Chapter II

REVIEW OF LITERATURE
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2.1 Multiple shoot induction by *in vitro* method

The *in vivo* clonal propagation of plants is often difficult, expensive and even unsuccessful. Tissue culture methods offer an alternative means of plant vegetative propagation. Micropropagation through tissue culture can be achieved in short time and space. Thus, it is possible to produce plants in large numbers starting from a single individual. Several efforts have been made on *in vitro* propagation of tree species (Razdan, 1993).

2.1.1 Sterilization

The success of micropropagation depends on prevention or elimination of microbial contamination in *in vitro* cultures which can be achieved through proper surface sterilization (Mondal, 2003). Haldeman *et al.*, (1987) showed significant reduction in bacterial and fungal contaminations in tea explants with 24-hour intermediate culture of the traditionally disinfected materials in liquid medium containing benomyl at 1, 2 or 4 gms/l and rifampicin at 10, 25 or 50 mg/l without any phytotoxic effects. Wala and Jasrai (2003) treated the explants with chloramphenicol (0.5%) and bavistin (0.25%) for 15-18 hrs prior to surface sterilization through the traditional method to avoid microbial contamination in *Cuculigo orchioides*. Das and Barman (1988) treated the tea explants with 0.1% mercuric chloride for 5-10 minutes and streptomycin sulphate for 1 minute prior to inoculation in order to prevent contamination. They also observed phytotoxic effect in the explants at higher concentration of mercuric chloride. Phukan and Mitra (1983) used apical buds from *in vitro* seedlings of *Albizia odoratissima* to avoid the problem of contamination. Later in 1992, Phukan used tissues of the transitional area between the hypocotyl and radicle of the sterilized seeds of *Albizia chinensis* as explant for multiple shoot proliferation to get rid of microbial contaminations.
Explants taken from field grown *Persoonia* spp., could not be freed from microbial contamination using all available sterilization techniques and the tissues were damaged due to leaching of toxic components to the culture medium from the explant (Anon., 1992). For micropropagation, the embryo culture technique was, therefore, opted as an alternative method for establishment of the explants in the culture media. Reports are also available on the use of greenhouse grown plants for isolation of explants to avoid contamination. Ogutuga and Northcote (1970) used 5-15mm stem segments from the green house-grown tea plant and surface sterilized with 70% ethanol followed by 7% sodium hypochlorite solution. Kato (1985 and 1989) took stems with 3-4 leaves from the green house-grown seedlings (cultivar Yabukita) and sterilized with 7% calcium hypochlorite for 20 minutes. Neidz and Bausher (2002) also found it easier to control contamination in the explants of greenhouse grown plants of Citrus plants as compared to the explants from field-grown trees. Ferrador *et al.* (2003) also reported about the heavy contamination of explants derived from the field grown *Castanea sativa* plants and could successfully minimize it using explants from green house plants.

2.1.2 Effect of hormones for multiple shoot proliferation

Multiple shoot proliferation in the presence of only a cytokinin like 6-benzyl aminopurine (BAP) was reported in leguminous trees like *Albizia odoratissima* (Phukan and Mitra, 1983), *Albizia chinensis* (Phukan, 1992), *Bauhinia variegata* and *Parkinsonia aculeata* (Mathur and Mukunthakumar, 1992), *Albizia falcatoria* (Sinha and Mallick, 1993), *Dalbergia sissoo* (Pradhan *et al.*, 1998b) and *Pterocarpus marsupium* (Anis *et al.*, 2005).

The concentration of BAP required for multiple shoot proliferation of the leguminous trees varied from species to species. Phukan and Mitra (1983) reported a concentration of BAP 1.0 mg/l for shoot proliferation of *Albizia odoratissima*. For *in vitro* shoot proliferation of *Albizia chinensis* a concentration of BAP 1.0 mg/l was required. In *Bauhinia variegata* and *Parkinsonia aculeata*, shoot proliferation was achieved using medium supplemented with 2.22-31.1 μM BAP. Again in *Albizia falcatoria*, BAP concentration required was 4.4 μM and 8.9 μM. In *Dalbergia sissoo*, high frequency shoot proliferation and maximum number of shoots were recorded with BAP at
an optimum level of 8.9 μM. In *Pterocarpus marsupium*, a BAP concentration of 5 μM was required for shoot proliferation. Similar findings where optimal shoot growth was obtained in the presence of only BAP was also reported in other non-leguminous species like *Tamarix gallica* (Lucchesini et al., 1993), *Berberis trifoliata* (Mackay et al., 1996), *Bambusa vulgaris* (Ndiaye et al., 2006) and *Citrus limon* (Rathore et al., 2007). In the non-leguminous species also, the BAP concentration used was different for different species. In *Tamarix gallica*, shoot proliferation was induced in a medium containing 3.3 μM 6-benzyl aminopurine. In *Berberis trifoliata*, the optimum level of BAP concentration required for shoot proliferation was 5.5 μM BAP. In *Bambusa vulgaris*, shoot proliferation was obtained in a medium containing 2.0 mg/l BAP whereas in *Citrus limon*, the BAP concentration required was 9 μM.

Shoot proliferation in other leguminous trees like tamarind (Mehta et al., 2000), *Albizzia lebbeck* (Varghese and Kaur, 1988), *Dalbergia latifolia* (Pradhan et al., 1998a), *Acacia seyal* (Al-Wasel-, 2000) and *Dalbergia sissoo* (Singh et al., 2002) were reported to be achieved in combination of BAP with Naphthalene acetic acid (NAA).

The efficacy of BAP in combination with other cytokinins or auxins for induction of in vitro shoot proliferation was also reported in other perennial woody plants like tea (Das and Barman, 1988), floribunda, ground cover and miniature roses (Douglas et al., 1989), apple (Belaizi et al., 1991), *Commiphora wightii* (Barve and Mehta, 1993), *Betula pendula* (Leege and Tripepi, 1993), *Petroselinum crispum* (Vandermoortele et al., 1996), *Citrus aurantifolia* (Al-Khayri and Al-Bahrany, 2001), *Salvadora persica* (Mathur et al., 2002), *Balanites aegyptiaca* (Ndoye et al., 2003), *Tectona grandis* (Shirin et al., 2005) and *Phellodendron amurense* (Azud et al., 2005).

Reports are also available on multiple shoot proliferation in the presence of other cytokinins. It was also reported that kinetin along with NAA are the appropriate requirement of auxin and cytokinin for shoot proliferation of leguminous trees like *Prosopis cineraria* (Goyal and Arya, 1981). In *Bauhinia vahlii* (Bhatt and Dhar, 2000), shoot proliferation was obtained in media supplemented with both kinetin and thiadiazuron (TDZ).
The presence of only kinetin was also reported to be the best condition for shoot proliferation in non-leguminous species like *Ficus carica* (Fraguas et al., 2004) and *Sesbania sesban* (Jha et al., 2003). There are reports on shoot proliferation in *Myrica esculenta* (Bhatt and Dhar, 2004) and *Cordia verbenacea* (Lameira and Pinto, 2006) in media with a combination of kinetin and NAA.

In certain species like *Psoralea corylifolia*, a two step method for shoot proliferation was followed (Baskaran and Jayabalan, 2008). In the first step induction of multiple shoot proliferation from apical bud explants was obtained in the presence of 5 μM benzylamino purine and 5 μm thidiazuron (TDZ) from apical buds explants of 1 week old cultures. The shoot buds were isolated and re-cultured in the medium supplemented with 2 μm benzylamino purine, 1 μm thidiazuron and 100 mg/l bavistin in second step which was effective in producing greater number of shoots per explants after 4 weeks of culture.

### 2.1.3 Effect of basal media for multiple shoot proliferation


Regeneration of shoots from leguminous trees like *Albizia amara*, *Albizia lucida* and *Albizia richardiana* (Tomar and Gupta, 1988), *Albizia julibrissin* (Sankhla et al., 1993) could be achieved in B5 medium.

The efficacy of MS media for better shoot proliferation was also reported in other non-leguminous woody species, such as tea (Das and Barman, 1988), floribunda, ground cover and miniature roses (Douglas et al., 1989), *Commiphora wightii* (Barve and Mehta, 1993), *Citrus aurantifolia* (Al-Khayri and Al-Bahrany, 2001), *Salvadora persica* (Mathur et al., 2002), *Balanites aegyptiaca* (Ndoye et al., 2003), *Tectona grandis* (Shirin
et al., 2005), Phellodendron amurense (Azad et al., 2005), Bambusa vulgaris (Ndiaye et al., 2006) and Citrus limon (Rathore et al., 2007).

2.1.4 Choice of explants for multiple shoot proliferation

Choice of the organ of the experimental material to use as explant after successful proliferation of microshoot is very important. Shoot proliferation from apical buds of the leguminous shade tree Albizia odoratissima (Phukan and Mitra, 1983) was obtained earlier. In 1992, Phukan obtained multiple shoot proliferation in Albizia chinensis from tissues of the transitional area between the hypocotyl and radicle of the sterilized seeds of Albizia chinensis. In Albizia lebbeck, on the other hand, Varghese and Kaur (1988) obtained proliferated shoots from the hypocotyl, cotyledon, root, leaf and rachis segments.

There are many reports on the use of hypocotyl, cotyledon, zygotic embryos, meristem, leaf etc. as explants for successful shoot regeneration in other leguminous trees like Prosopis cineraria (Goyal and Arya, 1981), tamarind (Mascarenhas et al., 1981; Mascarenhas et al., 1987; Mehta et al., 2000), Albizia amara, Albizia lucida and Albizia richardiana (Tomar and Gupta, 1988), Prosopis tamarugo (Nandwani and Ramawat, 1992), Albizia julibrissin (Sankhla et al., 1993), Albizia falcataria (Sinha and Mallick, 1993), Dalbergia latifolia (Pradhan et al., 1998a), Dalbergia sissoo (Pradhan et al., 1998b; Singh and Chand, 2003; Pattnaik et al., 2000; Singh et al., 2002), Acacia farnesiana and Acacia schauffneri (Cañedo Ortiz et al., 2000), Acacia mangium (Xie and Hong, 2001), Acacia sinuata (Vengadesan et al., 2002) and Bauhinia vahlii (Upreti and Dhar, 1996).

Successful in vitro mass propagation of Acacia seyal (Al-Wasel-, 2000) was possible using seedling shoot tips as explants. In Acacia chundra, Rout et al. (2008) obtained multiple shoots using shoot tip and nodal segments as explants. Successful shoot proliferation from apical buds of other non-leguminous species like floribunda, ground cover and miniature roses (Douglas et al., 1989), Penstemon serrulatus (Wysokinska, 1993), chickpea (Barna and Wakhlu, 1995), Morus nigra (Yadav et al., 1998), and Cordia verbenacea (Lameira and Pinto, 2006) was also obtained earlier.
Suitability of internodal segments as explants for successful \textit{in vitro} shoot proliferation of non-leguminous species like apple (Belaizi \textit{et al.}, 1991), \textit{Cephaelis ipecacuanha} (Yoshimatsu \textit{et al.}, 1991), \textit{Sesbania sesban} (Jha \textit{et al.}, 2003) and \textit{Scabiosa caucasia} (Hosoki \textit{et al.}, 2004) was observed and reported.

\textbf{2.1.5. \textit{In vitro} rooting}

In many leguminous tree species viz. \textit{Albizia odoratissima} (Phukan and Mitra, 1983), \textit{Albizia chinensis} (Phukan, 1992), \textit{Bauhinia variegata} and \textit{Parkinsonia aculeata} (Mathur and Mukunthakumar, 1992) and \textit{Bauhinia vahlii} (Uperti and Dhar, 1996; Bhatt and Dhar, 2000) and \textit{Dalbergia sissoo} (Singh \textit{et al.}, 2002) \textit{in vitro} rooting of the microshoots could be induced with the supplementation of a single auxin like naphthalene acetic acid (NAA), indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA), etc. in either of the MS, B\textsubscript{5} or White’s basal medium.

In \textit{Albizia lebbeck} (Varghese and Kaur, 1988), rooting was obtained in MS medium supplemented with BAP and NAA. In \textit{Dalbergia latifolia} (Pradhan \textit{et al.}, 1998a), regenerated shoots were rooted on MS (half) media supplemented with IAA, IBA and IPA. In tamarind (Mascarenhas \textit{et al.}, 1981; Mascarenhas \textit{et al.}, 1987; Mehta \textit{et al.}, 2000) and \textit{Dalbergia sissoo} (Pradhan \textit{et al.}, 1998b; Pattnaik \textit{et al.}, 2000), \textit{in vitro} rooting was reported in the combination of two or more auxins like IBA, IAA, IPA and NAA. In \textit{Prosopis cineraria} (Goyal and Arya, 1981), rooting could be induced in White’s medium supplemented with kinetin and IBA.

The single requirement of an auxin for \textit{in vitro} rooting were also reported in other woody species like floribunda, ground cover and miniature roses (Douglas \textit{et al.}, 1989), \textit{Tamarix gallica} (Lucchesini \textit{et al.}, 1993), \textit{Citrus aurantifolia} (Al-Khayri and Al-Bahenary, 2001), \textit{Salvadora persica} (Mathur \textit{et al.}, 2002), \textit{Myrica esculenta} (Bhatt and Dhar, 2004), \textit{Tectona grandis} (Shirin \textit{et al.}, 2005), \textit{Phellodendron amurense} (Azad \textit{et al.}, 2005), \textit{Bambusa vulgaris} (Ndiaye \textit{et al.}, 2006) and \textit{Citrus limon} (Rathore \textit{et al.}, 2007). The presence of IAA in the medium induced rooting in floribunda, ground cover, miniature roses and \textit{Citrus aurantifolia}. Again, IBA promoted rooting in \textit{Tamarix gallica}, \textit{Salvadora persica}, \textit{Phellodendron amurense} and \textit{Bambusa vulgaris}. NAA helped in rooting of \textit{Myrica esculenta}, \textit{Tectona grandis} and \textit{Citrus limon}. In these studies different
basal medium viz., Linsmaier and Skoog, MS and Woody Plant medium (WPM) were used where the above auxins were supplemented.

In some woody species like *Commiphora wightii* (Barve and Mehta, 1993) and *Pinus taeda* (Tang, 2001), a combination of hormones was required for induction of *in vitro* rooting of the micropropagated shoots. In *Commiphora wightii*, a combination of IAA and IBA could induce rooting. In *Pinus taeda*, adventitious shoots rooted on media supplemented with IBA, BAP and GA₃.

However, in *Cordia verbenacea* (Lameira and Pinto, 2006), *in vitro* rooting was obtained in MS basal medium without any growth regulators.

The efficacy of half MS basal media for *in vitro* rooting of leguminous trees was also studied in *Dalbergia sissoo* (Singh et al., 2002; Pradhan et al., 1998b; Pattnaik et al., 2000). *Bauhinia vahlii* (Upreti and Dhar, 1996). *Dalbergia latifolia* (Pradhan et al., 1998a). and tamarind (Mascarenhas et al., 1981; Mascarenhas et al., 1987; Mehta et al., 2000). Singh et al., (2002) could induce rooting from regenerated shoots derived from cotyledons of *Dalbergia sissoo* in half MS media supplemented with IBA. However, Pradhan et al. (1998b) and Pattnaik et al. (2000) could induce rooting in *Dalbergia sissoo* in half MS medium containing a combination of hormones like IAA, IBA and IPA. In micropropagation of *Bauhinia vahlii*, rooting of the regenerated shoots was achieved on half MS media supplemented with NAA. In *Dalbergia latifolia*, shoots derived from cell-suspension-derived callus were rooted on half MS media containing IAA, IBA and IPA. In tamarind, shoots developed roots on half MS liquid media supplemented with 2% sucrose following a 72 hour treatment with 2 ppm each of IAA, IBA, IPA and NAA.

Rooting success in half MS basal media was also reported in non-leguminous woody species like *Commiphora wightii* (Barve and Mehta, 1993), *Salvadora persica* (Mathur et al., 2002) and *Citrus limon* (Rathore et al., 2007). In *Commiphora wightii*, the presence of both IAA and IBA in half MS medium could induce rooting. In *Salvadora persica*, only IBA was necessary for rooting and in *Citrus limon*, rooting was obtained on half MS media supplemented with NAA. In *Pinus taeda* (Tang, 2001) rooting was induced in TE medium (Tang et al., 1998) supplemented with IBA, BAP and GA₃.
2.1.6 Hardening of the *in vitro* derived plantlets

Among leguminous trees, successful hardening and establishment of *in vitro* derived plantlets have so far been reported in *Albizia lebbeck* (Varghese and Kaur, 1988), *Prosopis cineraria* (Goyal and Arya, 1981), *Dalbergia latifolia* (Pradhan et al., 1998a), *Dalbergia sissoo* (Pradhan et al., 1998b; Singh and Chand, 2003), *Bauhinia variegata* and *Parkinsonia aculeata* (Mathur and Mukunthakumar, 1992), *Bauhinia vahlii* (Bhatt and Dhar, 2000), tamarind (Mehta *et al.*, 2000) and *Acacia mangium* (Xie and Hong, 2001). In hardening of the *in vitro* derived plantlets of *Albizia lebbeck*, sterile mixtures of sand, soil and farm yard manure (1:1:1) was used and irrigated regularly with nutrient solution (half MS) at regular intervals. In *Prosopis cineraria*, establishment of the plantlets was obtained in a mixture of sterile soil and vermiculite (3:1). In *Albizia lebbeck* and *Prosopis cineraria*, the plantlets which were covered initially with 1000 ml beaker to maintain humidity. In *Dalbergia latifolia*, hardening of the plantlets was established by transferring the rooted plantlets to vermi-compost. In *Dalbergia sissoo*, Pradhan *et al.* (1998b) transferred rooted plantlets to vermi-compost which were kept in a plant growth chamber for 3 weeks, followed by 2 weeks in shade and eventually established outdoors under full sun. Again, Singh and Chand (2003) kept rooted plantlets of *Dalbergia sissoo* in half and one-fourth liquid MS medium, each for 10 days, and then to plastic pots containing autoclaved peat moss and compost mixture (1:1). In case of *Bauhinia variegata* and *Parkinsonia aculeata*, hardening was accomplished by washing plants with well-developed roots free of agar and then planting them in soil-filled pots. In *Bauhinia vahlii*, plantlets were successfully acclimatized in 90 g (w/v) soilrite, sand and soil (2:1:1) in the shed house. In *Acacia mangium*, hardening was carried out by transferring rooted plantlets in pots containing peat : sand (3:1, v/v). In tamarind, plantlets were transferred to pots containing 3 parts soil to 1 part sand and covered with glass sheets in the hardening room before transferring to the greenhouse.

Success in hardening was also reported in other perennial woody species like tea (Konwar *et al.*, 1999), *Pinus taeda* (Tang, 2001), *Salvadora persica* (Mathur *et al.*, 2002), *Myrica esculenta* (Bhatt and Dhar, 2004), *Tectona grandis* (Shirin *et al.*, 2005) and *Eucalyptus tereticornis* (Prakash and Gurumurthi, 2005). In tea, success in hardening was obtained in a mixture of soil and sawdust. In *Pinus taeda* acclimatization was achieved in
a mixture of perlite, peat moss and vermiculite (1:1:1). Better success in hardening was reported in soil and vermiculite (3:1) mixture in *Salvadora persica*. Hardening in a mixture of soil, soilrite and sand (1:1:1) was also carried out in *Myrica esculenta*. In hardening of *Myrica esculenta* plantlets, the pots were covered with glass beakers or polyethylene bags to maintain humidity. In *Tectona grandis* acclimatization could be achieved in a mixture of soil, sand and farmyard manure (1:1:1). In *Eucalyptus tereticornis*, acclimatization of the plantlets was carried out in a mixture of soil, sand and farmyard manure (1:1:1).

2.2 Callus induction, somatic embryogenesis and organogenesis

Organogenic differentiation is an outcome of the process of dedifferentiation followed by redifferentiation of cells. Dedifferentiation favours unorganized cell growth and the resultant developed callus has meristems randomly divided. The callus then redifferentiates to develop into a complete plantlet (Razdan, 1993).

Somatic embryogenesis is the process of a single cell or a group of cells initiating the developmental pathway that leads to reproducible regeneration of non-zygotic embryos capable of germinating to form complete plants. Under natural conditions this pathway is not normally followed, but from tissue cultures somatic embryogenesis occurs most frequently and as an alternative to organogenesis for regeneration of whole plants (Razdan, 1993).

Among tree legumes, direct/indirect somatic embryogenesis has been reported in *Acacia koa* (Skoleman, 1986), *Cerecis canadensis* (Trigiano et al., 1988), *Robinia pseudoacacia* (Arrillaga et al., 1994), *Dalbergia latifolia* (Rao and Sita, 1996), *Hardwickia binata* (Chand and Singh, 2001), *Acacia mangium*, (Xie and Hong, 2001) and *Acacia sinuata* (Vengadesan et al., 2002).

Organogenesis from callus cultures of leguminous trees has so far been reported in *Prosopis tamarugo* (Nandwani and Ramawat, 1992) and *Dalbergia latifolia* (Pradhan et al., 1998a).
2.2.1 Effect of basal media and hormones for callus induction, somatic embryogenesis and organogenesis

Reports are available for callus induction in MS based medium supplemented with 2,4-Dichlorophenoxy acetic acid (2,4-D) and kinetin in other leguminous trees like *Acacia farnesiana* and *Acacia schaffneri* (Cañedo Ortiz et al., 2000), *Acacia mangium* (Xie and Hong, 2001) and *Dalbergia sissoo* (Singh and Chand, 2003). Callus was also obtained in *Prosopis tamarugo* (Nandwani and Ramawat, 1992), *Dalbergia latifolia* (Pradhan et al., 1998a) and *Dalbergia sissoo* (Pattnaik et al., 2000) on MS medium supplemented with NAA and BAP. In *Acacia sinuata* (Vengadesan et al., 2002) callus was induced in MS medium supplemented with 2,4-D and BAP.

Better callus induction in MS basal media supplemented with a combination of 2,4-D and kinetin was also reported in other woody species like tea (Das and Barman, 1988) and *Cephalotaxus harringtonia* (Wickremensinhe and Arteca, 1993).

Induction of callus in MS medium supplemented with only 2,4-D was achieved in non-leguminous species like *Parkia biglobosa* (Oluwaseun and Erhinmeyoma, 2005), rice (Islam et al., 2005), *Heliconia psittacorum*, *Strelitzia reginae* and *Camellia japonica* (Bora, 1998). In *Eucalyptus tereticornis* (Prakash and Gurumurthi, 2005), callus was induced in MS medium supplemented with NAA.

It was reported in *Plumbago rosea* (Das and Rout, 2002) that callus induction was achieved in MS medium supplemented with kinetin and NAA. Hoque et al. (2007) could induce callus in water chestnut using a combination of BAP, NAA and GA₃ in the medium.

In *Phyllanthus amarus* (Unander, 1991) callus was induced in MS media supplemented with BAP, 2,4-D or IBA. Callus induction in MS media supplemented with BAP and 2,4-D or only IBA was also reported in *Pappea capense* (Mng’omba et al., 2008). In *Mangifera indica* (Laxmi et al., 1999) callus induction was obtained in MS (full) media supplemented with BAP. In *Azadirachta indica* (Khalafalla et al., 2007), callus was induced in both half and full MS media supplemented with only IBA or in combination with BAP.
Induction of somatic embryogenesis in the presence of IAA alone or in combination with other hormones was reported in *Solanum carolinense* L. (Reynolds, 1987), banana (Ganapathi et al., 1999) and geranium (Slimmon et al., 1991). In potato (Rihova and Tupy, 1996), *Anacardium occidentale* (Ananathakrishnan et al., 1999), cassava (Sofiari et al., 1997) and wheat (Mahalakshmi et al., 2003), somatic embryogenesis was obtained in 2,4-D supplemented medium. In leguminous trees like *Dalbergia latifolia* (Rao and Sita, 1996) and *Hardwickia binata* (Chand and Singh, 2001) direct embryogenesis was obtained in MS medium supplemented with 2,4-D.

In *Eucalyptus globulus*, Nugent et al., (2001) observed somatic embryogenesis in the MS medium with only IBA. While, Zhang et al., (2005), could induce somatic embryogenesis of Golden Pothos in MS medium supplemented with TDZ and NAA and in MS medium containing kinetin and 2,4-D.

In *Plumbago rosea* (Das and Rout, 2002), somatic embryogenesis was achieved when MS medium was supplemented with kinetin, GA₃ and NAA. In *Lactuca sativa*, somatic embryogenesis was induced in MS medium containing 6-benzyl amino purine and NAA (Zhou et al., 1992). Canhoto et al., (2006), obtained somatic embryogenesis of *Ceratonia siliqua* on MS medium supplemented with BAP and IBA.

### 2.2.2 Choice of explant for somatic embryogenesis and organogenesis

Explants derived from seed organs were found to be suitable for somatic embryogenesis. Reports are available for induction of somatic embryogenesis/organogenesis from cotyledons of leguminous trees like *Prosopis tamarugo* (Nandwani and Ramawat, 1992), *Albizzia falcataria* (Sinha and Mallick, 1993), *Bauhinia vahlii* (Upreti and Dhar, 1996), *Albizzia julibrissin* (Burns and Wetzstein, 1998) and *Dalbergia sissoo* (Pradhan et al., 1998b; Singh and Chand, 2003; Singh et al., 2002). Similar results of somatic embryogenesis/organogenesis from cotyledons of other species like tea (Das and Barman, 1988), *Solanum aviculare* (Alizadeh et al., 1991), *Lactuca sativa* (Zhou et al., 1992), *Coffea arabica* and *Coffea canephora* (Giridhar et al., 2004) *Quassia amara* (Martin and Madassery, 2005), *Parkia biglobosa* (Oluwaseun and Erhinmeyoma, 2005) and *Ceratonia siliqua* (Canhoto et al., 2006) have also been reported.
2.2.3 Maturation and germination of somatic embryos

The importance of ABA (Cañedo Ortiz et al., 2000) and BAP (Rao and Sita, 1996; Jia and Chua, 1992; Compton and Gray, 1993) for further maturation and germination was reported. Faster maturation of somatic embryos of *Dalbergia latifolia* (Rao and Sita, 1996) was achieved in BAP supplemented MS medium. The efficacy of BAP along with auxins like NAA and ABA for maturation and germination of somatic embryos was also reported in other leguminous trees like *Tamarindus indicus* (Mehta et al., 2000) and *Hardwickia binata* (Chand and Singh, 2001). In leguminous trees like *Acacia farnesiana* and *Acacia schaffneri* (Cañedo et al., 2000), maturation of the embryos was achieved in ABA supplemented MS media. In *Dalbergia sissoo* (Singh and Chand, 2003), better maturation of embryos was reported in hormone free MS media.

In other non-leguminous species like *Quassia amara* (Martin and Madassery, 2005), *Eucalyptus tereticornis* (Prakash and Gurumurthi, 2005) and *Mangifera indica* (Laxmi et al., 1999) also maturation of somatic embryos could be achieved by culturing in MS (half) media supplemented with BAP. In *Fraxinus excelsior* (Capuana et al., 2007), however, maturation of the somatic embryos was done through culturing in the MS medium supplemented with 2,4-D and BAP.

There are reports on the development of plantlets through somatic embryogenesis in leguminous trees like *Dalbergia latifolia* (Rao and Sita, 1996), *Acacia farnesiana* and *Acacia schaffneri* (Cañedo Ortiz et al., 2000) and *Dalbergia sissoo* (Singh and Chand, 2003). Reports on somatic embryogenesis of other perennials, such as tea (Das and Barman, 1988) and *Fraxinus excelsior* (Capuana et al., 2007) are also available. In *Dalbergia latifolia*, somatic embryogenesis was obtained in MS medium supplemented with 2,4-D and kinetin. MS media supplemented with BAP was found to be effective for maturation and conversion of the somatic embryos to whole plantlets. In *Acacia farnesiana* and *Acacia schaffneri*, callus was induced in MS medium supplemented with 2,4-D and kinetin. Greatest number of somatic embryos were produced in media without growth regulators. But addition of ABA to the media helped the embryos to reach more advanced differentiation stages. Somatic embryos germinated on media containing adenine sulfate. In *Dalbergia sissoo*, callus was achieved on MS media supplemented with 2,4-D and kinetin and embryogenesis was achieved on transfer of the calli to half MS.
media without growth regulators. Somatic embryos converted into plantlets on half MS media containing 2% sucrose. In tea, embryogenic calli was achieved on media containing 2,4-D and kinetin. In *Fraxinus excelsior*, somatic embryogenesis was obtained on MS media supplemented with 2,4-D and BAP. Somatic embryos developed and matured in hormone free media and subsequent culture on medium containing BAP.

2.2.4 Prevention of oxidation of polyphenols and their leaching to the culture media

High polyphenol content of the explants and its leaching to the media make the media toxic and restricts the growth of cultured tissues. Therefore, prevention of leaching and oxidation of phenolic substances is very important for success of *in vitro* studies. Prevention of oxidation of polyphenols with ascorbic acid during *in vitro* experiments was reported by Konwar *et al.* (1999) in tea. Similarly the efficacy of PVP in the prevention of browning of explants was proved in *Tectona grandis* (Gupta *et al.*, 1980), guava (Amin and Jaiswal, 1988), *Cleistanthus collinus* (Quraishi and Mishra, 1988) and *Myrica esculenta* (Bhatt and Dhar, 2004).

2.3 DNA extraction

The effectiveness of CTAB for DNA isolation was showed for several species like cranberry, chestnut and peanut (Stewart and Via, 1993), *Pinus radiata* (Stange *et al.*, 1998), *Vitex pubescens*, *Nervilia aragoana*, *Gymnema sylvestre*, *Withania somnifera*, *Origanum majorana*, *Boswellia serrata*, *Saraca asoca* and *Gloriosa superba* (Padmalatha and Prasad, 2006).

It was shown that polysaccharides distort the results in many analytical applications and therefore, lead to a wrong interpretations (Kotchni *et al.*, 2003). Fang *et al.* (1992) reported that polysaccharides interfere with the polymerase chain reaction (PCR) by inhibiting *Taq* polymerase activity. Polysaccharide co-precipitation is avoided by adding a selective precipitant of nucleic acids, i.e. (CTAB) to keep polysaccharides in solution through SDS (Dellaporta *et al.*, 1983).

The presence of polyphenols, which are powerful oxidizing agents present in many plant species, can reduce the yield and purity by binding covalently with the
extracted DNA making it useless for most research applications (Katterman and Shattuck, 1983; Peterson et al., 1997; Porebski et al., 1997). Addition of PVP along with CTAB may bind to the polyphenolic compounds by forming a complex with hydrogen bonds and may help in removal of impurities to some extent (Padmalatha and Prasad, 2006).

Long term chloroform:isoamylalcohol treatment ensured removal of chlorophyll and other colouring substances such as pigments, dyes, etc. during DNA isolation procedures (Padmalatha and Prasad, 2006).

Many DNA isolation procedures yield large amounts of RNA, especially 18S and 25S rRNA (Doyle and Doyle, 1987; Padmalatha and Prasad, 2006). Large amounts of RNA in the sample can chelate Mg$^{2+}$ and reduce the yield of the PCR and therefore, a RNAse treatment is necessary that degraded RNA into small ribonucleosides that do not contaminate the DNA preparation and yield RNA-free pure DNA (Padmalatha and Prasad, 2006).

### 2.3.1 Optimization of RAPD protocol

Optimization of amplification can be achieved by varying PCR reaction parameters like DNA concentration, primer concentration, enzyme concentration, MgCl$_2$ concentration, dNTP concentration, and PCR cycle conditions and the effects of these variations can be monitored by examining the intensity and distribution of bands after electrophoresis on agarose (Munthali et al., 1992; Padmalatha and Prasad, 2006). In *Robinia pseudoacacia* (Bindiya and Kanwar, 2003) amplification was performed using 50 ng of genomic DNA with a PCR run for 45 cycles and the amplified products were separated in a concentration of 1.8 % agarose gel. In *Prosopis glandulosa* and *Prosopis velutina* (Bessega et al., 2000) amplifications were carried out using 50 ng of genomic DNA and with 40 PCR cycles and then the amplified products were resolved in 1.4% agarose gels. In *Tectona grandis* (Gangopadhyay et al., 2003), reactions were continued for 45 cycles using 100 ng of template DNA.

### 2.3.2 RAPD analysis

Genetic molecular markers are considered to be reliable in monitoring variability in the DNA sequences of plants. Several authors have applied the RAPD technique for investigation of genetic variability and it was found to be very efficient as
well as reliable (Brown et al., 1993; Munthali et al., 1996). Munthali et al. (1996) compared the results obtained from RAPD analysis with those obtained with restriction fragment length polymorphism (RFLP) and isozymes and found no difference in their results. Using RAPD technique, various authors have reported the absence of genetic variation in micropropagated plants like *Picea mariana* (Isabel et al., 1993), *Festuca pratensis* (Vallés et al., 1993), *Picea abies* (Heinze and Schmidt, 1995) *Pinus thunburghii* (Goto et al., 1998), *Acacia mangium* (Nanda et al., 2004), chestnut (Carvalho et al., 2004), almond (Martins et al., 2004) *Arachis retusa* (Gagliardi et al., 2007) and banana (Lakshmanan et al., 2007). However, some investigators, have shown genetic variations through RAPD analysis in the micropropagated bananas and plantains (Schoofs, 1992), *Populus deltoides* (Rani et al., 1995), *Beta vulgaris* (Munthali et al., 1996), Peach (Hashmi et al., 1997), Robinia pseudoacacia (Major et al., 1998), Angelica acutiloba (Watanabe et al., 1998), Populus tremuloides (Rahman and Rajora, 2001), tea (Mondal and Chand, 2002), Robinia pseudoacacia (Bindiya and Kanwar, 2003), Tectona grandis (Gangopadhyay et al., 2003) and ornamental pineapple (Santos et al., 2008).