Medicinal plants have been used as valuable sources of potent and powerful drugs to treat diseases all over the world since olden times (Singh et al., 2002). The conventional method is the principal means of propagation but takes a long time for multiplication because of a low rate of fruit set and poor germination. The clonal uniformity is not always maintained through seeds. With the increasing demand for the crude drugs, the plants are being overexploited resulting into threatening status of many rare species. Many medicinal plant species are disappearing at an alarming rate due to rapid agricultural and urban development, uncontrolled deforestation and indiscriminate collection. Advanced biotechnological methods of culturing plant cells and tissues provide new means for conserving and rapidly propagating valuable, rare, and endangered medicinal plants (Benson, 2003; Nalawade and Tsay, 2004).

Plant tissue culture is the technique of growing plant cells, tissues and organs in an artificial nutrient medium under aseptic conditions. Plant tissue culture is also employed in haploid production, production of disease free and resistant plants, selection of desirable traits, elimination of breeding barriers and biosynthesis of secondary metabolites (Pijut et al., 1990; Karp, 1994; Roja and Rao, 1998; Nalawade and Tsay, 2004). Micropropagation offers a rapid means of afforestation, multiplying biomass, conservation of elite and rare germplasm (Bajaj, 1986; Karp, 1994). The application of micropropagation techniques as an alternative mean of asexual propagation of important plant species has increased the interest of workers in various fields. The technique of plant cell and tissue culture has contributed in raising new plants through shortening germination and developmental phase of plants. So, it holds a place of unique importance in today’s world among plant biologists (Batra et al., 2000).

2.1. Micropropagation:

During the past decade, great progress has been made in this field to make it an industrial technology. Great advances in micropropagation have occurred since Harberlandt exploration of the concept presented in his landmark paper published in 1902. The pioneering experiments were initiated by the father of tissue culture, Gottlieb Haberlandt in 1898. He choose single cell, grew them on Knop’s (1865) salt solution and observed growth in the palisade cells but could not succeed because of handling with highly differentiated cells due to lack of proper techniques.
Haberlandt also perceived the concept of growth enzymes and felt that these are released from one type of cells to stimulate growth and developments in other cells. His pioneering experiments inspired other scientists to conduct further work on the morphogenetic potentialities of living cell, tissue and organ to develop into complete plant. Kotte (1922), a student of Haberlandt and Robbins (1922) were successful in the establishment of excised plant root tips under in vitro conditions. Success in continuously growing cultures of tomato root tips using sucrose, inorganic salts and yeast extract was achieved by White (1943).

Gautheret (1934) observed proliferation of callus by culturing cambium cells of Salix and Populous on Knops’ solution. Van Overbeek et al. (1942) studied the stimulatory effect of coconut milk on embryo development in Datura. These findings set the stage for the large advancement in research for tissue culture, such as the eradication of viruses through meristem culture (Morel and Martin, 1952), cell suspension cultures (Muir et al., 1954), auxin and cytokinin basis of organogenesis (Skoog and Miller, 1957), somatic embryogenesis (Reinert, 1959), large scale culture of cells (Tulecke and Nickell, 1960), regeneration of plants from single cell (Vasil and Hildebrandt, 1966), uptake of DNA by cells (Ledoux, 1965) and variability of cells in culture (Lutz, 1969). During in vitro culture, various intrinsic and extrinsic factors like culture medium (carbohydrates, growth regulators, agar concentration, and pH), cultural conditions (photoperiod, temperature) and explant type affect the growth and development of the culture.

A number of horticultural, medicinal and commercially important plant species have been successfully regenerated viz., Nicotiana tabacum (Nagata and Takebe, 1971), Atropa belladonna (Narayanaswamy and George, 1972), Solanum khasianum (Chaturvedi and Sinha, 1979), Glycyrrhiza glabra (Shah and Dalal, 1980), Catheranthus roseus (Constable et al., 1982), Ocimum sp. (Ahuja et al., 1982), Cinchona species (Kobitz et al., 1983), Urginea indica (Jha et al., 1984), Stevia rebudiana (Tamura et al., 1984), Asparagus racemosus (Kar and Sen, 1985), Anthurium patulum (Eapen and Rao, 1985), Mentha sp. (Rech and Pires, 1986), Papaver bracteatum (Day et al., 1986) Solanum nigrum (Agarwal and Bansal, 1987), Artemisia annua (Jha et al., 1988), Eucalyptus tereticornis (Das and Mitra, 1990), Tamarindus indica (Knopp and Nataraja, 1990), Anogeissus pendula (Joshi et al., 1991), Eucalyptus Sideroxylen (Cheng et al., 1992), Acacia nilotica (Singh et al., 1993), Acacia senegal (Gupta et
al., 1994), Fraxinus angustifolia (Perez-parron et al., 1994), Stryphnodendron polyphythum (Franca et al., 1995), Dalbergia sissoo (Gulati and Jaiwal, 1996), Terminalia arjuna (Kumari et al., 1998), Sapindus mukorossi (Philomina and Roa, 2000), Areca catechu (Mathew and Philip, 2000), Cardiospermum halicabum (Babber et al., 2001), Anogeissus latifolia and A. pendula (Saxena and Dhawan, 2001), Chukrasia tabularis (Nagalakshmi and Pullaiah, 2001), Melia azedarach (Shahzad and Siddique, 2001), Alnus nepalensis (Thakur et al., 2001), Tectona grandis (Gangopadhyay et al., 2003), Vitex negundo (Sharma et al., 2006), Anomum microstephanum (Thoyajaksa and Rai, 2006), Prosopis laevigata (Gonzalez et al., 2007), Acacia senegal (Khalafalla and Daffalla, 2008) Acacia chundra (Rout et al., 2008), Wrightia tomentosa (Purohit et al., 2009), Spondias mangifera (Tripathi and Kumari, 2010), Michelia champaca (Armiyanti et al., 2010) Acacia auriculiformis (Girijashankar, 2011), Streblus asper (Gadidasu et al., 2011), Terminalia catappa (Phulwaria et al., 2012) and Glycyrrhiza glabra (Yadav and Singh, 2012) have been propagated in vitro.

Various experimental conditions maintained during micropropagation are briefly reviewed as follows:

2.1.1 Nutrition:

The in vitro culturing of tissue mainly depends on the media composition. The main components of most plants tissue culture media are mineral salts, sugar and water. Other components include organic supplements, growth regulators and gelling agent (Gamborg et al., 1968; Gamborg and Phillips, 1995). The nutritional requirements for optimal growth of a tissue cultured in vitro vary with the species. Even tissues from different parts of the same plant may require different media for satisfactory growth (Murashige and Skoog, 1962). The various media formulated have been modified time to time to provide all the necessary requirements to the cultured tissue (Gautheret, 1939; White, 1943; Nitsch, 1951; Murashige and Skoog, 1962; Mc Cown and Lloyd, 1981). MS medium (Murashige and Skoog, 1962) is the most commonly and frequently used culture media. Media compositions have been formulated for the specific plants and tissues (Nitsch and Nitsch, 1969). Some tissues respond much better on solid media while others on liquid media. No single medium can be suggested as being entirely satisfactory for all types of plant tissues and organs. The pH of the medium is also an important factor for tissue culture. The pH of the medium is usually around 5.8 before autoclaving and extremes of pH are avoided. Each plant
species has different optimized conditions for growth of the cells and secondary metabolite production. So, it is necessary to optimize the conditions in each case. Humidity in the culture vessel and osmotic potential of the medium also affects the growth and development of plantlets in vitro in different ways (Brown et al., 1976; Ziv et al., 1983).

MS medium is used either originally or with minor variation and combination of phytohormones and vitamins in different plant species viz. Dalbergia latifolia (Raghava Swamy et al., 1992), Terminalia arjuna (Kumari et al., 1998), Sapindus mukorossi (Philomina and Rao, 1999), Melia azedarach (Shahzad and Siddique, 2001), Azadirachta indica (Shekhawat et al., 2002). Raghava Swamy et al. (1992) observed better axillary bud initiation in Dalbergia latifolia on MS medium while multiple shoot induction was better on Woody Plant Medium (WPM) or MS medium with reduced major salts. Bhargava et al. (2003) reported globular proembryonic mass of callus on MS medium after 40-50 days of incubation and later transferred it on B₅ medium for fragile snowy callus in Phoenix dactylifera. Sharada et al. (2003) used MS medium for shoot induction and B₅ or WPM medium for root development in Celastrus paniculatus.

Agar-agar is the most commonly used gelling agent to solidify the medium and assumed to be that of neutral support for callus growth and multiplication. Mostly 0.8% agar is used for culture medium. It has been reported that a higher concentration of solidifying agent in the medium reduced vitrification (Pasqualatto et al., 1986). But in certain cases an increase in agar-agar amount causes adverse effect as observed by Lal and Singh (1995). Selby et al. (1989) reported that combination of lower agar-agar (0.6%) and pH (5.6) showed better growth and proliferation in many coniferous plants. Suthar et al. (2011) reported that lowering of agar concentration and liquid media resulted in better micropropagation protocol for Boswellia serrata. Qin et al. (2012) observed that hypocotyls and stem explants regenerated buds directly on growth regulator free MS medium media containing 30.0 g/l sugar and 6.0 g/l agar in Cleome spinosa. Pant et al. (2012) placed roots explants horizontally on agar gelled (0.7% w/v) MS medium supplemented with 3% sucrose and different concentrations of BAP (4.44 to 22.20 µM) in combination with 2,4-D (0.90 to 4.52 µM) and obtained good callusing.

In addition to growth regulators, plant cell sometimes requires complex substances like coconut milk, adenine sulphate (AS) and casein hyrolysate (CH) and
activated charcoal (AC) for callus induction and regeneration. Van Overbeek et al. (1941) demonstrated the stimulatory effect of coconut milk on embryo development in *Datura*. Nadagouda et al. (1978) reported rapid clonal multiplication on MS medium supplemented with coconut milk and BAP in *Curcuma longa*. Mandal and Gadgil (1979) achieved callus culture of *Solanum nigrum* on MS medium supplemented with coconut water and 2, 4-D and studied the effect of purines, pyrimidines and growth regulators on bud-differentiation. Coconut milk of green nut is very effective in providing an undefined mixture of organic nutrients and growth factors (Gamborg and Phillips, 1995). Roja et al. (1991) cultured *in vitro* derived shoots on MS medium supplemented with coconut milk (10%) and 2,4-D and reported callus formation. Ray and Jha (2001) reported that culture in liquid regeneration medium containing 10% coconut milk not only favoured an increment in the number of micro shoots per explant but also withaferin A accumulation. Mathur et al. (2008) noted best rooting response on MS basal media containing 40 or 80 mg/l AS in *Chlorophytum borivilianum*. Nadha et al. (2011) cultured *in vitro* derived shoots on MS medium supplemented with BAP (2 mg/l) and ADS (10 mg/l) for further proliferation and multiplication in *Guadua angustifolia*. Mohanty et al. (2012) inoculated rhizome explants of *Zingiber zerumbet* on MS medium containing 30 g/l sucrose and different concentration of BAP, IAA, NAA, GA₃ and ADS (80-100 mg/l). Mohanty et al. (2012) transferred the elongated shoots of *Zingiber zerumbet* on MS medium containing BA, IAA and ADS for mass multiplication. Gopinath and Arumugam (2012) obtained maximum number of multiple shoots on half strength of MS medium with Kn 1.0 mg/l + BAP 1.5 mg/l + 20% coconut water in *G. superba*.

Fridberg et al. (1978) reported the importance of activated charcoal (AC) in absorbing toxic compounds released by inoculated explants during culture. Pierik (1987) noticed the promoting effect of AC on growth and organogenesis in plant species. AC has been used in regeneration medium in *Dalbergia sissoo* (Gulati and Jaival, 1996) and *Areca catechu* (Mathew and Philip, 2000) to prevent browning of culture due to phenolic exudation released by the explants. Addition of 15% (v/v) coconut water and 2g/l AC increased the number of shoots per shoot culture in *Gloriosa superba* (Hassan and Roy, 2005). Perera et al. (2007) achieved embryogenesis in *Cocos nucifera* by culturing unfertilized ovaries in presence of 2,4-D and AC. The use of activated charcoal to overcome phenolic exudation was also reported in *Dioscorea bulbifera* (Narula et al., 2003), *Tinospora cordifolia* (Gururaj et al.,

Carbohydrates act as an energy source required for growth, maintenance and synthesis of cell constituents during in vitro culture. Most commonly used carbohydrate source is sucrose, but other sugars like glucose, fructose, dextrose, mannitol, sorbitol etc. are also occasionally used. Sucrose is required for differentiation of xylem and phloem elements in the cultured cells (Aloni, 1980). Glucose and fructose are also known to support good growth of some tissues. Sucrose represents the major osmotic component of the medium and necessary for various metabolic activities. In most of plants, 2-3% sucrose is found to be very effective for optimal growth and morphogenesis. MS medium with 2% sucrose was optimal for culturing of shoot tips in *Tamarindus indica* (Knopp and Nataraja, 1990). MS medium with 4-6% sucrose caused more callusing on cultured axillary shoots in *Eucalyptus sideroxylon*, while 2-6 per cent sucrose supported roots development (Cheng *et al.*, 1992). It was found that 3% sucrose is effective for shoot initiation from cotyledonary node explants in *Stryphnodendron polyphythum* (Franca *et al.*, 1995). Twenty per cent sucrose concentration is more effective for development of globular embryos of *Terminalia arjuna* (Kumari *et al.*, 1998). In *Alnus nepalensis*, 1.5 % sucrose in WPM medium was optimal for shoot proliferation from terminal axillary buds (Thakur *et al.*, 2001). Shahzad and Siddiqui (2001) reported that 3% sucrose was required for callus induction and shoot proliferation in *Melia azedarach*. Shekhawat *et al.* (2002) also advocated the use of 2-3% sugar to obtain multiple shoots in *Azadirachta indica*. Hossain *et al.* (2005) reported that maltose containing MS media resulted in higher shoot number per explant in *Centella asiatica*. Chakradhar and Pullaiah (2006) also reported that 1.0 % sucrose was necessary for rooting of regenerated plantlets in *Wattakaka volubili*. Behera *et al.* (2009) tested different percentage of sucrose in the culture media as a source of carbon for microtubers induction. A positive effect of concentration of sucrose on size and weight of microtubers has also been observed in *Dioscorea nipponica* (Chen *et al.*, 2007), *Dioscorea cayenensis* (Ovono *et al.*, 2009) and *Habenaria bractescens* (Medina *et al.*, 2009). Rahman *et al.* (2010) noted maximum height in all the potato cultivars with maltose-media as compared to that of sucrose and glucose. Mahmoud *et al.* (2011) noticed that 2% concentration of sucrose gave significantly better results than 3% regardless type of growth regulator used. In *Solanum nigrum* L., fructose
at 4% proved to be better choice for multiple shoot regeneration followed by sucrose, maltose and glucose (Sridhar and Naidu, 2011).

2.1.2 Plant growth regulators:

Plant growth regulators play an important role in micropropagation, as they affect the growth and differentiation of plant tissue to a great extent. Several studies have been made on the influence of the concentration of various growth regulators. There are five known major classes of compounds with plant growth regulatory activity. They are auxins, cytokinins, gibberellins, abscisic acid (ABA) and ethylene. Among various growth regulators, auxins (NAA, IAA, IBA and 2,4-D), cytokinins (BAP, Kinetin, Zeatin), ABA, gibberellins and ethylene are very important. It has been reported that low concentration of auxins and high concentration of cytokinins in the medium favoured shoot induction whereas reverse proportion promoted root formation and an intermediate concentration caused callus development. Therefore, a critical role of auxin/cytokinin ratio is inevitable for inducing root and shoots proliferation (Murashige, 1974).

Among cytokinins, BAP is most commonly used in a variety of explants for shoot regeneration. Goyal and Arya (1979) observed the regeneration in Prosopis cineraria on MS medium supplemented with different concentrations and combinations of Kinetin, IAA, IBA and BAP. Gamborg’s medium was used by Mukhopadhayay and Mohan ram (1981) for culturing of Dalbergia sissoo. Rumary and Thorpe (1984) reported beneficial role of mixed cytokinins in some cases. Multiple shoots were observed in Eucalyptus grandis on MS medium supplemented with additional thiamine (Lakshmi Sita and Rani, 1985). It has been reported that the decrease in NAA ensured shoots formation (Rao et al., 1984; Sudha Devi and Natreja, 1987). Mittal et al. (1989) obtained multiple shoots from axillary buds of Acacia auriculiformis on Gamborg’s (B5) basal medium supplemented with coconut milk and BAP. Knopp and Nataraj (1990) regenerated plantlets by supplementing 2.0 mg/l BAP in Tamarindus indica. Multiple shoots were obtained from cotyledonal nodes of Dalbergia latifolia on MS medium fortified with BAP (2.0 mg/l) (Lakashmi Sita and Raghwa Swami, 1992). Abhyankar and Chinchani and Harikrishnan and Hariharan (1996) obtained direct shoot multiplication from nodes and tender leaves of Plumbago rosea on MS medium supplemented with a range of concentration.
of BAP and IAA. Anand and Hariharan (1997) observed multiple shoot formation on MS medium supplemented with BAP alone or in combination with auxin from excised rhizome bud of *Alpinia galangal*. Thidiazuron (TDZ) is a potent plant growth regulator which exhibit cytokinin like activity in regeneration system for many plant species (Faisal et al., 2005; Ahmad et al., 2006; Barik et al., 2007; Mukhtar et al., 2012). Johnson et al. (1997) cultured shoot tips of seedlings of *Saussurea leppa* on MS medium supplemented with TDZ. Gisbert et al. (2006) reported the decrease in development of shoots with the increase in the concentration of TDZ. Better response of BAP over Kn during in vitro studies has been reported by Behera et al. (2008) and Nayak et al. (2007). Higher concentration of TDZ resulted in shoot fascination (Debnath, 2006) in Strawberry and somatic embryogenesis in *Fragaria x ananassa* (Husaini and Abdin, 2007). MS media supplemented with BAP in combination with NAA gave highest per cent callus induction in *Leucaena leucocephala* (Singh and Lal, 2007) and *Albizia lebbeck* (Yadav and Singh, 2011a). In *Eucalyptus camaldulensis*, highest frequency of somatic embryos were produced from callus obtained on MS medium supplemented with BAP (0.5 mg/l) and NAA (0.1 mg/l) from mature zygotic embryos (Prakash and Gurumurthi, 2010). Ali et al. (2011) noted best shoot formation response (88%) was obtained on MS medium containing 1.0 mg/l BAP + 0.25 mg/l NAA. Lahiri et al. (2012) obtained highest shoot regeneration from the callus tissue on MS medium fortified with BAP at 1.33 µM level.

### 2.1.3 Selection of Explants:

The successful production of multiple shoots, callus induction, followed by subsequent plantlet regeneration partially depends upon the number of factors associated with explant used. Several factors influencing the behavior of the inoculum in culture (Murashige, 1974) include:

- The organ serving as tissue source
- Explant size
- Physiological and ontogenic stage of the explants
- Season in which explants is obtained
- Overall quality of plant from which explants are to be taken

Practically any plant part can be used as the explant source and can regenerate plantlets cultured in vitro. The explant should be obtained at the suitable physiological stage of development. Generally the immature tissues and organs are
invariably more morphogenetically plastic than the mature ones. The different explants viz. leaf segments, internodes and nodal segments showed the different morphogenic responses (Huda et al., 2007). Nodal segments initiated callus earlier than leaf segments and internode explants but higher amount of callus were obtained from leaf segments than internodes and nodal segments. The quality of explants primarily determines the establishment of in vitro culture (Keathley, 1984). Nodal explants from mature tree of Heavea brasiliensis failed to produce plantlets while explants taken from 6 to 8 weeks old plant regenerated plantlets (Rehman et al., 1981). Similarly explants from mature trees of Eucalyptus citriodora required pre-treatment for shoot induction but explants from seedlings did not require any pre-treatment (Gupta et al., 1981).

The size of explants plays a key role in expressing the morphogenetic potentiality. Okazava et al. (1967) reported that small explants are more likely to form callus while larger explants maintain greater morphogenetic potentiality. It may be due to the availability of food reserves and growth regulators proving useful in the initiation of new growth (Anderson, 1980). Orientation of the explants also plays an important role in giving morphogenic response. The horizontal position of the explants has been reported to promote adventitious shoot formation in many higher plants (Frett and Smagula, 1983; Pierik, 1987).

The age of explants source also influence the callus development and differentiation. The tendency for clonal propagation is closely linked with the genetic and physiological factors that control the transition from juvenile to mature growth in plant species (Bonga, 1982). In general, the more juvenile the tissue, the better it will respond to in vitro treatments leading to de novo primordium initiation and subsequent organogenesis. Gulati and Jaiwal (1996) reported that nodal explants taken from copped shoots of mature Dalbergia sissoo exhibited least phenolic exudation with better shoot regeneration. Callus formation and regeneration of plantlets from nodal explants was reported by Nandwani and Ramawat (1991) in Prosopis juliflora. Raghava Swamy et al. (1992) used nodal explants of in vitro grown root suckers from 60-80 years old Dalbergia latifolia for direct organogenesis. In Fraxinus angustifolia, shoot tips and nodal segments were used for micropropagation (Perez-parron et al., 1994). The maximum number of shoots (9/explant) was obtained in Aegle marmelos from nodal segments on MS medium supplemented with BAP (8.8µM) + IAA (5.7µM) (Pati et al., 2008). In Melia azedarach, multiple shoots were produced from nodal
segments on MS medium supplemented with 5 µM of BAP (Husain and Anis, 2009). Tripathi and Kumari (2010) obtained an efficient *in vitro* propagation of *Spondias mangifera* using nodal explants from seedlings. The young explant source showed higher growth and shoot length as compared to the old source. Many authors use *in vitro* mother plants for the juvenility of explants and the stability of environmental conditions (Nassour *et al*., 2003, Hassanein and Dorion, 2005; 2006). Mahmoud *et al.* (2011) reported that nodal explants obtained from young mother plants were more capable of growth as compared to those obtained from old ones. The frequency of *in vitro* shoot regeneration depends on the type of explants and the type and concentration of growth regulators used in the regeneration medium (Ishag *et al*., 2009). The use of nodes in preference of shoot tips has been reported in *Azadirachta indica* (Arora *et al*., 2010) and *Aegle marmelos* (Yadav and Singh, 2011a). In *Clitoria ternatea*, cotyledonary node explants produced considerably higher number of shoots at lower concentration of TDZ in comparision to nodal explants (Mukhtar *et al*., 2012).

Season also affects the shoot proliferation and explants contamination. Seasonal conditions at the time of explants collection influence the *in vitro* growth, phenolics exudation and degree of contamination. The nodal segments of *Eucalyptus tereticornis* collected during July to September were more responsive towards micropropagation due to negligible phenolic exudation as compared to those collected in October-November and May-June having higher amount of phenolic exudation (Das and Mitra, 1990). Similar effects of season have also been noticed in *Tectona grandis* (Gupta *et al*., 1980). Bonneau *et al.* (1994) observed higher percentage of embryonic callus production from the zygotic embryo explants in *Euonymus europacus* collected during May to September. Similarly, Thakur *et al.* (2001) observed optimal establishment of axillary and terminal buds of *Alnus nepalensis* cultured during February and March; thereafter, the percentage establishment showed a declining order. Singh and Goyal (2007) reported that August to October season was the best for explant collection in *Salvadora oleoides* throughout the year. The harvesting time of pods also showed a significant effect on *in vitro* germination of seeds. Yadav and Singh (2011a) recorded highest *in vitro* germination (83.3%) of seeds extracted from dark-yellow colored pods in *A. lebbeck*. Yadav and Singh (2012) observed highest bud-break percent from middle order nodal explants (3rd to 5th node from apex) collected between May to August in *Glycyrrhiza glabra*. 
2.1.4 Cultural Conditions

The major environmental factors of tissue culture are light and temperature. The intensity, quality and extent of daily exposure of light are the determining factors in tissue culture. Most of the cultures grow well within a wide range of photoperiods, light intensities and optimal temperature (White and Risser, 1964), whereas some cultures are temperature sensitive (Staritsky, 1970). Cultures are usually maintained at a constant temperature of 25±2°C and a photoperiod of 16hrs light (20 µmol m⁻² s⁻¹ photosynthetic photon flux intensity) followed by 8hrs of dark.

Gupta et al. (1981) reported multiple shoots production on MS medium at 15°C in continuous light followed by culture at 25°C with 16 hours photoperiod from the terminal buds of twenty years old Eucalyptus citridora. The effect of light and cytokinins interaction on cultured cotyledon explants of Radiata pine was studied by Victor et al. (1984). In Eucalyptus tereticonis, a higher multiplication was achieved on MS medium at a slightly higher temperature (30-32°C) (Das and Mitra, 1990). Calleberg and Johansson (1993) studied that direct regeneration was mostly stimulated when the anther cultured was incubated at 20°C. In Jatropha curcas multiple shoot formation occurred on MS Medium at 25±2°C under16 hours photoperiod (Rajore et al., 2002). Shekhwat et al. (2002) reported multiple shoots formation at 25±2°C and 16 hours photoperiod having light intensity of 3000–4000 lux. Azad et al. (2005) maintained the cultures at 25±1°C under an illumination of cool-white florescent tubular lamp with a light intensity of 50 µmol m⁻² s⁻¹ for 16–hours photoperiod. Geekiyanage et al. (2006) noticed the effect of photoperiods, light intensity and gibberellic acid (GA₃) on adventitious shoot regeneration from spinach cotyledons and observed that the shoot regeneration were significant at higher light intensity of 90-100 µmol m⁻² s⁻¹. The combined effect of optimum shoot regeneration and highest shoot multiplication was observed in explants of short day grown seedlings cultured under the short day condition at higher light intensity on MS medium supplemented with 1.0 mg/l 6-benzyladenine and 0.4 mg/l α- naphthalene acetic acid. As far as optimum temperature for micropropagation is concerned, different workers have reported different temperature conditions. This variation in temperature may be due to variation in genotype of explants used for micropropagation studies (Ali, 2008). Joshi et al. (2003) obtained optimum results at 23±2°C while Thirunavoukkarasu et al. (2010) reported

2.1.5 Organogenesis

Organogenesis is a comprehensive term encompassing direct and indirect pathways (analogous to the organized partial system and unorganized system of Durzan and Campbell, 1974). It involves the formation of organized structure like shoot and root from pre-existing structure i.e. unorganized mass of cells known as callus. The controlled organogenesis under *in vitro* was given by White (1939), who obtained shoots from callus of *Nicotiana glauco* and *N. longsodorffii* hybrid on a agar-agar solidified medium. Later, a number of reports approved depicting the formation of shoots and roots either directly from the explants or indirectly i.e. through callus formation.

Organogenesis deals through two pathways i.e. direct pathway and indirect pathway. Direct pathway occurs through the continuous development of shoot meristematic activity from lateral or axillary buds in shoot cultures. Indirect pathway deals with the shoot formation *via* callus formation. Either pathway culminates with a root induction phase. However, in many cases, efforts are made to achieve *de novo* organogenesis directly on the explant without an intervening callus phase (Thorpe and Patel, 1984).

2.1.5.1 Direct Organogenesis

The lateral and axillary bud production system is described as conservative due to its relative ability to produce true to type plants/clones without genetic variability. Because of this reason, it is often a preferred technique in laboratories where commercial production of economical plants is carried out (Dunstan and Thorpe, 1984).

Micropropagation without an intervening callus phase is advantageous over conventional vegetation propagation in terms of quantity, quality and economics (Altmann and Loberant, 1998). In general three modes of *in vitro* plant regeneration have been in practice: organogenesis, embryogenesis and axillary proliferation. The difference mainly matters when it relates to the genetic stability of the resulting micropropagated plants; the obvious option would be axillary and adventitious shoot proliferation. In *Eucalyptus grandis*, multiple shoots induced from nodal segments (Cresswell and Nitsch, 1975). Multiple shoots were also obtained from terminal buds of 20 year old trees of *Eucalyptus citriodora* (Gupta et al., 1981). Mittal
et al. (1989) observed the formation of multiple shoots from axillary buds from in vitro grown seedling of Acacia auriculiformis. A rapid in vitro propagation of Aloe barbadensis through axillary bud development and adventitious bud formation was achieved by Meyer and Staden (1991). Rout and Das (1993) induced bud break and multiple shoots in apical and axillary meristems derived from seedlings of Madhuca longifolia on MS medium. Patnaik and Debata (1996) developed a protocol for in vitro propagation of Hemidesmus indicus from nodal segments. Kumar and Seeeni (1998) achieved rapid clonal multiplication of Aegle marmelos by enhanced axillary bud proliferation in single node segment of a twenty five years old tree on MS medium supplemented with BAP (2.5 mg/l) in combination with IAA (1.0 mg/l). Komalavalli and Rao (2000) established micropropagation of Gymnema sylvestre - a multipurpose plant. MS medium fortified with growth regulators such as BAP (0.5 mg/l) in combination with NAA (0.01mg/l) has been reported to give optimum results in Utleria salcifolia (Gangaprasad et al., 2003). Kameri et al. (2005) reported multiple shoot formation from nodal and shoot tip explants in Wedelia chinensis under in vitro conditions. Rathore et al. (2005) developed protocol for in vitro propagation of Maerua oblongifolia using nodal shoots segments on MS medium and achieved higher rate of shoots multiplication. An efficient, rapid and reproducible plant regeneration protocol was successfully developed for Cassia angustifolia using nodal explants on MS medium supplemented with BAP and TDZ by Siddique and Anis (2007). Rathore and Shekhawat (2009) reported 35–40 shoots per culture vessel on MS medium supplemented with 4.44 lM BAP in combination with 0.57 lM IAA and additives in Pueraria tuberosa. Multiple shoot formation was observed to be highest in MS medium fortified with BAP (2.0 mg/l) and NAA (0.1 mg/l) in Acacia auriculiformis (Girijashankar, 2011). Murti et al. (2012) also tested different concentrations of TDZ combined with IBA were supplemented in MS medium to induce direct organogenesis in Fragaria ananassa.

2.1.5.2 Indirect Organogenesis

Organogenetic regeneration from callus, as with axillary and adventitious bud cultures involves separate shoot and root induction phase, similar to adventitious shoot induction. Callus organogenesis involves meristematic induction.

Laksami Sita and Vaidyanathan (1979) raised plantlets from cotyledonary callus of Eucalyptus citriodora. Buds and root formation was observed in hypocotyl callus of
Broussonetia kazinoki (Ohyama and Oka, 1980). Organogenesis was observed in the form of roots and shoots from the calli of seedling origin in Dalbergia latifolia. Shoot segments, hypocotyl and leaves were also proved useful in regenerating plants through callus (Rao et al., 1984; Sudhadevi and Nataraja, 1987). Inamdar et al. (1990) reported formation of somatic embryos via callusing from culture of shoot apices of Crataeva nurvala on MS medium containing 2, 4-D. Callus cultures and plantlet formation in vitro was reported in Prosopis juliflora by Nandwani and Ramawat (1991). Joshi and Dhar, (2003) obtained maximum shoots from epicotyle explants, cultured on MS medium supplemented with NAA (0.25 µm) and Kn (1.0 µm) in Saussurea obvallata. Thomas and Philip (2005) reported high frequency shoot organogenesis from leaf derived callus in Dalbergia sissoo. Similarly, a protocol has been developed for high frequency shoot regeneration and plant establishment in Tylophora indica from petiole derived callus by Faisal and Anis (2005). In vitro shoot proliferation from callus cultures of Aconitum heterophyllum was reported by Jabeen et al. (2006). Pareek and Kothari (2007) reported plant regeneration via callus formation in Dianthus caryophyllus on MS medium supplemented with BAP, 2, 4-D and NAA. It should be noted that regeneration from callus may lead to genetic variation in regenerated plants (Smith and Drew, 1990). Wang et al. (2004) reported that MS + 2 mg/l BAP + 0.05 mg/l NAA was the best culture medium to induce buds from cotyledon-derived calluses. Albarello et al. (2006) observed callus formation at the basal end of leaf explants which failed to regenerate the media, perhaps because of different genotype of plant material or combinations of concentrations of growth regulators used. Erisen et al. (2009) noted 23 shoots per callus on MS medium supplemented with 4.0 mg/l BAP + 0.5 mg/l NAA in Astragalus cariensis. Jeong et al. (2009) noted differentiation of organogenic callus into plantlets in Hovenia dulcis on MS medium supplemented with 0.23 µM GA₃ + 0.46µM Kn. Similarly maximum number of shoots was achieved from leaf callus of Hyptis suaveolens on MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l GA₃ (Mandal, 2011). Mandal and Laxminarayana (2012) observed that TDZ treatment was superior to BAP + NAA for inducing multiple shoots from organogenic callus derived from leaf explants of Quisqualis indica. Qin et al. (2012) subcultured leaf-derived calluses on MS + 0.5 mg/l Kn + 0.5 mg/l BAP and achieved the highest differentiation rate of 100%. Pant et al. (2012) obtained best results of shoot organogenesis via root derived callus on MS medium supplemented with BAP and 2, 4-D.
The occurrence of genetic variation is a matter of great concern whereas commercial success in micro propagation depends mainly on the maintenance of clonal uniformity (Bonga, 1987). However abnormalities in tissue culture and in the plants produced from them often increase in frequency with increase culture passages.

2.1.6 Rooting of in vitro regenerated shoots:

For the development of a perfect plantlet, it is essential that the regenerated shoots must develop the roots. The shoots must be transferred to a rooting medium having different hormonal and salt composition from the shoot multiplication medium. However, the nutritive medium for rooting, vary from tissue to tissue as well as species to species. Shoot multiplication was induced on full strength MS medium whereas for in vitro rooting, the salt concentration was reduced to half (Garland and Stoltz, 1981; Zimmerman and Broome, 1981) or a quarter (Skirvin and Chu, 1979). Most often IAA, IBA and NAA have been used for this purpose but IAA and IBA are found to be more effective (George and Sherrington, 1984). Reports on some species of the family Apiaceae, such as cumin (Tawfik and Noga, 2001; Ebrahimie et al., 2003), fennel (Anzidei et al., 2000) and Thapsia gargarica (Makungu et al., 2005) showed the successful use of a medium devoid of plant growth regulators for root induction. Medium fortified with IBA was found effective for rooting in Dalbergia latifolia (Raghava Swamy et al., 1992), Centella asiatica (Banerjee et al., 1999) and Rhinacanthus nastulus (Sudhakar et al., 2006). In Moringa pterygosperma, half strength MS medium supplemented with GA\textsubscript{3} (0.2 mg/l) produced roots within 7 days in 25% cultures. However, better rooting was developed with 3% sucrose and IBA (0.2 mg/l) only (Mohan et al., 1995). Sharma and Padhya (1996) observed root induction in Crataeva nurvala within 7 days on MS medium with low dose of NAA (0.5 µm). Mustafa and Harihara (1997) got limited root in hormone free medium whereas NAA alone was better than the combinations of NAA and IBA in Alpinia galangal. Sharada et al. (2003) achieved 85% rooting on McCown medium (WPM) containing IBA (5 x 10\textsuperscript{-6}M) in Celastrus paniculatus. Higher percentage of root regeneration in adventitious shoots was obtained on MS medium supplemented with 0.1 mg/l IAA in Populus ciliata (Thakur et al., 2005). Ahmed et al. (2007) used different concentrations of IBA, NAA and IAA for rooting and highest rooting percentage (97.66%) was reported on MS medium with IAA (0.1 mg/l). Vadodaria et al. (2007) observed rooting on medium containing 1% sucrose and fortified with NAA (0.1mg/l).
in *Glycyrrhiza glabra*. Sayd *et al.* (2010) reported that, all strengths of MS-medium with 1.0 g/l AC produced rooted shootlet but quarter strength of MS-medium produced the highest rooting, leaf number and shootlet length. *In vitro* developed microshoots were rooted on MS half strength medium supplemented with 2.46 µM IBA in *Streblus asper* (Gadidasu *et al.*, 2011). Thirunavoukkarasu *et al.* (2010) recorded maximum rooting percent with ½ strength MS medium supplemented with 7.35 µM IBA. *In vitro* developed microshoots were rooted on MS half strength medium supplemented with 2.46 µM IBA in *Streblus asper* (Gadidasu *et al.*, 2011). Regardless of IBA concentrations, plantlets in 40.9 µM TDZ produced the best rooting system in *Fragaria ananassa* (Murti *et al.*, 2012).

2.1.7 Hardening and acclimatization of regenerated plantlets:

Most of the species grown under *in vitro* conditions require proper acclimatization process in order to ensure maximum survival rate and vigorous growth when transferred to soil (Hazarika, 2003). The special conditions during *in vitro* culture result in the formation of plantlets of abnormal morphology, anatomy and physiology. After *ex vitro* transfer, these plantlets might easily be impaired by sudden changes in environmental conditions and so need a period of acclimatization to correct abnormalities. Various types of substrates had been used during acclimatization such as soil vermiculites mixture, sterilized sand and soil (Goyal and Arya, 1981; Gulati and Jaiwal, 1996; Philomina and Rao, 1999; Thakur *et al.*, 2001; Sunaina and Goyal, 2000). Hardening of plantlets in *Jatropha curcas* had been achieved by using soil and vermiculate (3:1) by Rajore *et al.* (2002). Sharma *et al.* (2004) transferred *in vitro* raised plantlets of *Ruta graveolens* into pot containing sterile vermiculite and covered with polythene bags to ensure high humidity in order to ensure to acclimatize the plants to the field conditions. The serving plants were transplanted to field after 2 months. In *Leptadenia reticulata*, hardening has been done by transferring the rooted shoots to pasteurized mixture of soil, sand and coco peat (made up from coconut coir) in equal proportions filled in plastic cups (Parabia *et al.*, 2007). Mathur *et al.* (2008) used sterilized soil: sand: organic manure mixture (1:1:1) for acclimatization in pots in the glass house. In micropropagated plantlets of *Tylophora indica*, soil: vermicompost: *Azotobacter* (N2 fixer): *Pseudomonas* (1:1:1:1) showed the highest percentage survival (92%) upon transplantation of plants to the field conditions (Kaur *et al.*, 2011). Girijashankar (2011) used coco peat as hardening medium
and got 75 % survival rate during hardening phase. Faisal *et al.* (2012) noted highest survival rate of *Rauvolfia serpentine* plantlets in soil-rite and lowest in garden soil. Arumugam and Gopinath (2012) transferred the rooted plantlets of *G. superba* into small plastic nursery tray containing vermi compost, sand and red soil (1:2:2) and kept in a mist house. Akram and Aftab (2012) transferred the four weeks old plantlets of *Morus macroura* to plastic pots containing soil: sand: peat moss mixture (1:1:2) under glasshouse conditions.

The most crucial step in the micropropagation is the hardening and acclimatization as it is the process which makes the plantlets capable of tolerating the natural environmental conditions. Successful acclimatization and field transfer with good survival rate of *in vitro* regenerated plantlets have also been reported in *Tamarindus indica* (Knopp and Nataraja, 1990), *Eucalyptus tereticornis* (Das and Mitra, 1990), *Prosopis juliflora* (Nandwani and Ramawat, 1991), *Dalbergia latifolia* (Raghava Swamy *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 disticho-phyllum* (Karuppusamy and Pullaiah, 2007), *Prosopis cineraria* (Kumar and Singh, 2009), *Spondias mangifera* (Tripathi and Kumari, 2010), *Streblus asper* (Gadidasu *et al.*, 2011) and *Glycyrrhiza glabra* (Yadav and Singh, 2012). Hardening and acclimatization of plantlets was done because the plantlets raised under *in vitro* conditions on synthetic carbohydrate supplemented medium under artificial light fail to acclimatize abruptly to rigor of natural environments. So, a careful transfer of plantlets in the soil after hardening and acclimatization is required.

### 2.2 Mycorrhizal inoculation:

The term mycorrhiza was coined by A.B. Frank, a German Forest Pathologist in 1885. Mycorrhiza is the combination of two words, one Greek “mykes” (Mushroom/Fungus) and other latin “rhiza” (roots), it literally means fungus root. It is a general term for fungi forming mutualistic symbiotic association between plant and fungus. About 95% plant species harbour mycorrhizal association.

Plants having branched, fine, long roots with numerous root hairs are less dependent on mycorrhiza for nutrient acquisition (Manjunath and Habte, 1992; Hetrick *et al.*, 1992) than plant with coarse roots (Graham *et al.*, 1991). More than 6000 fungal species are capable of establishing mycorrhizal association of
approximately with 2, 40,000 plant species (Singh, 2007). At least seven different types of mycorrhizal association have been recognized, involving different groups of fungi and host plants with distinct morphological patterns: Ectomycorrhiza, Ectendomycorrhiza, Arbutoid, Monotropoid, Ericoid and Orchidoid mycorrhiza (Harley and Smith, 1983; Mukerji and Mandep, 1998).

2.2.1 **Arbuscular Mycorrhizae:**

Arbuscular mycorrhiza is endomycorrhiza and has got its name from the fungal tree shaped, short lived structure that develops in plant root cells (arbus tree). Earlier, the name Vesicular Arbuscular Mycorrhizae (VAM) fungus was used, but since not all the groups produce vesicles, the term AM fungi is preferred (Friberg, 2001; Smith and Read, 1997; Walker, 1995).

AM fungi are ubiquitous, important for terrestrial ecosystem and have potential applications. AM fungi are found under all climates and in all ecosystems regardless of the type of soil, vegetation or growing conditions. It is found in all angiospermic families, except some families such as Betulaceae, Commelinaceae, Urticaceae etc (Gerdemann, 1975). Families that rarely form arbuscular mycorrhiza are Brassicaceae, Chenopodiaceae, Cyperaceae and Polygonaceae (Gerdemann, 1975; Tester et al., 1987; Meney et al., 1993).

AM fungi are characterized by the presence of intracellular hyphae in the primary cortex which form specific structures called vesicles and arbuscules later on. Arbuscules are small tree like, hyphae filled, invagination of the cortical cells, which provide intimate contact between plasmalemmae of the two symbiotic partners and are presumably the point of material exchange between host plant and fungus. Arbuscules are named by Gallaud (1905) because they looked like trees. Vesicles are thin or thick-walled, globose to subglobose irregular shaped structures terminally or intercalary having inter or intracellular hyphae within the roots. Some of genera of AMF are *Glomus, Acaulospora, Gigaspora, Scutellospora, Entrophospora* and *Sclerocystis*.

Arbuscular mycorrhizal (AM) fungi are one of the most important beneficial micro-organisms in the rhizosphere. AM fungi infect almost all the plants. The AM fungi penetrate the living cells of plants without harming them and form the typical
organs such as vesicles and arbuscules in the root. AMF colonization have great potential in nutrient uptake, water absorption, bicontrol of pathogens, synthesis of growth hormones and establishment of plantlets in adverse soil and climatic conditions (Rai, 2001; Kapoor et al., 2007; Chaudhary et al., 2008; Parkash and Aggarwal, 2009; Kaushih et al., 2011; Parkash et al., 2011a; Singh et al., 2012). Secondary metabolite accumulation in plants under AM symbiosis definitely impels the development of attractive strategies to bring medicinal plants cultivation into a new era for pharmaceutical purposes (Radhika and Rodrigues, 2010).

The plantlets generated in vitro under aseptic conditions, eliminate all microbes including natural symbions also (Gaur and Adholeya, 1999). High mortality is observed when these cultured plants having functionally impaired stomata, poorly developed cuticle and root system ultimately come in association with either symbiotic or pathogenic organisms under ambient condition during ex vitro acclimatization (Yadav et al., 2012). The establishment of mycorrhizal associations during the acclimatization stage can result in reducing the stress of acclimatization and enhance growth of micropropagated plantlets (Lovato et al., 1996; Kapoor et al., 2008; Folli-Pereira et al., 2012). According to Vazquez et al. (2000), inoculation with AMF induced significant impacts on biochemical attributes of in vitro raised plantlets. The increase in contents of secondary metabolites in inoculated plants has also been noticed by Tejavathi et al. (2011). The benefits of VAM fungi have also been demonstrated in various other micropropagated plants of medicinal interest, Scutellaria integrifolia (Joshee et al., 2007), Artemisia annua (Kapoor et al., 2007; Chaudhary et al., 2008), Curculigo orchioides (Sharma et al., 2008), Acorus calamus (Yadav et al., 2011), Spilanthes acmella (Yadav et al., 2012), Punica granatum (Singh et al., 2012), Jatropha curcas (Folli-Pereira et al., 2012). To our knowledge, there is no information available about the role of AM fungi on micropropagated plantlets of G. superba.

2.3 Biochemical attributes:

Plant regeneration from cultured plant tissues is a prerequisite for the application of in vitro techniques for crop improvement. It is often achieved by altering hormone composition and other media components (Kumar et al., 2010). Different tissues are specifically adapted for specialized functions, such as secretion, storage, mechanical support and protection. Differentiation in such tissues involves differences in basic metabolic pathways (Singh et al., 2006). Hence, there is a need to study biochemical aspects underlying initiation of organized development in vitro. The increase in the content of various biochemical parameters in
Micropropagated plants may be due to the effect of different phytohormones in *in vitro* raised plants (Mohapatra *et al.*, 2008).

**Table 2.1 Arbuscular mycorrhizal fungi used as biohardening agent in various micropropagated plantlets**

<table>
<thead>
<tr>
<th>Mycorrhizal species</th>
<th>Host plant</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>G. clarum</em></td>
<td><em>Jatropha curcas</em> L.</td>
<td>Folli-Pereira <em>et al.</em> (2012)</td>
</tr>
<tr>
<td><em>G. mosseae</em>, <em>A. laevis</em> and <em>G. manihotis</em></td>
<td><em>Punica granatum</em> L.</td>
<td>Singh <em>et al.</em> (2012)</td>
</tr>
<tr>
<td><em>G. mosseae</em> and <em>A. laevis</em></td>
<td><em>Spilanthes acmella</em> Murr.</td>
<td>Yadav <em>et al.</em> (2012)</td>
</tr>
<tr>
<td><em>G. mosseae</em> and <em>A. laevis</em></td>
<td><em>Acorus communis</em> L.</td>
<td>Yadav <em>et al.</em> (2011)</td>
</tr>
<tr>
<td><em>Acaulospora laevis</em>, <em>Glomus fasciculatum</em> and</td>
<td><em>Gerbera jamesonii</em></td>
<td>Anisha <em>et al.</em> (2010)</td>
</tr>
<tr>
<td><em>Scutellospora dussii</em></td>
<td></td>
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<tr>
<td><em>G. geosporum</em> and <em>G. microcarpum</em></td>
<td><em>Curculigo orchioides</em></td>
<td>Sharma <em>et al.</em> (2008)</td>
</tr>
<tr>
<td><em>G. aggregatum</em></td>
<td><em>Chlorophyllum borivilianum</em></td>
<td>Mathur <em>et al.</em> (2008)</td>
</tr>
<tr>
<td><em>Glomus mosseae</em>, <em>Sclerocystis</em> and <em>Acaulospora sp.</em></td>
<td><em>Chrysanthemum cinerariifolium</em></td>
<td>Yaseen <em>et al.</em> (2006)</td>
</tr>
<tr>
<td><em>G. mosseae</em>, <em>G. manihotis</em>, <em>G. deserticola</em>,</td>
<td><em>Vitis vinifera</em> L.</td>
<td>Krishna <em>et al.</em> (2005)</td>
</tr>
<tr>
<td><em>Gigaspora gigantia</em> and <em>A. laevis</em></td>
<td></td>
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</tr>
<tr>
<td><em>Glomus</em> and <em>Scutellospora</em> sp.</td>
<td><em>Bacopa monnieri</em></td>
<td>Dhami (2005)</td>
</tr>
<tr>
<td><em>Glomus</em> and <em>Scutellospora</em> sp.</td>
<td><em>Centella asiatica</em></td>
<td>Dhami (2005)</td>
</tr>
<tr>
<td><em>G. fasciculatum</em> and <em>G. etunicatum</em></td>
<td><em>Musa acuminate</em></td>
<td>Rizzardi (1990)</td>
</tr>
<tr>
<td><em>Scutellospora fulgida</em> and <em>Entrophospora</em></td>
<td></td>
<td></td>
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<tr>
<td><em>colombiana</em></td>
<td></td>
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<tr>
<td><em>G. mosseae</em></td>
<td><em>Citrus limon</em> (L.) Burm.</td>
<td>Quatrini <em>et al.</em> (2003)</td>
</tr>
<tr>
<td><em>G. macrocarpum</em> and <em>G. fasciculatum</em></td>
<td><em>Coriandrum sativum</em></td>
<td>Kapoor <em>et al.</em> (2002)</td>
</tr>
<tr>
<td><em>Glomus</em>, <em>Gigaspora</em> and <em>Scutellospora</em> sp.</td>
<td><em>Syngonium podophyllum</em></td>
<td>Gaur and Adholeya (1999)</td>
</tr>
<tr>
<td><em>Glomus</em>, <em>Gigaspora</em> and <em>Scutellospora</em> sp.</td>
<td><em>Draceana sp.</em></td>
<td>Gaur and Adholeya (1999)</td>
</tr>
<tr>
<td><em>G. intraradices</em></td>
<td><em>Fragaria ananassa</em></td>
<td>Hernandez Sebasta <em>et al.</em> (1999)</td>
</tr>
<tr>
<td><em>G. fasciculatum</em> and <em>G. etunicatum</em></td>
<td><em>Alocasia sp.</em></td>
<td>Sivaprasad <em>et al.</em> (1999)</td>
</tr>
<tr>
<td><em>G. deserticola</em></td>
<td><em>Persea Americana</em></td>
<td>Azcon Aguilar <em>et al.</em> (1997)</td>
</tr>
<tr>
<td><em>G. mosseae</em></td>
<td><em>Actinidia delicosa</em></td>
<td>Gribaudo <em>et al.</em> (1996)</td>
</tr>
<tr>
<td><em>G. fasciculatum</em></td>
<td><em>Persea americana</em></td>
<td>Vidal <em>et al.</em> (1992)</td>
</tr>
<tr>
<td><em>Acaulospora sp.</em>, <em>G. mosseae</em> and <em>G. fasciculatum</em></td>
<td><em>Ananas comosus</em></td>
<td>Guillemin <em>et al.</em> (1991)</td>
</tr>
<tr>
<td><em>G. epigaeus</em></td>
<td><em>Vitis sp.</em></td>
<td>Kuo and Li (1987)</td>
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</table>
Sivanesan et al. (2008) noted the increment in total chlorophyll content of the micropropagated *Scorphylaria takeimensis* by the iron source (FeSO₄ and Na₂EDTA) supplied in MS medium. Kumara Swamy et al. (2010) documented the increase in chlorophyll content in the micropropagated plantlets of *Pogostemen cablin* than the mother plant. Similarly, Indra et al. (2011) observed higher level of chlorophyll, protein, starch, carbohydrate and free amino acids in the micropropagated plantlets of *Cassia siamea*. Suganthi Deva Manohari et al. (2011) also observed higher primary biochemical contents in *in vitro* raised plants of *Adenanthera pavonina* as compared to mother plant. Yadav and Singh (2012) also observed significant difference in the chlorophyll content in the leaves of *in vitro* regenerated and wild plants.

Primary metabolites are considered to be building blocks needed for general growth and primary physiological function. They are also considered as the precursors of secondary metabolites. The *in vitro* techniques have gained importance as methods for enhancing the production of secondary metabolites also (Vanisree et al., 2004). AM fungi also have an influence on the primary and secondary metabolism in the host (Maier et al., 1997; Peipp et al., 1997). Tejavathi and Rao (1998) recorded higher chlorophyll content, total proteins and amino acids in micropropagated plants inoculated with *G. fasciculatum*. However, Matsubara et al. (2009) attributed the increase in amino acid contents in strawberry plants in response to *G. mosseae* due to some unknown factors other than mycorrhizal association. There are many reports on the enhancement of secondary metabolism in micropropagated plantlets than the source of explants (Giulietti and Ertola, 1999; Tejavathi and Rao, 1998; Leal et al., 2009). In addition, various studies have demonstrated that AMF can influence phytohormones levels of jasmonate (Hause et al., 2002), terpenoids and carotenoids (Akiyama and Hayashi, 2002; Fester et al., 2002) and phenols (Zhu and Yao, 2004). The sesquiterpenoid ABA reaches considerably higher level in *Glomus* colonized plants over control, indicating the influence of AMF association on the enzymes involved in terpenoid biosynthesis (Danneberg et al., 1993). Kapoor et al. (2002, 2004) also observed enhanced concentration and quality of essential oils on mycorrhizal inoculated *Coriandrum sativum* L. and *Foeniculum vulgare* Mill. For *Mentha arvensis* L. mycorrhizal colonization significantly increases oil content and yield over control (Gupta et al., 2002).

Gupta (1990) compared the production of different colchicinic substances among *G. superba* and *C. autumnale*. He reported extensive range of colchicinic compounds like
colchicines (0.9%), dimethyl-3-colchicine (0.19%), colchicoside (0.82%) and their formyl derivatives in *G. superba*. While these values were found to be less in *C. autumnale* having 0.62%, 0.9%, and 0.39% values respectively. Sivakumar *et al.* (2004) noticed the role of phenylalanine and tyrosine in colchicine production in calluses induced from corm buds. Jha *et al.* (2005) also reported production of forskolin, withanolides, colchicine and tylophorine from plant source using biotechnological approach. Ghosh *et al.* (2009) analysed the colchicine content in the tubers of five different populations of *G. superba* L. Kavina *et al.* (2011) noticed the effect of gibberellic acid and *Psedomonas aerugunosa* in improving the content of colchicine in seeds and tubers.

Secondary metabolite accumulation in plants under AM symbiosis definitely impels the development of attractive strategies to bring medicinal plants cultivation into a new era for pharmaceutical purposes (Radhika and Rodrigues, 2010). To our knowledge, there is no information available about the role of AM fungi on micropropagated plantlets of *G. superba*

**2.4 Genetic Fidelity:**

Genetic fidelity is the maintenance of the genetic constitution of a particular clone through its life span (Lattoo *et al.*, 2006). However, micropropagation protocol is severely hindered due to incidences of somaclonal variations (Kumar *et al.*, 2011). Somaclonal variation mostly occurs as response to the stress imposed on the plant in culture conditions and is manifested in the form of DNA methylations, chromosome rearrangements, and point mutations (Phillips *et al.*, 1994, Martin *et al.*, 2006). The application of sub and supra-optimal levels of growth regulators and the recurrent subculture for indefinite period hinders maintenance of genetic fidelity in the tissue cultured clones (Martins *et al.*, 2004). It is, therefore, imperative to establish genetic uniformity of micropropagated plants to confirm the quality of the plantlets for its commercial utility. Molecular techniques are at present powerful and valuable tools used in analysis of genetic fidelity of *in vitro* propagated plants. Various morphological, cytological and protein markers have been used for the detection of variations in tissue-cultured raised plantlets. Polymerase chain reaction (PCR) techniques like randomly amplified polymorphic DNA (RAPD) (Williams *et al.*, 1990) and inter simple sequence repeats (ISSR) (Zietjiewicz *et al.*, 1994) markers have been favored because of their simplicity, cost-effectiveness, stability, sensitivity, highly reproducible and reliability (Ray *et al.*, 2006). RAPD has proven to be quite efficient in assessing genetic fidelity even in closely related organism such as near isogenic lines
and ISSR technique is also a simple, fast, cost effective and highly reliable technique (Reddy et al., 2002) in which a single simple sequence repeat motif is used as a primer for amplification of region between adjacent but palindromic microsatellites (Zietkiewicz et al., 1994). The use of two types of markers have been successfully applied to amplify different regions of the genome allowing better analysis of genetic stability in several micropropagated crops, such as Musa spp. (Venkatachalam et al., 2007); Amorphophallus albus (Hu et al., 2008); Saussurea involucrata (Yuan et al., 2009); Bacopa monnieri (Antony Cesar et al., 2010); Pogostemon cablin (Paul et al., 2010); Sapindus trifoliatus (Asthana et al., 2011); Citrus jambhiri (Savita et al., 2012); Malus domestica (Pathak and Dhawan 2012).

Bhowmik et al. (2009) detected maximum of 88 and 90% genetic similarity between in vitro raised hardened plantlets and mother stock of Mantisia spathulata and Mantisia wengeri using RAPD markers. Chandrika and Rai (2009) screened thirty two ISSR primers in Ochreinauclea missionis, of which twenty nine primers generated 183 clear, distinct and reproducible bands resulted that all amplicons generated were monomorphic and similar to mother plant. Kumar et al. (2011) screened a total of 48 (32 RAPD and 16 ISSR) primers, out of which 24 RAPD and 13 ISSR primers produced a total of 191 clear, distinct and reproducible amplicons in Simmondsia chinensis. Ghosh et al. (2009) analysed five different populations of G. superba L. using twenty-four arbitrary sequence decamers for comparative assessment of genetic relationships among them. There has been no report on the assessment of genetic fidelity of micropropagated plants of G. superba.