperma* (Khan *et al,* 2011), *Terminalia bellerica* (Phulwaria *et al,* 2012),*Semecarpus anacardium* (Panda *et al,* 2016).

Feyissa *et al* (2005) have reported that performance of cultures remained same in either WPM or MS medium in *Hagenia abyssinica*. The highest number of shoots per explant was obtained on MS medium containing agar plus gelrite or on WPM containing only agar. Husain *et al* (2008) used different basal media ie.MS, ½ MS, WPM and B5 to identify the most suitable medium formulation whereas Rajanna *et al* (2011) reported MS as a suitable medium for shoot induction, proliferation, elongation and rooting although both MS and WPM medium induced 6-8 shoot buds from cotyledonary node and shoot tip explants.

2.3.3 Role of plant growth regulators in regeneration of shoots

Determination of optimal concentrations of plant growth regulators in medium is another important parameter for successful plant regeneration (Peeters *et al,* 1991; Ruzic and Vujovic, 2008) as they regulate the growth and developmental processes (Sharma, 2017). Plant hormones like cytokinins and auxins play a key role for *in vitro* regeneration as the cytokinin affects the formation of shoots (Mahadevappa *et al,* 2014) and their interaction controls the *in vitro* growth and differentiation (Sharma, 2017). The cytokinin-to-auxin ratio differentiate shoots at high cytokinin with low auxin concentrations and vice versa induces root formation (George, 1984).

2.3.3.1 Effect of individual cytokinins

Cytokinins are known to play a key role in cell division and its presence in the medium is required for multiple shoot induction, but the type and its optimal concentration varies with the plant species (Park *et al,* 2008; Ruzic and Vujovic, 2008).

Effect of three different cytokinins BAP, Kn, TDZ on regeneration have been studied in *Albizia amara* (Indravathi and Pullaiah, 2013),*Sapindus emarginatus* (Srinivas *et al,* 2015) ,*Sterculia urens* (Devi *et al,* 2011) ,*Butea monopserma* (Ratnaprabha *et al,* 2017) and obtained varied results.

- **BAP(6-Benzylaminopirine)**

BAP is a frequently used cytokinin in plant tissue cultures and there are many reports which states that BAP as the suitable cytokinin for shoot proliferation.

Superiority of BAP was reported in many woody tree species (Joshi *et al*, 1991; Gupta *et al*, 1993; Purohit and Dave, 1996). Sunnichan *et al* (1998) have recorded the highest frequency number of shoots from axillary bud in BAP than Kn in *Sterculia urens.* BAP is the most effective synthetic cytokinin for stimulating organogenesis in various plant species (Kane *et al*, 1993; Manjula *et al*, 1997; Hoque *et al*, 2000; Jenks *et al*, 2000;Anis *et al*, 2009;Husain and Anis, 2009;Siddique and Anis, 2009). Superiorty of BAP over other cytokinins has already been established in trees (Murashige, 1974) and in neem (Joshi and Thengane, 1996; Quarishi *et al*, 2004).

Anitha and Pullaiah (2002) have reported in *Sterculia foetida* that the presence of BAP in the medium enhanced the shoot bud proliferation as compared to Kn and TDZ. Mathur and Batra (2014) and Phulwaria *et al* (2012) have also documented that BAP was effective for shoot multiplication resulting in maximum frequency. Others reports with similar results includes *Pterocarpus marsupium* (Anis *et al*, 2005), *Psidium guajava* (Shah *et al*, 2008), *Pistacia atlantica* (Safari *et al*, 2013), *Boucerosia diffusa* (Ramadevi *et al*, 2012).

Benjamin *et al* (1987) has shown that 6-Benzylaminopurine (BAP), at high concentration stimulates the development of the axillary meristems and shoot tips of *Atropa belladona.* Whereas low concentration of BAP has been found to be effective in adventitious shoot proliferation in Guava (Loh and Rao, 1989) and *Rubus* (Fiola *et al*, 1990). Other reports also obtained multiple shoots formation in MS medium with 2.0 mg/L BAP in forest tree *Wrightia tinctoria* (Purohit and Kukda, 2004) while the MS medium supplemented with 1.0 mg l-1 BA has led to the vegetative growth of axillary buds in *Ananas comosus* (Ibrahim *et al*, 2013).

- **Kinetin**

Another important cytokinin widely used in tissue culture is Kinetin and is utilized for micropropagation of many ornamental plants (Jain and Ochatt, 2010).

There are reports where Kn has proved to be an effective cytokinin as compared to BAP.

de Oliveira *et al* (2003) reported higher multiplication rate on media with Kn than BAP in *Tabernaemontana fuschsiaefolia* L. Similarly in *Ananas comosus* nodal segments cultured

in MS medium supplemented with Kinetin at 1.0 mg/1 proved superior than other treatments of MS media (Ibrahim *et al,* 2013).

Effect of different concentrations of Kinetin has also been documented in *Matthiola incana* (Hesar *et al,* 2011), *Vigna mungo* (Srilatha *et al,* 2014) and in *Sapindus emarginatus* (Srinivas *et al,* 2015).

Ruzic and Vujovic (2008) have reported that Kn mainly influenced shoot growth of cherry whereas it made little impact on multiplication. While in *Pterocarpus marsupium* only elongation of single shoot took place and no significant response was observed in presence of Kn (Anis *et al,* 2005)

- **TDZ (Thiadiazuron)**

The widely used cytokinin nowdays for woody trees is TDZ (Thiadiazuron) which has gained a considerable attention during past decades due to its efficient role in plant cell and tissue culture. It has been proved that TDZ, unlike traditional phytohormones, individually fulfilled the requirements of various regenerative responses of many different plant species. TDZ has shown both auxin and cytokinin like effects, although, chemically, it is totally different from commonly used auxins and cytokinins (Murthy *et al,* 1998). It exhibits strong cytokinin like activity and promotes the proliferation of axillary shoots (Faisal *et al,* 2005) as well as releases the lateral bud dormancy and stimulates adventitious organ regeneration (Mroginski *et al,* 2004). It has shown to induce high bud regeneration rates than purine based cytokinins in a number of woody plants (Jones *et al,* 2007). Huetteman and Preece (1993) also states TDZ as the most active cytokinin like substance for woody plant tissue culture and it has been considered to be more potent than most of the commonly used cytokinins.

TDZ is a substituted phenylurea (N-phenyl-N-1,2,3-thiadiazol-5-ylurea) used as a synthetic herbicide and a plant growth regulator to stimulate high rate of axillary shoot proliferation in many woody plant species (Malik and Saxena, 1992). It is highly effective than all adenine type cytokinins in shoot organogenesis. Thidiazuron has stimulated shoot formation in wide variety of plant species (Fiola *et al,* 1990; Malik and Saxena, 1992). According to Capelle *et al* (1983), TDZ directly promotes growth due to its own biological activities in a fashion similar to that of an N- substituted cytokinin or it may induce the synthesis and accumulation of an endogenous cytokinin.

In woody plant species, low levels of TDZ induce the axillary shoot proliferation but higher levels may inhibit it. Higher levels, on the other hand, promote callus and somatic embryo formation (Huetteman and Preece, 1993).Whereas according to Husain *et al,* (2007);Corredoira *et al,* (2008);Feng *et al,* (2010) this synthetic compound has been found to be effective at very low as well as at higher concentrations for micropropagation of several species.

TDZ has shown to induce response in many recalcitrant woody species like *Quercus robur* (Chalupa, 1988) ,*Cassia augustifolia* (Siddique and Anis, 2007; Parveen and Shahzad, 2011),*Pterocarpus marsupium* (Husain *et al,* 2007) and *Cardiospermum halcaabum* (Jahan and Anis, 2009).

Varshney and Anis (2012) tried different concentration of TDZ for inducing axillary shoot proliferation in *Tecomella undulata* and found that it is useful in multiple shoot regeneration. Similarly Verma and Bansal (2014) reported that TDZ (1.0 mg/l) induced multiple shoots in *Hedychium coronarium*.Karuppusamy and Kalimuthu (2010) also obtained multiple shoots from nodal explants cultured on Murashige and Skoog's medium supplemented with TDZ in *Andrographis neesiana.*

2.3.3.2 Synergistic effect of cytokinins

Medium fortified with a combination of cytokinins are used for *in vitro* multiplication of shoots in many plants.

There are various reports which documents the significant effect of cytokinins in combinations on shoot proliferation. Kathiravan and Ignacimuthu (1999) found the efficiency of BAP and Kn together in the medium in *Canavalia virosa.* Appa Rao (2004) observed the synergistic effect of BAP and Kn in enhancing the shoot buds proliferation in *Sapindus trifoliatus.*

The other reports where BA and Kn in combination induced maximum numbers of multiple shoots has been reported in *Bauhinia racemosa* (Rajjanna *et al,* 2011), *Withania coagulans* (Jain *et al,* 2009), *Eucalyptus globules* (Bennett *et al,* 1994), and in *Soymida febrifuga* (Chiruvella *et al,* 2013). Similalry synergistic effect of BA and Kn in promoting shoot intiation has been reported in *Acacia catechu* (Kaur and Kant, 2000;Thakur *et al,* 2002).

Kalimuthu and Prabakaran (2014) tried BAP and TDZ combination for shoot induction in *Syngonium podophyllum*. Similar results with BAP and TDZ as effective combination was proved in *Boucerosia diffusa* (Ramadevi *et al,* 2012) while in *Bauhinia vahlii* Kn and TDZ combination was proved to be effective (Bhat and Dhar, 2000).

2.3.3.3 Synergistic effect of cytokinins and auxins

Many aspects of cell growth, cell differentiation and organogenesis in tissue and organ cultures have been found to be controlled by an interaction between cytokinins and auxins. Cytokinins in combination with an auxin are essential for the onset of growth and the induction of embryogenesis (Fujimura and Komamine, 1980). The effects of auxins and cytokinins on shoot multiplication of various medicinal plants have been reported (Tripathi and Tripathi, 2003;Beena *et al,* 2003; Faisal *et al,* 2007).

Mathur and Batra (2014) obtained multiple shoot formation when nodal segments were inoculated on MS medium supplemented with BAP (4.0mg/1) and IAA (0.5mg/1).The multiple shoots were produced only when *in vitro* shoot apices were cultured on MS medium supplemented with lower concentration of cytokinin and auxin.

In *Pterocarpus marsupium* addition of IAA (0.5µM) significantly enhanced the shoot regeneration capacity (Hussain *et al,* 2008). Similar results where the addition of low-level of auxin with cytokinin promoted shoot proliferation as reported in *Acacia catechu, Eucalyptus grandis* and *Lagerstromia parviflora* (Kaur *et al,* 1998; Luis *et al,* 1999; Tiwari *et al,*2002) and higher concentration of auxin (IAA) reduced the regeneration percentage and resulted in the callus formation at the base of shoots.

Mizuri *et al* (2011) also states that cytokinin and auxins are commonly used to regulate *in vitro* plant growth and studied the effect of BA and IAA on shoot number in 3 types of citrus.

In *Pterocarpus marsupium* IAA and NAA(0.25-1µM) was added along with BA to observe the synergistic effect of auxin and cytokinin where BA(5µM)+IAA(0.25µM) resulted in maximum number of shoots(Anis *et al,* 2005).The synergistic effect of BA and IAA on axillary bud proliferation has also been reported in *Myrica esculenta*(Bhatt and Dhar, 2004),*Pterocarpus marsupium* (Chand and Singh, 2004),*Aegle marmelos*(Pati *et al,* 2008) and *Malus zumi* (Xu *et al,* 2008). *Gardenia latifolia* (Reddy and Saritha, 2013).Jafari

et al (2011) also reported incorporation of IAA to the media in enhancing shoot proliferation and elongation in *Musa acuminata*.

The role of cytokinin and auxin- cytokinin combinations on direct plant regeneration and adventitious bud induction from cotyledon explants was studied by Srinivas *et al* (2015) in *Sapindus emarginatus* where the combination of TDZ and IAA induced highest number of plantlet regeneration among all hormonal combinations and concentrations were used and in *Pistacia atlantica* the TDZ and IAA combination induced taller shoots (Safrai *et al,* 2013).

The synergistic effect of auxin and cytokinin combination was also found to be effective in inducing maximum number of shoots in *Vigna mungo* L. where TDZ + IAA gave better results as compared to Kn + IAA and BAP+ IAA (Srilatha *et al,* 2014).

Rasool *et al* (2013) studied the synergistic effect of auxins and cytokinins on propagation of *Artemisia amygdalina* where BAP and NAA favored multiple shoot regeneration via callus redifferentiation. Similarly Mackay and Kitto (1988); Nin *et al* (1996) also reported BAP and NAA good for multiple shooting and callus formation in *A. dracunculus* and *A. Absinthium* respectively.

Gebeyehu (2015) evaluated the effects of different concentrations of BAP and NAA on Banana (*Musa spp.)* cv. Giant Cavendish Shoot Proliferation and obtained highest proliferation. BAP (5.0 mg/l) + NAA(0.5 mg/l). Indravathi and Pulliah (2013) also used various combinations of cytokinins and auxins at different concentrations for rapid shoot proliferation where cytokinin in combination with NAA was found to be more effective in rapid shoot proliferation. Other reports with similar results includes in *Albizia procera* (Kumar *et al,* 1998), *Albizia chinensis* (Sinha *et al,* 2000) and *Albizia amara* (Ramamurthy and Savithramma, 2003). Maximum growth and multiplication rates were also obtained in treatments of BAP and NAA in Damask rose (Salekjalali, 2012), *Salix tetrasperma* (Khan *et al,* 2011), and Teak (Shirin *et al,* 2005).

According to Faria and Illg(1995), the addition of BA (10 mM) along with IAA (5 mM) or NAA (5mM) induces a high rate of shoot proliferation of *Zingiber spectabile.* Mahadevaapa *et al* (2014) used combinations of cytokinins and auxins for direct organogenesis and found MS medium supplemented with TDZ(1.5 mg/l) and NAA(0.5 mg/l) and BAP(2.0mg/l) with NAA(0.25 mg/l) as effective combinations. According to him a low concentration of auxin is often beneficial in conjunction with high levels of

cytokinin when shoot multiplication is required. Whereas Barna and Wakhlu (1988) has indicated that the production of multiple shoots is higher in *Plantago ovata* on a medium having kinetin along with NAA.

Sathe *et al* (2013) studied the effect various concentrations of cytokinins and auxins and obtained maximum shoot proliferation in media supplemented with Kn along with IBA. Whereas in sweet cherry effect of BA/Kn/TDZ combined with IBA was studied and medium with BAP (5 µM) and IBA(0.5µM) gave the highest multiplication index (Ruzic and Vujovic, 2008).

2.3.4 Rooting of microshoots

Root induction in microshoots is associated with factors like medium strength, type of auxins and their concentrations. The auxins like NAA and IBA stimulates induction of adventitious roots in *in vitro* shoots (Copes and Mandel, 2000).

Devi *et al* (2011) used half strength MS medium fortified with IAA, IBA and NAA for root induction and observed that IBA and NAA were effective in differentiating roots in *Sterculia urens*. Whereas in *Cinammomum camphora* root induction was done in WPM medium fortified with different concentrations of IBA and NAA supplemented with additive like activated charcoal (Nirmal babu *et al*, 2003) and in *Aquilaria crassna* WPM basal medium supplemented with IAA, IBA, or NAA, roots developed on media with IBA or IAA at 0.3mg/l (Van Minh, 2005). Addition of IBA and NAA to half strength MS medium facilitated rhizogenesis in *Cassia sophera* and an optimum rooting response (93.6%) was obtained in presence of IBA where maximum number of roots per shoot were formed (Parveeen and Shahzad, 2010).

In *Pterocarpus marsupium* shoots were transferred to rooting medium containing different concentration of MS salts (1/4, 1/2 and full strength) and different auxin (IAA, IBA and NAA) (Anis *et al*, 2005). Similarly in *Terminalia bellerica* different concentrations (0.1-1mg/l) of IAA, IBA and NAA induced rooting when incorporated in the medium containing ¼ MS salts. Best rooting was observed on medium containing quarter strength strength MS salts and IBA(0.1mg/l) (Rathore *et al*, 2008).Purohit and Dave (1996); Sunnichan *et al* (1998);Hussain *et al* (2007);Hussain *et al* (2008) showed maximum rooting response on IBA using half and quarter strength MS medium. Whereas Khan *et al* (2011) carried out rooting of *in vitro* raised shoots by transferring the healthy elongated

shoots to rooting media composed of either full or half strength WPM with NAA, IAA and IBA and rooting of shoots was best achieved in half strength WPM containing IBA (0.5µM).

IBA has been used and has stimulatory effect on *in vitro* root induction in many tree species *Morus indica* (Chand *et al,* 1995), *Sterculia urens* (Hussain *et al,* 2008), *Balanites aegyptica* (Mansor *et al,*2003), *Albizia odoratissima* (Rajeswari and Paliwal, 2008),*Punica granatum* (Chikkalaki *et al,* 2017), *Alpus glutinosa* (Perinet and Halonde, 1983),*Tecomella undulata* (Varshney and Anis, 2012), *Parkia biglobosa* (Sambe *et al,* 2010),*Holarrhena antidysentrica* (Kumar *et al,* 2005), *Santalum album* (Singh *et al,* 2016).

NAA has also been proved to be equally effective for root induction. Microshoots were successfully rooted on half strength MMS medium supplemented with IBA(1.5mg/)l and NAA(0.5mg/l) where NAA(0.5mg/l) was most responsive in Guava (Shah *et al,* 2008).A varied effect of auxins on rooting was observed with auxins like NAA,IBA, IAA and IPA in MS medium where NAA proved to be the most effective auxin for *in vitro* rooting of teak shoots(Shirin *et al,* 2005).Other reports where NAA was effective for *in vitro* rooting includes *Saccharum officinarum* (Tolera, 2016),Citrus (Chatzissavvidis *et al,* 2010) and *Pinus roxburghii* (Arya *et al,* 2014).

2.3.5 Hardening of plantlets

In vitro propagation system provides an alternative method for the rapid production of plants but its ultimate success depends upon the successful transfer and establishment of these plants in the field conditions. The transfer of plants from a sterile environment to a greenhouse is known as acclimatization, which corresponds to the Stage IV in the process of micropropagation. It constitutes an important factor in the adaptation of *in vitro* raised plants to natural conditions (Van Huylenbroeck *et al,* 1998).

Plants produced under *in vitro* conditions which are in high humidity diffused light and constant temperature need to be acclimatized because transferring of these plant from *in vitro* to ex vitro conditions is the most traumatic experience for them(Kaur *et al,* 2011).Therefore the development of successful hardening technique is prerequisite for micropropagation method(Mishra *et al,* 2011) and also due to the technical requirements it is considered one of the most expensive method and consequently a possible limiting factor on a commercial scale (Lewandowski, 1991). Direct transfer to sunlight causes

charring of leaves and wilting of the plants (Hiren *et al,* 2004;Lavanya *et al,* 2009) and therefore it is necessary to accustom the plants to a drier or natural atmosphere(Deb and Imchen, 2010).

The microenvironment inside the culture vessels normally used in plant micropropagation is characterized by high relative humidity and poor gaseous exchange between the internal atmosphere and its surrounding environment that may induce physiological disorders. These contribute a culture induced phenotype that cannot survive the environmental conditions when directly placed in greenhouse or field (Hazarika, 2003). Similarly Kaur *et al* (2011) also stated that direct transfer of tissue culture raised plants to field conditions is not possible due to high mortality rate as the plants kept under controlled environmental conditions have heterotrophic mode of nutrition and show uncontrolled loss of water. It is therefore necessary to transfer the plants to field through various weaning stages in order to increase the survival percentage. He has reported that using various hardening and acclimatization stages the survival rate was found to be much higher than as compared with the direct transfer of the plantlets to the field.

To acclimatize the micropropagated plants different worker have employed different approach toward successful establishment of *in vitro* raised plants under ex vitro condition. Several protocols have been developed for the micropropagation of woody trees but only a few have been demonstrated at field level. These protocols involve several steps each of which may be critical in deciding the success of micropropagation of a particular plant species; Rooting and acclimatization are two such crucial steps. The *in vitro* plantlets not only live in a 100% relative humidity but they also depend upon the medium which supplies sugars and other nutrients(Chabukswar and Deodhar, 2005). In many plant species the leaves formed *in vitro* are unable to develop further under ex vitro conditions and they are replaced by newly formed leaves (Preece and Sutter, 1991; Diettrich *et al,* 1992).Different potting substrates have been employed for hardening of *in vitro* raised plants by various workers like soilrite for *Carica papaya* (Agnihotri *et al,* 2004),soaked cotton for *Saccharum offinarum*(Gill *et al,* 2004),cocopeat and sand for *Garcinia indica* (Chabukshwar and Deodhar, 2005) and soil:vermicompost for *Tylophora indica* (Kaur *et al,* 2011). Autoclaved garden soil mixed with vermiculite and sand (1:1:1) was filled in plastic cups covered with perforated polyhtene bags to maintain high humidity(70%) in *Sterculia urens* (Devi *et al,* 2011).

Acclimatization of rooted plantlets was achieved by transferring them into plastic cups containing soil and farmyard manure using a mist chamber. The % survival decreased to 90 after 3 months (Purohit *et al*, 2012).

Among the three planting substrates (vemiculite,soilrite and garden soil) highest percent of plantlets survived in soilrite and after this transferred to field showed 80% survival rate in *Tecomella undulata* (Varshney and Anis, 2012).

Sterculia urens plantlets were transferred to plastic pots containing vermiculite kept under culture room conditions and later on transferred to garden under shade and later on to normal conditions (Hussain *et al*, 2008). Similalry in *Cassia sophera* regenerated plantlets were successfully hardened off inside the culture room and then transferred to greenhouse conditions with 90% survival rate (Parveen and Shahzad, 2010). Phulwaria *et al* (2012) reported that plantlets of *Terminalia bellerica* rooted *in vitro* as well as ex vitro were acclimatised successfully under the greenhouse conditions.

Successful acclimatization and field transfer of *in vitro* regenerated plantlets have also been reported in *Tamarindus indica* (Kopp and Nataraja, 1990), *Eucalyptus tereticornis* (Das and Mitra, 1990), *Prosopis juliflora* (Nandwani and Ramawat, 1991), *Dalbergia latifolia* (Raghavaswamy *et al*, 1992), *Thevetia peruviana* (Kumar, 1992), *Alpinia galangal* (Anand and Hariharan,1997), *Terminalia arjuna* (Kumari *et al*, 1998), *Sapindus mukorossi* (Philomina and Rao, 2000), *Salvadora persica* (Mathur *et al*, 2002), *Bupleurum distichophyllum* (Karuppusamy and Pullaiah, 2007), *Spondias mangifera* (Tripathi and Kumari, 2010), *Acacia auriculiformis* (Girijashankar, 2011), *Streblus asper* (Gadidasu *et al*, 2011).

2.4 Synthetic seed studies

Seeds are derived from sexual recombination of male and female gametes and thus are not genetically identical to their parents. Many important plant species are sterile and do not set viable seeds. Synthetic seeds also called as artificial seeds are seen as a means of overcoming these limitations and allowing the clonal propagation of large number of disease free propagules (Redenbaugh *et al*, 1991a,b; Mckersie and Bowley,1993; Mckersie *et al*, 1995).

A synthetic seed is a functional mimic seed that has the ability to convert into a plant under *in vitro* or ex vitro conditions (Capuano *et al,* 1998).Ara *et al* (2000) defined synthetic seeds as artificially encapsulated somatic embryos,shoot buds,cell aggregates or any other tissue that can be used for sowing as a seed and has the ability to convert into a plant under *in vitro* or ex vitro conditions and retain this potential also after storage. While according to Manoj *et al* (2009) synthetic seeds or artificial seeds made by encapsulating somatic embryos,shoot buds or any other meristematic tissues help minimize the cost of micropropagated plantlets for commercialization.

Synthetic seeds have multiple advantages for propagation as they are ease to handle, potential for long term storage at low production costs (Redenbaugh *et al,* 1987).It is used for propagation of transgenic plants,non-seed producing plants, and one with problems in seed propagation.It has been suggested as a powerful tool for mass propagation of elite plant sps with high commercial value. Currently system of artificial seed production have progressed substantially the most advanced being in seeding under ex vitro or field conditions, obtaining high percentages of conversion to plants(Saiprasad, 2001).

The first synthetic seed was reported by Kitto and Janick (1985) who successfully germinated carrot somatic embryos. However synthetic seeds can often be produced more conveniently using unipolar structures such as apical or axillary buds(Ahmad and Anis, 2010;Singh *et al,* 2010;Mishra *et al,* 2011).Vegetative propagules of uninodal cuttings have been used for encapsulation (in woody plants like *Punica, granatum*, tree sps like *Santalum album* and medicinal plant like W*ithania* sp.

Encapsulation is expected to be the best method to provide protection & to convert the *in vitro* derived propagules into 'synthetic seeds' or synseeds or 'artificial seeds'.This technology is an exciting and rapidly growing area of research in plant cell & tissue culture (Ara *et al,* 2000) as it offers an effective means for exchange of plant germplasm between laboratories,short or long term storage and direct transfer of *in vitro* material to ex vitro conditions(Standardi and Piccioni, 1998;Ara *et al,* 2000;Micheli *et al,* 2003;Rai *et al,* 2009;Germana *et al*,2011). It has been applied to produce synthetic seeds of a number of plant species belonging to angiosperms and gymnosperms(Ara *et al*,2000).The composition of the gel matrix is an important factor that significantly affects the conversion performance of encapsulated tissue.

Reports for synthetic seed production in forest tree species has been documented two decades back in *Santalum album* (Bapat and Rao, 1988), *Betula platyphylla* var.japonica (Kinoshita and Saito, 1990), *Picea glauca* (Attree *et al,* 1994), *Betula pendula* (Piccioni and Standardi, 1995). Till date the same technology has been successfully used in many tree species utilising various explants and encapsulated in different matrix as listed in (Table 2).

Table 2. Synthetic seed with various encapsulation matrices

Tree Species	**Explant**	**Encapsulation matrix**	**Conc. of $CaCl_2$**	**References**
Dalbergia sissoo	In vivo nodal segment	3%SA+1/2 MS	75mM	Chand and Singh, 2004
Vitex negundo	*In vitro* nodes	2,3,4 and 5% SA+MS	25,50,75,100 and 200mM	Ahmad and Anis, 2010
Acacia hybrid	*In vitro* shoot buds and axillary buds	2,3,4 and 5%SA+MS(Ca salt free)	25,50,75,100 mM	Asmah *et al,* 2011
Decalepis hamiltonii	Nodal segments	1,2,3,4,5%SA+MS/DDW	25,50,75,100, 200mM	Sharma and Shahzad, 2012
Oxalis triangularis	*In vitro* shoots	3%SA+MS BM	75mM	Taha *et al*,2013
Ficus carica	*In vitro* nodes	4%SA+MS BM	100mM	Sharma *et al,* 2014
Salvia splendens	*In vitro* shoots	4%SA+DDW/MS+BM/MS +BA(5µM)/MS+BA(5 µM)+IAA(2.5 µM)	100mM	Sharma *et al,* 2014
Balanites aegyptica	*In vitro* node	2,3,4 and 5 % SA+MS+BM	25,50,75,100 and 200mM	Varshney and Anis, 2014
Rhinacanthus nasutus	Shoot tips and nodal segments	2-5% SA+MS	25,50,75,100 and 200mM	Elangomathavan *et al,* 2017