Chapter 5 - DISCUSSION

*Pterocarpus marsupium* is a high value tree species with immense commercial potential. Attempts have been made in the past to propagate this species from seedlings. Present investigation describes the *in vitro* propagation of *Pterocarpus marsupium* from nodal segments. *In vitro* regeneration protocols have been reported in *P. marsupium* using different explant sources including cotyledonary nodes, nodes and hypocotyl (Chand and Singh, 2004; Tiwari *et al*., 2004; Anis *et al*., 2005; Husain *et al*., 2007, 2008, 2010).

Present study deals with important factors influencing shoot proliferation, shoot multiplication, *in vitro* and *ex vitro* rooting, hardening of *P. marsupium*. *In vitro* studies attempted through axillary shoot proliferation were mainly focused on i) Shoot proliferation: effect of plant growth regulators, different basal media, season, effect of Additives, ii) Shoot multiplication: effect of plant growth regulators, effect of different basal medium, additives, carbon source and concentration, medium strength, semi-solid and liquid medium, subculture cycle, propagule Size, pH of the medium iii) *In vitro* rooting: Effect of auxins, strength of MS medium, iv) *Ex- vitro* rooting.

**AXILLARY BUD PROLIFERATION**

Micropropagation as a part of biotechnology, can contribute to genetic improvement and propagation of improved planting stock material. Plant tissue culture offers the unique opportunity not only for realizing totipotency of cells into whole plants but also provide conditions under which physiological manipulations can be carried out with the objective of overcoming endogenous controls inherent in the intact plants. It comprises a range of technologies integral to both commercial plant production and research investigation leading towards genetic manipulation and genetic engineering (LilaSmith, 1994, Zimmerman, 1996). World wide there have numerous efforts to effectively utilize plant tissue culture for commercial propagation of plants. Micropropagation through nodal segments is a method which could help in the regeneration of large number of plants in a relatively short time.
The issue at stake has been rigorously discussed by several reviewers (Farnum et al., 1983; Powledge, 1984; Bajaj, 1986; Burley, 1987; Haissing et al., 1987; Boulay, 1987; Krikorian, 1988; Fraclet and Boulay, 1989; Mascarenhas and Murlidhran, 1989; Bhojwani, 1990; Thorpe, 1990, 1991; Hammatt, 1992; Gupta et al., 1993).

In vitro propagation from mature trees has always been difficult due to several inherent problems, such as the establishment of aseptic cultures, severe microbial contamination, seasonal morphogenic variations, and browning of explants due to accumulation of secondary metabolites (Rathore et al., 1992; Shekhawat et al., 1993; Purohit and Kukda, 1994; Quraishi and Mishra, 1998; Agarwal et al., 2002). The present investigation was undertaken with a view to improve the methods of propagation by developing an efficient, reproducible, reliable and routinely available technology for micropropagation.

The present study deals with the application of plant tissue culture technology for the micropropagation of *Pterocarpus marsupium*. The results obtained are discussed stepwise in the light of existing literature. In the present study for *in vitro* propagation of *Pterocarpus marsupium*, plant material was collected from AFRI nursery, Jodhpur.

Heavy bacterial and fungal contamination of nodal segments collected from tree was one of the major problems in the *in vitro* propagation of *Pterocarpus marsupium*. Out of various sterilizing agents tried 0.1% HgCl$_2$ for 7 min. proved to be most effective treatment for sterilization of nodal segments and gave maximum aseptic bud break. These results are in agreement with those of other workers who found 0.1% HgCl$_2$ as a powerful sterilant for sterilization of nodal explants (Das and Rout, 1991 and 1994; Arya and Sharma, 1998; Mishra et al., 2001; Arya et al., 2002; Arya et al., 2006; Kappor and Rao, 2006).

Shoot bud differentiation in cultured tissue is dependent on the auxin/cytokinin ratio in the medium (Bonga and Von Aderkas, 1992). Presence of
cytokinin in the medium leads to the promotion of bud differentiation and development.

Comparing BAP and Kn, in the medium, Kn proved better than BAP alone for axillary bud proliferation of *P. marsupium*. MS medium supplemented with 13.95µM Kn, was found to be optimal for axillary shoot proliferation. Maximum bud break response of 64.44% was obtained with 13.95µM Kn in MS medium where 2.51 axillary shoots developed. Similar to our findings, Dhar and Uperti, (1994) found that Kn is better than BAP, TDZ and Zeatin in MS medium for shoot proliferation of *Bauhinia vahlii*. This result is contrasting with reports on leguminous species where BAP found to be superior to Kn as reported in *Dalbergia sissoo* Roxb. and *Macrotyloma uniflorum* (Arya et al., 2013 and Bhisht et al., 2013) where BAP gave maximum shoot proliferation. The efficiency of BAP for shoot proliferation is also reported in *Dendrocalamus asper* (Arya et al., 2002); *Dendrocalamus gigantenus* (Arya et al., 2006) and *Drepenaostachyum falatum* (Arya et al., 2008). The role of cytokinins in shoot differentiation from seedlings nodal explants has been emphasized in different *Acacia* species such as *A. nilotica* and *A. senegal* (Dewan et al., 1992; Badji et al., 1993).

Incorporation of auxins (NAA or IAA or IBA) with Kn intensified callusing in *P. marsupium*. Similar result reported in *A. nilotica* (Singh et al., 1993), where addition of auxins with cytokinin led to the basal callusing. Use of auxin in shoot induction medium is also known to induce callus and lowering the rate of induction in other leguminous tree species such as in *Dalber gia latifolia* (Lakshmisita and Raghavaswamy, 1992) from hypocotyls segments.

Nutritional medium plays a vital role in propagation through tissue culture. The suitability of basal media differs not only from one plant to another but also amongst different species of a single genus. It was revealed from the studies that nutrient media had significant effect on frequency of shoot initiation, number of shoots per explants and shoot length. MS medium proved to be the best and exhibited highest bud break (64.44%) with maximum number of shoots (2.51) and
shoot length (1.47 cm). B<sub>5</sub> medium proved least effective and exhibited low rate of bud break response of (37.78%) with 1.1 shoots. The findings are in agreement with earlier reports on many woody tree species including *Swartzia madegascariensis* and *Lagerstromia parviflora* (Berger and Schaffer, 1995; Tiwari et al., 2002). Whereas, Warakagoda and Subsinghe (2013) suggested that B<sub>5</sub> medium was superior to MS for plant regeneration of *Pterocarpus santalinus*.

It was found that the season in which the explants is collected had its profound effect on the bud break and culture establishment in *Pterocarpus marsupium*. The physiological condition of explants sources varies with season, which in turn influences *in vitro* response of explants. In the present study, based on the preliminary experiments, it was revealed that during March–May proved the best period for collection of nodal segments for high frequency shoot proliferation with minimum problem of contamination. Severe contamination problem was encountered during monsoon (June-August) and non availability of right stage of explants (dormant buds for axillary shoot proliferation) was a limiting factor in winter season. Seasonal effect on bud break in mature tree’s explants has also been reported in *Myrica esculenta* (Bhatt and Dhar, 2004), *Acacia sinuta* (Vengadesan et al., 2003) and *Sapium sebiferum* (Siril and Dhar, 1997).

Incorporation of additives to the shoot induction medium has resulted in significant enhancement in axillary shoot proliferation. Additives were incorporated in the medium to further improve the rate of response and quality of shoots. Results revealed that various additives (ascorbic acid, citric acid, ammonium sulphate and adenine sulphate) were added in MS medium help in controlling shoot tip necrosis as well as browning and leaching. Similarly a positive effect of ascorbic acid, citric acid and adenine sulphate was reported by Rathore et al., (1992) in *Maytenus emarginata*. Kuar et al., (1998) reported that addition of adenine sulphate showed a synergistic effect on shoot proliferation in *Acacia catechu*. Auxillary effect of adenine sulphate in MS medium with Kn has been observed by Dhar and Upreti (1999) in *Bauhinia vahlii* for proliferation of axillary bud. Similarly additives have been
successfully used for axillary shoot proliferation in *Tectona grandis* (Devi et al., 1994).

In accordance to our results adenine sulphate did not enhance shoot proliferation at higher concentration. Similar to our results Raghawaswamy et al., 1992, reported that high concentration of adenine sulphate is toxic in *Dalbergia latifolia*. This may be attributed to supra optimal concentration of adenine sulphate in the medium.

Sharma and Chandel (1992) found that addition of ascorbic acid to the growth regulator supplemented medium was essential for bud break in *Tylophora indica* and no bud break was observed in MS medium without ascorbic acid. Enhancement of bud break response upon inclusion of ascorbic acid may be due to some product of its oxidation, which might presumably had increased the shoot number through ascorbate protection of endogenous phytohormones as implicated for tobacco, *Pinus picea* (Berlyn and Beack, 1980; Richard et al., 1988). Auxillary effect of ascorbic acid and citric acid in the medium on bud break response, shoot number and length may be attributed to anti-oxidative properties of these chemicals.

Incorporation of ammonium sulphate in medium reduced hyperhydricity, shoot tip necrosis and promoted growth of shoots. Similar observations have also been made in *Melia azedarach* (Husain and Anis, 2009). Several lines of evidences also document the sulphate ions promote absorption of nitrate and buffered the culture medium (Ivanova and Staden, 2008; Kopriva et al., 2009).

**IN VITRO SHOOT MULTIPLICATION**

Shoot multiplication is the major criterion for successful micropropagation. The rate of multiplication is affected by numerous factors, such as physiological status of plant material, culture medium and culture environment. Exogenous requirements of plant growth regulators may be same as of the shoot proliferation medium or it may vary with subsequent passage during shoot multiplication stages.
In tissue culture one of the main functions of cytokinin is to release axillary buds from suppression by apical dominance thus initiating shoot proliferation. Cytokinin is essential for axillary bud proliferation and in vitro shoot multiplication.

Auxin and cytokinin source, concentration and effectiveness of these plant growth regulators either alone or in combination may vary with the plant species. In the present investigation various cytokinins i.e. BAP, Kn or TDZ were used either alone and in combination with auxins for in vitro shoot multiplication. Maximum rate of shoots multiplication was obtained in medium containing Kn, which was significantly higher than multiplication rate obtained in medium supplemented with BAP. BAP at higher concentrations resulted in high intensity of callusing from the shoot base, which has not only hindered shoot multiplication, but shoot length too. Medium consisted of Kn performed better than BAP and TDZ at all concentrations tested. Medium consisted of TDZ at all concentrations induced extensive callusing from the shoot base at all the concentrations tested. Intensity of callus was more at higher concentration of TDZ in the medium.

In contrast to our findings incorporation of BAP in medium resulted in high rate of shoot multiplication in *Aegele marmelos* (Ajeethkumar and Seeni, 1998). Huang *et al*., (1998) and (Arya *et al*., 2012) revealed that maximum rate of shoot multiplication in *Cinnamomum camphora* was on MS medium with BAP. Kumar *et al*., (2005) found maximum rate of shoot multiplication with BAP in MS medium in *Holarrhena antidysenterica*.

The inclusion of cytokinin and auxin in the culture media stimulated the in vitro multiplication and growth of shoots in several plant species (George, 1993). It was revealed that medium consisted of Kn with NAA was most effective with multiplication rate (3.11) with shoot length (1.67cm) in present study. The results substantiate with earlier findings of several workers, where the addition of low level of auxin with cytokinin promoted shoots in *Acacia catechu*, *Eucalyptus grandis* and *Lagerstromia parviflora* (Kaur *et al*., 1998; Luis *et al*., 1999; Tiwari *et al*., 2002).
Inclusions of higher concentration of auxin (IAA or NAA) into the cytokinin-rich medium inhibited not only shoot multiplication but also produced some compact callus at the base of the explants. Similar observations were made in *Acacia auriculiformis* (Mittal et al., 1989); *Pterocarpus santalinus* (Lakshmi Sita et al., 1992), and *Acacia mangium* (Nanda et al., 2014).

Incorporation of additives in shoot multiplication medium, favored auxillary effect on shoot growth in the cultures. Additives like; ascorbic acid, citric acid are used to improve the rejuvenation and rate of shoot multiplication and growth. Additves like ascorbic acid and citric acid in shoot multiplication medium favoured overall shoot number and growth with reduced brwoning problem from the culture of *Psedoxytenanthera stocksii* (Sanjaya et al., 2005).

The ascorbic acid is able to scavenge oxygen radicals produced when the plant tissue is wounded and hence protecting the cells from oxidative injury (Titov et al., 2006). Citric acid is used for establishment of cultures for cloning of woody plants has also been worked out by other researchers (Rathore et al., 1992; Shekhawat et al., 1993; Pandey et al., 2006). A positive effect of ascorbic acid, citric acid and adenine sulphate was observed by Rathore et al., (1992) in *Maytenus emarginata*. Auxillary effect of adenine sulphate in MS medium with Kn has been observed by Dhar and Upreti (1999) in *Bauhinia vahlilii* for establishment of cultures from nodal explants.

Combination of cytokinin and Adenine is favourable for shoot formation (Skoog and Miller, 1957; Pierik, 1987). In *Bauhinia vahlilii*, maximum shoot multiplication was achieved in MS medium supplemented with Kn along with AdS by (Dhar and Upreti, 1999). Vengadesan et al., (2003) achieved better shoot multiplication in A. *sinuata* by using adenine sulfate in the medium. Husain et al., 2006, also reported shoot tip necrosis of *Pterocarpus marsupium*, which is controlled by incorporation of ammonium sulphate and adenine sulphate in MS medium.
It is concluded from the study that additives like ascorbic acid, citric acid, ammonium sulphate and adenine sulphate have important role in \textit{in vitro} propagation of \textit{P. marsupium}. These additives promote auxillary effect with cytokinins in shoot proliferation and multiplication and its importance is well established.

Nutrient media has shown significant effect on shoot multiplication rate and growth. Different plant species usually vary in their nutritional requirements, therefore they respond differently to various basal media. Effect of various nutrient media viz. MS, B$_5$ and WPM were tested. It was observed in our study, that the shoot multiplication and shoot growth was better on MS medium as compared to WPM and B$_5$ medium. MS medium supports high rate of multiplication on account of it being rich in nitrogen and potassium containing compounds are lower in the other media types tested. The better growth of shoots of \textit{P. marsupium} on MS medium may be because of its higher mineral concentrations together with better interaction of its different constituents. These results are in agreement with the findings of other workers who have also noted the effectiveness of MS medium for optimal shoot multiplication.

In our results MS medium supplemented with 9.30 µM Kn + 0.54 µM NAA + additives proved to be best for \textit{in vitro} shoot multiplication. The results substantiate with earlier findings of several workers, where MS medium better in different bamboo species (Chamber \textit{et al.}, 1991; Das and Rout, 1991; Prutpongse and Gavinlertvatana, 1992; Chaturvedi \textit{et al.}, 1993; Arya and Arya, 1997; Arya and Sharma, 1998, Ramanayake \textit{et al.}, 2001; Arya \textit{et al.}, 2002; Das and Pal 2005; Rathore and Rai, 2005; Arya \textit{et al.}, 2006) and in Eucalyptus species, (Arya \textit{et al.}, 2009). In contrast to our findings, Warakagoda and Subsinghe (2013) suggested that B$_5$ medium was superior to MS for plant regeneration of \textit{Pterocarpus santalinus}. It is evident from the above cited discussion that MS medium has been widely used and proved better than other media for shoot multiplication and shoot growth in large number of tree species.
Medium strength was found to influence the multiplication rate of in vitro shoots, as observed in case of Dalbergia latifolia (Raghwa Swamy et al., 1992). They observed that reducing the concentration of MS major salts in the medium to ¾ of their original strength increases the multiplication rate. In the present investigation, out of the various strength of MS medium tested, best shoot multiplication and maximum shoot was observed on full strength MS medium. This indicates the absolute requirement of higher concentrations of salts and vitamins for multiplication and growth of P. marsupium.

Physical status (liquid or agarified) of media also influences on frequency of shoot initiation and shoot growth as documented in many reviews. Some other workers also evaluated the effects of liquid and solidified (agar) medium on shoot initiation in different species. Agar is of popular use as a solidifier for most in vitro cultures. This may be attributed to its high clarity and inert nature that limits its intervention with metabolism (Jain and Babbar, 2002; Ozel et al., 2008).

In vitro shoot multiplication was carried out on semisolid MS medium routinely. In liquid MS medium shoot multiplication rate found to be less. Due to number of problems liquid medium was not preferred for regular shoot multiplication. Falling of shoots propagules in the medium, evaporation of medium during autoclaving, unfolding of paper bridges are the medium problems encountered that affected shoot multiplication. Also, in liquid medium prolong culture make the shoots brittle and vitrified. More-over, use of liquid medium had been frequently reported to enhance shoot and root growth in many plant species (Ziv, 1989; Sandal et al., 2001; Preece 2011; Arya et al., 2006 and Rathore et al., 2014). However, plants in liquid medium or in a medium having low concentration of the gelling agent suffer hyper-hydricty in some species (Pierik, 1997).

Specific carbohydrates may have different effects on morphogenesis under in vitro conditions. As carbohydrates, sucrose indispensable ingredient of all the culture media, as the photosynthetic ability of the cultured tissue is limited because of low irradiance and limited gas exchange (Kozai, 1991) and it is also required as an
osmotic agent (Thorpe, 1985). Shoot dormancy break requires high exogenous source of sugars that are essential to initiate and sustain shoot growth. Shoot response is regulated by interaction of phytohormones cytokinins and auxins with the disaccharide/hexose ratio (Tang et al., 1999).

Sucrose happens to be the carbohydrate of choice for most studies mainly due to being easily translocate and resistant to enzymatic degradation on account of its non reducing nature (Pontis, 1978). Poor growth of shoots at low concentration is attributed to the fact of inadequate availability of carbon source. Sucrose is the most commonly used carbohydrates and it has metabolic as well as osmotic functions in tissue culture. It is indispensible for cell growth and the cultures are dependent on it till they are ready for acclimatization (Bonga and Von Aderkas, 1992). In the present study use of sucrose at 3% level was essential for the development and growth of shoots, as the culture died or very less number of shoots developed on sucrose free medium.

The results of the present investigation are compatible with the reports of many workers who used 3% sucrose as a source of carbohydrate for shoot multiplication of different plant species (Chamber et al., 1991; Das and Rout, 1994; Huang and Huang, 1995; Ansari et al., 1996; Arya et al., 2002; 2006). However, there are many reports on successful multiplication of different plant species with 2% sucrose (Nadgir et al., 1984; Nadgauda et al., 1990; Saxena et al., 1990; Joshi and Nadgauda, 1997; Yasodha et al., 1997; Ramnayake et al., 2001; Kapoor and Rao, 2006). Saxena (1990) had reported that increased level of sucrose at 3-4% did not affect shoot number but caused albinism. In the present study, at high levels of sucrose (5-6%) no such instance of albinism were noted but the shoot multiplication rate decline. It was revealed from the results that MS medium supplemented with 3% sucrose is the best for in vitro shoot multiplication.

Tissues that are initially green gradually lose their green pigments in culture if no external carbon source in provided. Thus, it is essential to add utilizable source of carbon to the culture medium (Bhojwani and Rajdan, 1996). Although sucrose is
the most commonly used carbohydrates in majority of in vitro studies on shoot multiplication and development in woody species, sometimes alternative source are also used. The most commonly used carbon source is sucrose, which serves as the main mobile, hydrolysable carbohydrates in plants that also acts as energy source and osmoticum. In the present study, effect of sugars (glucose and sucrose) were tested and added in MS medium. Results illustrated that sucrose as a source of carbon in the medium was better than glucose and fructose. During the present investigation, 3% sucrose gave the best multiplication rate as well as healthy cultures.

As pH is defined as the negative logarithm of hydrogen ion concentration, acid solution have low pH values (< 7) and alkaline solution have high values (>7). Pure water, without any dissolved CO$_2$, has a neutral pH of 7.0. The pH of the culture medium must be such that it does not disrupt the plant tissue. This means that the effective range of pH for media is restricted. In culture media, detrimental effects of an adverse pH are generally related to ion availability and nutrient uptake rather than cell damage. The pH of the medium has an effect on the availability of many minerals (Scholten and Pierik, 1998). The uptake of ammonium and nitrate ions is markedly affected by pH (George and Sherrington, 1984). The pH of the medium changes rapidly especially, if the cultures are growing fast. The rapid change in pH of the medium is often caused due to depletion of ammonium in the medium and reasonable control of pH is achieved by frequent subculturing (Bonga and Von aderkas, 1992).

The pH of a plant tissue culture medium has pronounced effect on growth of tissues in vitro and it influences some plant developmental process (Owen et al., 1991). Plant cells and tissues require optimum pH for uptake of nutrient from the medium for growth and development in cultures. The pH affects uptake of ions, and for most of the culture media pH 5.0 to 6.0 before sterilization is considered optimal. Most of the tissue culture media are poorly buffered, resulted in pH fluctuations which could prove detrimental to long term survival and growth of single cells or cell
populations at low density. Therefore, standardization of pH for specific species for its rapid shoot multiplication and growth is essential.

Results of pH experiment revealed that increase of pH from 3.8 – 5.8 favored rate of shoot multiplication. Maximum rate of multiplication was observed at pH 5.8 of the MS medium supplemented with 9.30 µM KN + 0.54 µM NAA + additives. At pH of the medium higher than 5.8 resulted in drastic reduction in shoot numbers. It was reported that low pH (lower than 4.5) and high pH (higher than 7.0) generally stop growth and development in vitro (Pierik, 1987).

Subculturing is important to remove the dead and necrotic portion of the culture and to prevent the toxic effect of phenolics. Thus the subculture duration affect the response of the explants in vitro. Subculturing leads to the renewal of depleted nutrient medium to tissue, maintains the pH and thus helps in increase multiplication rate (Bonga and Aderkas, 1992). Frequent renewal also prevents the accumulation of phenolic exudates, which causes necrosis of tissue. A subculture frequency of four weeks was found to be most suitable for the yield of maximum numbers of shoots and also better overall growth of the culture. During the present investigation subculture duration of four weeks was found to be most suitable for the yield of maximum number of in vitro shoots and for the growth of the cultures. Four weeks subculture cycles in micropropagation of some species have been used by earlier workers also. Huang and Huang, (1995) employed 4 weeks subculture cycle for shoot culture in Bambusa ventricosa. Arya and Sharma (1998) obtained 5 fold multiplication by subculturing after every 4 weeks of subculturing. Four weeks subculture cycles in micropropagation have been used by earlier workers in Dendrocalamus asper (Arya and Arya, 2009) and Pterocarpus santalinus (Warakagoda and Subasinghe, 2013).

Cultures which were left without subculture beyond 5 weeks showed necrosis, older shoots turned brown, brittle and eventually died. Only new small green shoots survived that did not elongate when subculture at fresh medium. Prutpongse and Gavinlertvatana (1992) overcome browning of shoots by
subculturing at intervals of 3-4 weeks. Presently during each subculturing the necrotic parts of the shoots were removed to overwhelm the healthy shoots with toxic phenolics.

**IN VITRO ROOTING**

Rooting of *in vitro* multiplied shoots is a very important step of any *in vitro* propagation. Comparatively, it is easy to induce rooting from the shoots of seedling origin and quite often frequency of rooting reduce with increase in age of the source plants, particularly in tree species and woody plants. The induction of rhizogenesis usually requires an adjustment in the level of auxins and cytokinins or with auxin alone. Rooting frequency also depends on the level of rejuvenation during multiplication stage and quality of the shoots. Frequency and quality/quantity of rooting depends on factors like; species/genotype, source of auxin and their concentration, nutrient media, sucrose concentration and incubation condition.

Auxins are known to promote adventitious root formation at higher concentrations. This action is also coupled with stimulation of cell division. Exogenous requirement of auxin and concentration may vary with the plant species. Root induction is brought about by the polar transport from auxin exporting tissue to the rooting zone (Ford *et al*., 2002). The ability of plant tissue to form roots depends on interaction of many endogenous and exogenous factors. The role of auxins in root development is well established and has been reviewed by Torry (1976) and Scott (1972). Usually, there is sufficient residual cytokinin in shoots, thus little or no cytokinin is required in rooting medium (Hu and Wang, 1983). The presence of endogenous auxins has been found to be important during rooting even if there are supplied exogenously (Tartoura *et al*., 2004).

In woody species, micro shoots show some difficulties in *in vitro* rooting. Variability occurs in responsiveness of microshoots towards rooting in tissue culture. As a woody perennial, *P. marsupium* is difficult to root. The ability of plant tissues to form roots depends on interaction of many endogenous and exogenous factors. A varied effect of auxins IBA, NAA was observed by incorporating them in MS medium
at different concentrations. Our observation on root induction in *in vitro* shoots of *P. marsupium* reveals that IBA is more effective than any other auxin on root induction.

Efficacy of IBA on root induction is well reported in *Dendrocalamus asper* (Arya et al., 2002); in *Drepanostachyum falcatum* (Arya et al., 2008) and in *Gycyrrhiza glabra* L. (Arya et al., 2009). Report of Rout and Das (1993) on rooting of shoots of *Madhuca longifilia* mentions effectiveness of IBA over NAA and IAA for root induction. Effect of IBA for *in vitro* rooting has also been reported in leguminous species like *Acacia auriculiformis* (Rangarao and Prasad, 1991) and *Prosopis tamurago* (Nandwani and Ramawat, 1992). Similar reports were found on many tree and plant species (*Acacia sinuate, Hagenia abyssinica, Eclipta alba, Melicanna baccifera and Azadiracta indica*), where IBA has been used as the most suitable auxin for *in vitro* rooting (Vengadesan et al., 2002; Feyissa et al., 2005; Husain and Anis 2006; Kant et al., 2009 and Gehlot et al., 2014).

The *in vitro* raised shoots were first rooted on high auxin supplemented MS medium (two-step treatment) and later these *in vitro* grown shoots were transferred on half strength MS medium without auxin. With the two-step procedure 53.33% rooting was obtained. These results are supported by earlier reports where high concentrations of auxin were used to get significant rooting in *P. marsupium* (Husain et al., 2008). Two step rooting procedure has been used to advantage in several plant species (Shekhawat et al., 2004; Husain et al., 2004 and Arya et al., 2012).

In earlier publications concerning rooting of different plant species, MS macro and micro elements at full strength were the component of the media usually used (Boxus, 1971; Mehra and Mehra, 1974; Gupta et al., 1983; Arya and Shekhawat, 1986). Later, the full salt concentrations of macro elements of this formulation was lowered by half, one-third and quarter as basal rooting medium, (Quoirin et al., 1977; Lane, 1979). In the present case on half strength MS medium yielded best rooting response. Successful rooting at the medium of lower strength has been reported in other leguminous tree species, *Dalbergia latifolia* (Ravishanker Rai and Jagdhishchandra, 1989) and *Delonix regia* (Rahman et al., 1992). Effectiveness of
lower media strengths for rooting has also been seen in *Piper longum* L. (Bhat et al., 1992), *Campanula isopyilla* (Brandt, 1992) and *Dalbergia sisso* (Arya et al., 2013).

**EX-VITRO ROOTING**

*Ex vitro* rooting reduce one step of *in vitro* rooting, cost of production and improvement in survival rate of plantlets. Auxin type, its concentration and duration of treatment, rooting medium and incubation conditions are the factors, which influence *ex vitro* rooting. Various concentrations of auxins (IBA, NAA and IAA) used for pulse treatment for *ex vitro* root induction. None of the shoots rooted in any of the treatment tested.

**HARDENING AND ACCLIMATIZATION**

The hardening of *in vitro* raised plantlets is essential for better survival and successful establishment. Direct transfer of tissue culture raised plants to outer environment is not possible as the regenerates in the culture condition has been confined in very humidity, varied light and temperature conditions and being protected from the attack of microbes and other agents. Micropropagated plants are generally susceptible to transplantation shock due to delicate root system, reduced amount of epicuticular wax and reduced or abnormal stomata. Such micropropagated plants thus cannot be benefit from light intensifies until the photosynthetic mechanism improves. This can be resulted in excessive dehydration, poor control of gaseous exchange and also poor absorption of nutrients by the root system. Earlier reports on dicots, that leaves of plantlets produced in vitro often have an inadequate photosynthetic apparatus. The sucrose in the medium or their heterotrophic mode of nutrition is responsible for it. The sucrose in the shoot multiplication medium is partly responsible for this because it suppresses the formation of RUBISCO a key enzyme in photosynthesis. After reduction of sucrose concentration in the medium, the photosynthetic mechanism slowly recovers. Such, micropropagated plants thus cannot benefit from high light intensities until the photosynthetic mechanism improves. Therefore, it is customary to expose the tissue cultured plantlets from low to high intensity stepwise over a period of several weeks.
The transfer of plants from culture vessel to the soil requires careful, stepwise procedure, as the plants growing under *in vitro* conditions are continuously exposed to a unique microenvironment that is selected to provide minimal stress and nearly optimal conditions for plant multiplication. Use of vermiculite or soilrite like inert substance for hardening has been reported by many workers (Saxena, 1990; Yasodha, *et al*., 1997; Arya and Sharma, 1998; Arya *et al*., 1999; 2000). Vermiculite is an inert material and absorbs large quantity of water. It has a relatively high cation exchange capacity and thus can hold the nutrients in reserve and release them. It contains enough magnesium and potassium to supply most of the plants. Rooted plantlets were shifted to sand: soil: FYM mixture for hardening and enriched with ½ x nutrient solution for few weeks and is reported by many workers (Saxena, 1990; Arya and Arya, 1997; Arya and Sharma, 1998; Arya *et al*., 1999; 2002). The plantlets growing on a medium rich in minerals, sugar develop a heterotrophic mode of nutrition. They are not able to survive in the outside environmental conditions. Therefore, the plants should be properly acclimatized when they are transferred from the *in vitro* conditions.

Acclimatization presents challenges at least equal to those posed by the initiation of cultures because it marks the end of artificial control and the beginning of autonomous plant growth. The heterotrophic mode of nutrition and poor mechanism to control water loss render micropropagated plants vulnerable to transplantation shocks.

Acclimatization and hardening depends on the relative humidity and temperature therefore misting is preferred which maintains temperature of 30°C and relative humidity of 80-85%. It has been reported by many workers that survival percentage increases if the plants are transplanted to soil in rainy season (Saxena, 1990, Chaturvedi *et al*., 1993; Arya and Sharma, 1998; Arya *et al*., 2002).

Although considerable efforts have been directed to optimize the conditions for the *in vitro* stages of micropropagation, scant attention have been paid to understand the process of acclimatization of micropropagated plants to the soil
environment. Consequently the transplantation stage continues to be a major bottleneck in the micropropagation of many plants (Ziv, 1986; Corner and Thomas, 1981).

The success of any micropropagation protocol depends upon the rapid acclimation and survival of plantlets under outer environmental conditions. Plants rooted under in vitro conditions are partial autotrophs and lack epicuticular wax that ensures water regulation from stomata. Some research has been done on hardening/acclimatization of tissue culture plants to understand and improve the protocols efficiency (Fabbri et al., 1986; Drew, 1992; Deng and Donnelly, 1993; Bolar et al., 1998; Pospisilova et al., 1999; Hazarika, 2000; Deb and Imchen, 2010; Kaur et al., 2011).

Plantlets inside culture room were observed daily and after attaining certain growth these plantlets were transferred to the mist polyhouse. Similar, procedure was adopted by many researchers in Sterculia urens (Purohit et al., 1994), Wrightia tomentosa (Purohit and Dave, 1996) and Annona squamosa (Nagori and Purohit, 2004).

IN VITRO CALLUS CULTURE

Majority of reports on callus induction in leguminous tree is based on reproductive tissues like seeds, embryo, inflorescence or anthers etc. Plant regeneration from cultured explants involved the initiation of basal callus and then shoots bud differentiation. Establishment of callus growth with subsequent organogenesis has been obtained from in many species of plants and from numerous explants like cotyledon, hypocotyls, stem, leaf, shoot apex, root, young inflorescence, flower petals, petioles, embryo, etc. Callus is produced in vitro as a result of wounding and in response to hormones, either endogenously or exogenously supplied in the medium (Chawla, 2002).

In the present study, callus culture was investigated for P. marsupium. Callus was induced from in vitro raised leaves and cotyledonary explants derived from
seeds which was germinated in vitro on MS medium. Results illustrated that leaves obtained from in vitro raised shoots were not responsive in any treatment and only the cotyledons showed callus induction. Callus induction was maximum (55.55%) at 4.52 µM 2, 4-D.

A similar promoting effect of 2, 4-D on callusing was earlier reported in Dracaena sanderiana species (Ilah et al., 2002). However, in Dracaena fragran young stem segments produced calli on medium supplemented with 2, 4-D alone or in combination with BA (Vinterhalter, 1989). Additionally, in many other plants profuse callusing was observed on 2, 4-D added medium (Khan et al., 2002). For cultured tissues, the requirement for exogenous hormones depends on the endogenous level of plant tissue which varies with organs, plant genotype and the phase of plant growth (Chand and Singh, 2004). Nodal explants along with high callus biomass also exhibited high regeneration potentiality compared to other tested explants. Pua et al., (1989) have reported in Brassica napus that the nodal segment was more responsive for callusing and regeneration compared to other explants.

In the present study, type of calli obtained was soft and friable after 4 weeks. Several attempts were made for multiplication of callus by using different concentrations of 2,-4 D supplemented with BAP/Kn but the species was recalcitrant for somatic embryogenesis.