Due to increasing demand of high quality planting material and marketing competition necessitates, concerted efforts must be made to evolve new methods for mass propagation and production of plant species which are high yielding, resistant to pest and disease associated with increased photosynthetic efficiency. Conventional breeding is rather slow and less productive and cannot be used efficiently for the mass multiplication and genetic improvement of medicinal plants. The technology of plant tissue culture offers advantages over conventional methods of propagation for a rapid and large scale multiplication of important plants under \textit{in vitro} conditions irrespective of season with conservation of space and time (Nehra and Kartha, 1994 and Rao \textit{et al.}, 1997). In this chapter an attempt has been made to review the available literature related to micropropagation and integration of arbuscular mycorrizal fungi (AMF) to tissue culture raised plantlets and the impact of NaCl on micropropagation.

2.1 Micropropagation

In nature, the methods of plant propagation may be either asexual or sexual. Sexually propagated plants demonstrate a high amount of heterogeneity since their seed progeny are not true-to-type unless they have been derived from inbred lines. Asexual reproduction, on the other hand, gives rise to plants which are genetically identical to the parent plant and thus permits perpetuation of the unique characters of the cultivars. Multiplication of genetically identical copies of a cultivar by asexual reproduction is called clonal propagation. When clonal propagation is through tissue culture, it is popularly called micropropagation.

Advanced biotechnological methods of culturing plant cells and tissues provide new means for conserving and rapidly propagating valuable plants. 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 conventional propagation method is the principal means of propagation and takes a long time for multiplication because of a low rate of fruit set, poor germination and also sometimes clonal uniformity is not maintained through seeds. \textit{In vitro} methods can be used to produce genetically uniform plants in large numbers, maintain, multiply and transport pathogen free plants safely and economically. This
technology is being extensively used for large-scale production of elite planting material of desired characteristics.

The pioneer experiment was conducted by the great German physiologist G. Haberlandt who is regarded as father of plant tissue culture. He put forward the idea of totipotency of plant cells. Haberlandt in 1898 attempted to grow vegetative cells on Knop’s (1865) salt solution with sucrose and observed obvious growth in the cells but could not succeed because of handling with highly differentiated cells and lack of proper techniques. Hanning (1904) successfully cultured embryos of *Raphanus sativus*, *Raphanus landra*, *Raphanus caudatus* and *Cochlearia danica* on Tollen’s medium and obtained transplantable seedlings.

Although Haberlandt failed, Gautheret, Nobecourt and White in 1939 reported the formation of continuous callus cultures in carrot and tobacco independently of each other. Steward *et al.* (1958) demonstrated the totipotency of higher plant cells in unambiguous terms with their success in forming somatic embryos from cultured root phloem cells. Haberlandt’s hypothesis has now, flowered into a vigorous discipline—“Tissue culture”. This broad term refers to the growth of cells, tissues and organs in synthetic medium under aseptic conditions. Haberlandt had also perceived the concept of growth hormones which he called “growth enzymes” and felt these are released from one type of cells and stimulate growth and developments in other cells.

Van Overbeek *et al.* (1941) demonstrated first time the stimulatory effect of coconut milk on embryo development and callus formation in *Datura*. The possibility of chemical regulation of *in vitro* organogenesis was indicated for the first time by Skoog (1944). Concept of rising of whole plants of *Lupinus* and *Trapaeolum* by shoot tip culture was given by Ball (1946). Morel and Martin (1952) for the first time developed the method for obtaining virus-free dahlia plants from diseased individuals by culturing healthy shoot tips. Muir (1954) reported that it was possible to obtain single cells by transferring callus tissue of tagetes and *Nicotiana* to liquid medium and agitating the cultures on a shaking medium. The nurse culture method to culture single cells was carried out by Muir *et al.* (1954). Skoog and Miller (1957) put forth the concept of hormonal control of organ formation. They demonstrated the discovery of the regulation of organ formation by changing the ratio of auxin to cytokinins. Regeneration of somatic embryos from the nucellus of *Citrus ovulus* was reported by Maheshwari and Rangaswami (1958). The growth of dormant axillary buds can be initiated by exogenous applications of cytokinins (Wickson and Thimann, 1958). The first successful report on the formation of somatic embryos from carrot tissue was achieved by Steward
(1958) and Reinert (1959). Virus free orchid plants were obtained by the process of micropropagation (Morel, 1960). Murashige and Skoog (1962) developed the MS medium which is one of the most widely used nutrient media for the plant tissue culture techniques.

Haploid plant production was reported for the first time by Guha and Maheshwari (1964) from anther culture of *Datura innoxia*. Vasil and Hildebrandt (1965) demonstrated that a single, isolated cell can divide and ultimately give rise to whole plant of *Nicotiana*. Callus formation from excised roots, stems, leaves and cotyledons of *Rauwolfia serpentina* on white’s and MS medium supplemented with coconut milk and 2,4-D was reported by Mitra *et al.* (1965). The origin and development of callus in *Salvadora persica* was studied by Kant and Arya (1967).


curcas (Rajore et al., 2002), Cassia alata (Ramamurthy and Savithramma, 2002), Gloriosa superba (Sachdev et al., 2002), Mentha piperita (Wali and Siddiqui, 2003), Cinnamomum camphora (Babu et al., 2003), Eclipta alba (Gawde and Pratkar, 2004), Syzygium cumini (Rathore et al., 2004), Desmodium ooeijense (Kumari and Shivanna, 2005), Terminalia bellirica (Ramesh et al., 2005), Tylophora indica (Faisal et al., 2005), Santalum album (Sanjaya et al., 2006), Peristrophe bicalyclata (Sharma and Devi, 2006), Ricinus communis (Ahn et al., 2007), Dianthus caryophyllus (Pareek and Kathari, 2007), Vitex agnuscastus (Balaraju et al., 2008), Taxus wallichiana (Datta and Jha, 2008), Lillium longiflorum (Tang et al., 2009), Paederia foetida (Alam, 2010), Picrorhiza kurroa (Jan et al., 2010), Alternanthera sessilis (Gnanaraj et al., 2011), Aegle marmelos (Yadav and Singh, 2011a), Bacopa monnieri (Rout et al., 2011), Mentha piperita (Sujana and Naidu, 2011), Glycyrrhiza glabra (Yadav and Singh, 2012), Stevia rebaudiana (Rathore et al., 2013), Fritillaria imperialis (Rahimi et al., 2013), Achyranthes aspera (Sen et al., 2013) etc. have been propagated through tissue culture.

A large number of horticultural plantations, forest species, important fruit trees and medicinal plants are being propagated in vitro on commercial scale. There are number of factors that affect the success of in vitro regeneration are as follows:

2.1.1 Selection of explant

The maniputability of organ formation in tissue culture is often determined by the choice of the explants. While it is generally accepted that totipotency is characteristic of all the plant cells. Several plant parts can be used as explants. However, each plant organ differs in its growth and regeneration rate because the cells in that organ exist in a particular developmental stage. Organs also differ in their metabolic activity and capacity to transport and utilize growth regulators. Generally, meristematic tissues such as the root tips, stem tips and axillary buds are good explants because they show the most rapid rate of cell division. Also, these tissues have a greater ability for the uptake of growth regulators. The regeneration potential of explants is attributed by the physiological state, age and cellular differentiation among the constituent cells (Murashige, 1974).

The influence of plant material on the growth and development in tissue culture are related to many factors such as genotype, age of the plant, age of the tissue and organ, physiological state of the explants, the state of health of the plant, effects of season throughout the year such as winter and summer, growth conditions such as photoperiod, position of explants within the plant, size of the explants, wound surface area, method of inoculation etc. The use of young and meristematic tissue as in many cases enabled raising of
regenerative cultures when mature and differentiated explants failed to give such response. Juvenility is one of the most important factors influencing the in vitro response of many plant species (Bonga, 1982).

The choice of apical region to initiate in vitro cultures replaces the addition of exogenous auxins and is an alternative to obtaining the highest quantity of plants (Victório et al., 2012). The explant source is a way to take advantage of the plant potential to synthesize auxins in plant apices (Peer and Murphy, 2007). Additionally, the endogenous auxins are mainly biosynthesized at the young developing leaves, shoot apical meristems and plant stems (Bouillé et al., 2007). The regenerated plantlets from cultured callus of Anthurium patulum by culturing leaf, padicle, sapthe and petiole segments were demonstrated by Eapen and Rao (1985). Lal and Ahuja (1989) reported in vitro propagation of Rheumamodi by culturing shoot tip and leaf explant. Clonal multiplication under in vitro conditions of Curcuma sp. and Zingiber officinale via rhizome buds was developed by Balachandran et al. (1990). Desai et al. (2004) reported plant regeneration through somatic embryogenesis from inflorescence explants of Saccharum spp.

Nodal explants from mature plants have been used for regeneration in many plant species, Madhuca latifolia (Bansal and Chibbar, 2000), Melia azedarach (Shahzad and Siddique, 2001), Stevia rebaudiana (Sivaram and Mukndan, 2003), Michelia champaca (Iyer et al., 2005), Stevia rebaudiana (Uddin et al., 2006 and Kumar and Singh, 2009), Cassia angustifolia (Siddique and Anis, 2007), Morus alba (Balakrishnan et al., 2009), Balanites aegyptiaca (Siddique and Anis, 2009), Arbutus unedo (Gomes and Canhoto, 2009), Azadirachta indica (Arora et al., 2010), Spondias mangifera (Tripathi and Kumari, 2010) and Dalbergia sissoo (Ali et al., 2012).

Barthakur and Bordoloi (1992) attempted micropropagation of Curcuma amada from rhizome explants. Rout and Das (1993) reported multiple shoots induction from apical and axillary meristem explants derived from seedlings of Madhuca longifolia. Vegetative buds from young rhizome of Alpinia calcarata was used by Agretious et al. (1996) for direct shoot induction. Mustafa and Hariharan (1997) regenerated Alpinia galangal from excised rhizome bud cultured on MS basal medium supplemented with BAP alone or in combination with auxins, produced multiple shoots. Multiple shoots formation from nodal explants of field grown Canavalia virosa was obtained by Kathiravan and Ignacimuthu (1999). Regeneration via somatic embryogenesis in leaf explant culture of Jatropha curcas has been reported by Sardana et al. (2000). Seeja et al. (2000) reported micropropagation in Cinnamomum verum from mature nodal segments. Indirect organogenesis from nodal explants of Melia azedarach
was reported by Shahzad and Siddiqui (2001). Multiple shoots were initiated from cotyledonary node segments of Acacia catechu (Sahni and Gupta, 2002). Ramamurthy and Savithramma (2002) obtained multiple shoots from axillary buds of Cassia alata. Multiple shoots from nodal segments were developed in Jatropha curcas by Rajore et al. (2002). Ramamo et al. (2002) has developed an in vitro propagation protocol based on axillary bud proliferation from mature female trees of Ceratonia siliqua in MS medium supplemented with BAP and Zeatin. Rubin Jose et al. (2002) obtained multiple shoots from young rhizome segments of Kaempferia galangal. Protocol for callus induction and plant regeneration from hypocotyl, root and cotyledonary segments in Withania somnifera was reported by Rani et al. (2003).

Method for micropropagation of Baliospermum montanum through nodal explants was demonstrated by Johnson and Manikam (2003). A tissue culture propagation system for Curcuma zedoaria was developed by Loc et al. (2005). Shou et al. (2005) developed a protocol for micropropagation of Cinnamomum camphora by the formation of adventitious shoots from the delicate caudex. Arnebia euchroma a highly valued, critically endangered medicinal plant was propagated via somatic embryogenesis (Manjkhola et al., 2005). Pandey et al. (2006) developed a protocol for the regeneration of complete plantlets from nodal explants of Terminalia arjuna. Pasqual and Ferreira (2007) developed pathogen free plantlets of Ficus carica through micropropagation. Multiple shoots were produced from mature nodal segments of Syzigium cumini (Remashree et al., 2007). Soulange et al. (2007) reported multiple shoot induction in Cinnamomum camphora. Sujatha and Hazara (2007) used MS basal medium for induction of multiple shoots from mature tree-derived axillary meristems of Pongamia pinnata. Khalafalla and Daffalla (2008) reported multiple shoots from cotyledonary node segments of Acacia senegal. Micropropagation protocol has been developed by different workers for efficient plantlet regeneration through nodal segments of different plants viz. Eucalyptus polybractea (Goodger et al., 2008), Phoenix dactylifera (Aslam and Khan, 2009), Melia azedarach (Hussain and Anis, 2009), Arbutus unedo (Gomes and Canhoto, 2009) and Oroxylum indicum (Gokhale and Bansal, 2009). Mostafa et al. (2010) developed a protocol for micropropagation of Arbutus andrachne using explants from seedlings. Similarly, Tripathi and Kumari (2010) developed an efficient protocol for Spondias mangifera using cotyledonary nodes. Micropropagation from nodal explants from epicormic shoots of Dalbergia sissoo was developed by Thirunavukkarasu et al. (2010). Khalafalla et al. (2011) also reported the intact embryos cultures of Bascia senegalensis. Clonal propagation of Acacia auriculiformis through axillary buds was reported by

Seasonal conditions at the time of explants collection may influence the in vitro growth of explants, phenolics exudation and degree of contamination (Siril and Dhar, 1997). There is increasing evidences that seasonal differences influence the regulation of cell cycle and this can affect morphogenetic processes (Anderson *et al.*, 2001). Gupta *et al.* (1980) noticed the seasonal effects on regeneration of *Tactona grandis*. The nodal segments of *Eucalyptus tereticornis* collected during July to September were more responsive because of negligible phenolic exudation from explants as compared to that collected in October-November and May-June due to high amount of phenolic exudation (Das and Mitra, 1990). Excellent regeneration has been reported in plant species during spring season (March-May) when reserve food material is made available and helps the plants to sprout and bloom (Bhatt and Todaria, 1990). The nodal explants harvested during the months of March-April and August-October was found to be the best for cultures establishment of *Capparis deciduas* (Deora and Shekhawat, 1995). Bansal and Chibbar (2000) observed best response from nodal segments of *Madhuca latifolia* in the month of May. The collection of exapnts during a relatively milder weather condition (December-March) was best for promoting survival of explants in *Acacia sinuate* (Vengadesan *et al.*, 2003). Sharma *et al.* (2003) also observed best explants response in the month of October and November with maximum number of shoot buds in *Crataeva adansonii*. The best shoot initiation response was reported from November-February when the trees produced fresh sprouts and it was rare in explants collected during other periods in *Callophyllum apetalum* (Nair and Seeni, 2003). Nodal explants of *Myrica esculenta* collected during winter (November-December) gave the maximum response (Bhatt and Dhar, 2004). The nodal segments of *Wrightia tinctoria* collected during March-June from young lateral branches showed maximum bud break response (Purohit and Khuda, 2004). In *Holarrhena antidysnterica*, nodal explants showed maximal morphogenic response from May-July, and declined in subsequent months from October-February (Kumar *et al.*, 2005). Singh and Goyal (2007) observed that the season between August-October was best for explants collection in *Salvadora oleoies*. Pati *et al.* (2008) observed that nodal explants of *Aegle marmelos* excised during September-October was found ideal because most of the explants showed bud break whereas bud break frequency reduced in other months. The
cultures of *Melia azedarach* initiated during March exhibited the best response not only in terms of the frequency of bud break but also in shoot vigor (Hussain and Anis, 2009). The explants of *Maerua oblongifolia* collected during the months of July-August responded best *in vitro* as compared to explants harvested in any other months of the year (Rathore and shekawat, 2011). The nodal, inter-nodal segments and shoot apices of *Ficus religiosa* collected in May-June gave maximum response (Siwach *et al.*, 2011).

The variation in regenerative behaviour among explants is sometimes attributed to the age of the tissue or organ and the extent to which constituent cells are differentiated. Thorpe and Biondi (1984) also reported that the physiological state of the tree has been known to influence the behaviour of explants in culture. The capacity for clonal propagation is closely linked with the genetic and physiological factors that control the transition from juvenile to mature growth in plant (Bonga, 1982).

The orientation of explants also plays an important role in regeneration potential. The horizontal position of the explants has been reported to promote adventitious shoot formation in many higher plants (Pierik, 1987). McClelland and Smith (1990) reported that the horizontal orientation of explants produced the more shoots per explants in plant species viz. *Ameifanchier spicata, Acer rubrum, Border forsythia* and *Betula nigra*. Similar observations on the influence of explants orientation have also been made for other plant species including *Pyrus communis* (Lane, 1979) and tamarindus (Jaiswal and Gulati, 1991). The horizontal orientation of nodal explants of *Fraxinus angustifolia* reported the highest multiplication rate (Perez-Parron *et al.*, 1994). The orientation of disc in the floal stem was the most important factor affecting shoot regeneration in *Crinum macowanii* (Slabbert *et al.*, 1995). Sometimes the vertically placed nodal explants differentiated more shoots than explants placed horizontally. Bansal and Chibbar (2000) observed that vertically placed nodal segments of *Madhuca latifolia* differentiate more shoots than explants placed horizontally. This could be possible due to the influence of polarity on growth regulators transport and direct supply of nutrients (Durzan, 1984). Shimada *et al.* (2007) reported that the frequency of adventitious bud formation in begonia was dependent on the position of leaf explants on the medium. The abaxial orientation of leaf explants of *Bacopa monnieri* on medium can induce fast shoot bud regeneration (Joshi *et al.*, 2010).

There is a large importance of the size of explants to be cultured. Larger the explants, poor will be the response. The small explants are more easily directed by the substances contained in the medium. Okazova *et al.* (1967) reported that small explants are more likely to form callus while larger explants maintain greater morphogenetic potentiality. This may be
due to the available food reserves and growth regulators which proved useful in the initiation of new growth (Anderson, 1980).
The reproductive parts were also used for the regeneration. Regeneration through inflorescence calli was observed in *Aerva tomentosa* (Murgai, 1959). Vazquez and Short (1978) reported the callus induction from floral parts of *African violet*. Immature inflorescences of ginger resulted into plantlets directly without intervening callus phase when cultured on MS medium (Babu *et al.*, 1992). Zhong *et al.* (1993) developed the regeneration protocol from inflorescence pieces of *Beta maritima*. The highest numbers of shoots were regenerated from immature floral stems of *Crinum macowanii* (Slabbert *et al.*, 1995). Hannweg *et al.* (1996) cultured the pieces of inflorescence stems of *Bowiea volubilis*. Tyagi *et al.* (2005) induced direct somatic embryogenesis from mature zyotic embryos of *Capparis deciduas*. Asnita and Norzulaani (2006) reported shoot-like structures from the male inflorescence of *Musa acuminate* cultured on MS medium supplemented with BAP. Shankramurthy and Krishna (2006) observed the luxuriant mass of callus on MS medium supplemented with IBA and Kinetin from immature ovaries of the inflorescence segments of *Embelia ribes*. Sharma and Mohan (2006) developed a novel method of shoot regeneration of *Chlorophytum borivilianum* from immature floral buds. Inflorescence apices are suitable explants for the rapid in vitro propagation of *Musa* species (Resmi and Nair, 2007). Nirmal and Sehgal (2010) reported the regeneration of *Ocimum sanctum* using young inflorescences of mature plants.

### 2.1.2 Culture media

Growth and morphogenesis of plant tissues in vitro are largely governed by the composition of the culture media. Although the basic requirements of the cultured plant tissues are similar to those of whole plants, in practice, nutritional components promoting optimal growth of a tissue under laboratory conditions may vary with respect to the particular species. Media compositions are therefore formulated considering specific requirements of a particular culture system and a number of media have been devised for specific tissues and organs. In vitro growth of plants is largely determined by the composition of the culture medium. The importance of nutrition in plant tissue culture is initially reported by Gautheret (1955). The main components of most plants tissue culture media are mineral salts, sugars as carbon source and water. Other components may include organic supplements, growth regulators and a gelling agent (Gamborg *et al.*, 1968 and Gamborg and Philips, 1995). Although, the amounts of the various ingredients in the medium vary for different stages of culture and
plant species. During the past decades, many types of media have been developed for plant tissue culture (Torres, 1989). 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. As such, no single medium can be suggested as being entirely satisfactory for all types of plant tissues and organs. Different culture media are proposed by the different scientists from time to time and varies in salt concentrations. Some of the earliest plant tissue culture media were developed by White (1934). All subsequent media formulations are based on White’s and Gautheret’s media. Some common media used to fulfill the requirements of cultured tissue are MS (Murashige and Skoog, 1962), Gamborg (1968), Nitch and Nitch (1969), Gresshoff and Doy (1972), Eeuwens (1976) and Lloyd and McCown (1980). Murashige and Skoog (1962) is the most widely used medium, especially in procedures where plant regeneration is the main objectives.

There are some examples where modified MS medium have also been used viz. *Moringa pterygosperma* (Mohan et al., 1995), *Hovenia dulcis* (Echeverriegaray et al., 1998), *Lagerstroemia reginae* (Sumana and Kaveriappa, 2000) and *Bambusa vulgaris* (Ndiaye et al., 2006). Adventitious shoots were induced from the hypocotyls explants of *Sesbania rostrata* on Nitch’s medium (Nitch, 1969). Mukhopadhyay and Mohanram (1981) used Gamborg’s B5 medium for the multiplication of *Dalbergia sissoo*. Whereas, Datta and Datta (1983) obtained multiple shoots from nodal explants of *Dalbergia sissoo* on MS medium supplemented with vitamins of Gamborg’s B5 medium and NAA. Reddy et al. (1997) also used MS and B5 media for callus initiation in *Ricinus communis*. Muralidharan and Mascarenhas (1987) reported somatic embryogenesis in *Eucalyptus citriodora* on semisolid agar based B5 medium supplemented with NAA and increased sucrose concentration (5%). Dewan et al. (1992) also observed the higher number of shoots on B5 medium in *Acacia nilotica*. Sarasan et al. (1994) used MS and B5 medium for induction of callus and somatic embryogenesis in *Hemidesmus indicus*. Agretious et al. (1996) observed better shoot multiplication on MS medium as compared on B5 and White medium for regeneration in *Alpinia calcarata*. Babu et al. (2000) cultured nodal explants of *Murraya koengii* for regeneration on woody plant basal medium. Bhargava et al. (2003) reported the formation of globular proembryogenic mass of callus on MS medium and then transfer to B5 medium for fragile snowy callus in *Phoenix dactylifera*. The highest frequency of shoot bud proliferation from the cultures of *Crataeva adamsonii* was observed on MS medium followed by LS medium (Sharma et al., 2003). Purohit and Kukda (2004) reported the maximum number of shoots on MS medium followed by WPM, B5 and White’s media in *Wrightia tinctoria*. Among the five different basal media viz. B5, Nitch,
WPM, MS and Knop’s media used for *in vitro* shoot multiplication from nodal explants of *Holarrhena antidysenterica* MS medium was the most effective (Kumar *et al.*, 2005). Moreover, shoots of *Mucuna pruriens* were much longer and more vigorous and produced more in number on half-strength MS medium than in B5 medium (Faisal *et al.*, 2006). Chorabik (2007) used the two types of media (MS and SH) containing macro and microelements, enriched with myoinositol glutamine, casein hydolysate, vitamins and sucrose for the micropropagation of *Abies grandis*. Multiple shoots were induced from the nodal segments of *Syzigium cumini* inoculated on WP medium (Ramashree *et al.*, 2007). Tamta *et al.* (2008) reported the highest number of shoots regenerated on *Quercus semecarpifolia* on WP medium. Hazeena and Sulekha (2008) used MS medium for callus induction and plantlet regeneration using cotyledons explants of *Aegle marmelos*. Park *et al.* (2008) reported adventitious shoot formation in *Salix pseudolasiogyne* in WP medium supplemented with BAP, Zeatin and GA3. Lamrioui *et al.* (2009) tried MS (1962) and Knop (1965) medium for the *in vitro* germination of *Prunus ovium*. Thirunavoukkarasu *et al.* (2010) used MS and WP medium for plant regeneration through nodal segments of *Dalbergia sissoo*. The best *in vitro* root development in *Warburgia ugandensis* was observed on half strength WP medium (Kuria *et al.*, 2012).

The major problem during establishment phase is the incidence of heavy microbial contamination, especially when the explants are collected form field grown material. Therefore, surface sterilization is the pedestal of aseptic culture initiation. Contamination in tissue culture can originate from two sources, either through carry over microorganism on the surface of the explants or in the tissue itself (endophytic microorganisms). Although in meristem culture, depending on meristem size most of the microorganisms are eliminated. Whereas in leaf, petiole and stem explants, the infection is carried over to the cultures. Before establishment of the culture the explants are surface-sterilized for the removal of its external contaminants. These can be easily removed from the explant by washing in running tap water for 30-90 min. (Khosh-khui and Sink, 1982). Other sterilants like sodium hypochlorite (NaOCl) have also been utilized (Joshi *et al.*, 20102). Pre-culture dipping of axillary buds in carbendazim and 8-HQC for 3 to 4 hour reduced both bacterial as well as fungal contaminations (Prasad, 1993). Fabiola (2006) disinfected seeds of *Piper auritum* by using 70% ethanol and HgCl2 or commercial bleach (Yuhan Clorox, 4% sodium hypochlorite). The twigs of *Clematis gouriana* were thoroughly washed under running tap water for 25-30 min. and then rinsed in a solution containing the surfactant tween-20 (2 drops in 100 ml solution). Subsequently, they were surface sterilized with 0.1% (w/v) HgCl2 solution for 2-3 min.,
followed by 3 to 5 rinses with sterile distilled water in a clean air cabinet (Rajanaika and Krishna, 2008). Haque et al. (2009) used 0.05% fluconazole, 0.1% bavistin and 0.1% endosulphan and 1% sodium hypochlorite for sterilization. Hossain et al. (2010) disinfected the seed of *Perilla frutescens* L. Britton by using 70% ethanol for 1 min. then washed several times with distilled water. The seeds were treated with different concentrations of HgCl$_2$ or commercial bleach (Yuhan Clorox, 4% sodium hypochlorite). Gailite et al. (2010) washed the seeds of *Saussurea esthonica* with soap, soaked in 0.2% KMnO$_4$ solution for 40 min., sterilized in bleach: distilled water (1: 1) for 6 to 10 min. followed by three rinses of sterile distilled water. Rhizome buds of *Zingiber montanum* were selected as the initial explants by Hamirah et al. (2010), immersed rhizome buds in 75% (w/v) ethanol for one min. without rinsing. These were agitated in 20, 30 or 40 % (w/v) clorox (5.25 % w/v sodium hypochlorite). Sarin and Bansal (2011) applied HgCl$_2$ (0.1%) for 3-5 min. with continuous shaking to sterilize leaf, node and internode of *Adhatoda vasica* and *Ageratum conyzoides*. Mehta et al. (2012) applied tween-20 to *Mentha piperita* to remove the superficial dust particles as well as fungal and bacterial spores from node explants and were washed in running tap water and then washed again thoroughly by adding a few drops of tween-20. They were surface sterilized with 0.1% HgCl$_2$ for 5 min. followed by rinsing them five times with double distilled water inside the laminar air flow chamber. Sen et al. (2013) applied bavistin at different concentrations to sterilize leaves, internodes and roots of *Chyranthes aspera* followed by treatment with 10% teepol/tween-20 for 2 min. Then the explants were sterilized in 70% ethanol for 45 seconds and finally with 0.1% HgCl$_2$ for 2-3 min and washed 3-4 times with sterile double distilled water. The dry bulbs of *Fritillaria imperialis* were immersed in 70% ethanol for 1-2 min. then were soaked in benomyl (0.5g/l) for 30 min, followed by 5 min. rinses in sterile distilled water. They were immersed in 20% sodium hypochlorite (NaOCl) for 15 min. Rahimi et al. (2013).

One of the most common problems associate with the *in vitro* establishment of plant species is the deleterious effects of oxidized phenols (Zeweldu and Ludders, 1998). Phenols are ubiquitous compounds produced in plant system are known to increase or decrease with growth and maturity stages and also due to stimulus of biotic stresses (Compton and Preece, 1986). Tissue browning and blackening are also one of the major problems for *in vitro* culturing of many economically important plants. Oxidized phenolic compounds may inhibit enzyme activity and result in darkening of the culture medium and subsequent lethal browning of explants (Yildiz et al., 2007). Liquid media can be used to reduce phenolic oxidation. Several authors have also described the phenolics are negatively related to *in vitro*
proliferation (Ozyigit et al., 2007). Another group of authors pointed out the positive correlation between phenolics and totipotency (Thomas and Ravindra, 1999). They stated that phenolics affect plant tissue culture response (shoot induction) positively. Muscolo et al. (2001) showed that phenolics affect respiratory enzymes in seed germination and total phenols amount change in different stages of germination of *Fagus sylvatica* and *Pinus laricio*. *In vitro* growth of explants, phenolic exudation and degree of contamination may be influenced by seasonal conditions at the time of explants collection. The nodal segments of *Eucalyptus tereticornis* collected during July to September were more responsive to micropropagation because of negligible phenolic exudation from explants as compared to that collected in October-November and May-June due to high amount of phenolic exudation (Das and Mitra, 1990). Phenolic concentration is frequently affected by several internal and external factors (Zapprometov et al., 1989). Some stress factors like drought, water, radiation, and pathogen infection from injured surfaces affect concentrations of the phenolics in plants (Kefeli et al., 2003). Like other woody species, *Stevia* tissues exhibit high levels of polyphenols (Shukla et al., 2009). Oxidative stresses, which can be directly caused by various aspects of *in vitro* culture, may contribute to failure of the explants development. Moreover, explants contain endogenous substances which exude from the cut surfaces in to the medium are capable of inhibiting development and causing browning of uncontaminated excisions (Hartmann and Kester, 1983). Yu and Meredith (1986) observed a strong negative correlation between *in vitro* survival and pre-existing phenolic content of the explants. Browning can be minimized by adding antioxidants or phenol absorbents e.g. ascorbic acid, glutathione, activated charcoal and polyvinylpyrrolidone (Matkowski, 2000) or by transferring explants into new culture media on regular intervals.

Plant cells and tissues in the culture medium lack autotrophic ability and therefore, need external carbon sources for energy. Even tissues, which are initially green or acquired green pigments under special conditions during culture period, are not autotrophs for carbon. The addition of external carbon source to medium enhances proliferation of cells and regeneration of green shoots. Sucrose is the most widely used carbon source for tissue culture. Sucrose as a carbon source supports growth of plant cells in culture (Gamborg and Phillips, 1995). A sucrose concentration of 1-5% is generally used for *in vitro* tissue culture because it is also synthesized naturally by the tissue (Pierik, 1987). The photosynthetic activity of the plantlets of rose (Langford and Wainwright, 1987), carnation (Kozai and Kubota, 1988) and strawberry (Hdider and Desjardins, 1994) grown *in vitro* depends on the amount of carbon in the medium. Jan et al. (2010) reported that the highest net
photosynthetic rate was obtained when *Picrorhiza kurroa* plantlets were grown in media containing 3% sucrose. MS medium supplemented with 2% sucrose was optimal for culturing of shoot tips of *Tamarindus indica* (Kopp and Nataraja, 1990). Marino *et al.* (1993) reported *in vitro* proliferation and rooting capacity of *Prunus armeniac* on modified MS medium enriched with sucrose (58.4 mM) or sorbitol (116.8 mM) as main carbon energy sources. Bennett *et al.* (1994) reported that 2% sucrose is sufficient for multiplication and rooting in *Eucalyptus globules*. Franca *et al.* (1995) found that 3% sucrose is effective for shoot initiation from cotyledonary node of *Strychnodendron polyphyllum*. Julkunen-Tiitto (1996) reported that willow plantlets cultured *in vitro* with 8% sucrose died within a few days without any growth. Kumari *et al.* (1998) observed that 2% sucrose has been found more effective for the development of globular embryos in *Terminalia arjuna*. Khalafalla *et al.* (2007) found that 3% sucrose is required for multiple shoot induction in *Vernonia amygdalina*. Deb (2001) reported the germination of somatic embryos of *Melia azedarach* on MS medium containing 2% sucrose. Rout *et al.* (2011) reported the induction of maximum number of shoots from leaf explants of *Bacopa monnieri* L. on MS medium containing 3% sucrose. The medium containing 4% sucrose significantly increased the number of secondary embryos in *Juglans regia* (Vahdati *et al.*, 2008). Jain *et al.* (2008) reported that 3% sucrose was preferred carbon source both in terms of growth and preventing shoot tip necrosis compared glucose, maltose and fructose at equimolar concentration in *Harpagophytum procumbens*. Although sucrose has been the carbohydrate of choice in the vast majority of work on *in vitro* shoot induction and shoots development, glucose supports equally good growth, while fructose is less efficient. Other carbohydrate such as lactose, galactose, raffinose, maltose, cellobiose, melibiose and trehalose are generally poor source of carbohydrates (Razdan, 2003). Further, Tiwari (2010) investigated the effect of sucrose (3% and 1.5%) on shoot proliferation. In *Stevia*, most studies on micropropagation were undertaken with 3% sucrose (Hossain, 2008). Rathore *et al.* (2013) studied the effect of various concentration of sucrose on shoot regeneration in *Stevia* cultivars.

Growth additives such as activated charcoal (AC), silver nitrate, silver thiosulphate, ascorbic acid, jasmonic acid and polyamines can not strictly be defined as plant growth regulators but they exert growth modulating effects and may play a novel mean of overcoming recalcitrance problems of plants (Benson, 2000). Mittal *et al.* (1989) obtained multiple shoots from axillary buds of *Accacia auriculiformis* on Gamborg’s (B5) basal medium supplemented with coconut milk and BAP. Sita and Swamy (1992) also supplemented growth adjuvants like coconut milk, casein hydrolysate and adenine sulphate to
the media for direct organogenesis and somatic embryogenesis in *Dalbergia latifolia*. Multiple shoot induction was reported on MS medium supplemented with coconut milk (5-15%) in *Alpinia galangal* (Mustafa and Hariharan, 1997). Deshpande *et al.* (1998) observed that the MS medium supplemented with 1-2mg/l of adenine sulphate is sufficient for bud break and multiple shoot induction in *Ficus religiosa*. Deb (2001) used casein hydrolysate (200 mg/l) for the induction of embryogenic callus from imbibed seeds of *Melia azedarach*. Kaur *et al.* (1996) reported maximum shoot bud induction from the cotyledonary nodal explants of *Acacia senegal* on MS medium supplemented with adenine sulphate (25.0 mg/l), ascorbic acid (10.0 mg/l) and glutamine (146.0 mg/l). Nandagopal and Kumari (2006) used MS medium supplemented with adenine sulphate for the organogenesis of *Cichorium intybus*. Srivastava *et al.* (2006) reported shoot differentiation from the cotyledonary nodes and leaves segments of *Cassia senna* when cultured on MS medium supplemented with BA, adenine sulphate and coconut milk. Maximum shoot proliferation was achieved from nodal explants on MS medium supplemented 135.7 mM adenine sulphate (Vengadesan *et al.*, 2003). Addition of adenine sulphate improved shoot multiplication in *Acacia catechu* (Kaur *et al.*, 1998) and *Dracaena marginata* (El-Sawy *et al.*, 2000). Reddy *et al.* (2006) obtained the maximum shoot proliferation from nodal explants of *Azadirachta indica* inoculated on MS medium supplemented with 40mg/l adenine sulphate, 100 mg/l glutamine and 10 mg/l thiamine HCl. The addition of casein hydrolysate significantly increased the number of shoots per explants in *Crataeva nurvala* (Babber *et al.*, 2009) and *Pongamia pinnata* (Belide *et al.*, 2010). Negi *et al.* (2011) observed enhanced shoot growth in *Cassia auriculata* by adding adenine sulphate (25.0 mg/l), ascorbic acid (20.0 mg/l) and L-glutamine (150.0 mg/l).

Activated charcoal is often added to plant tissue culture media because of its beneficial effects on many aspects of *in vitro* regeneration (Thomas, 2008). The beneficial effects of activated charcoal may be attributed to its irreversely adsorption of inhibitory compounds in the culture medium and substantially reduce the toxic metabolites, phenolic exudation and exudates accumulation (Thomas, 2008). Gulati and Jaiwal (1996) used 1% activated charcoal in medium for root induction in *Dalbergia sissoo*. Deshpande *et al.* (1998) also observed the effects of activated caharcaol (0.3% w/v) on shoot elongation in *Ficus religiosa*. Mao *et al.* (2000) also reported the effect of activated charcoal on *in vitro* root induction of *Litsea cubeba* (Sharada *et al.*, 2003) in *Celastrus paniculatus*. Similarly, Dibax *et al.* (2010) found that addition of activated charcoal to the culture medium for regenerating *Eucalyptus* enhanced the elongation of shoots and made the leaves dark green and vigorous. Activated charcoal effects have been attributed to various factors; darkening of the media...
(Proskauer and Berman, 1970), removal of inhibitory compounds (Fridborg et al., 1975) and adsorption of growth regulators (Johansson et al., 1982). The positive effects of AC on the root formation ability of the microshoots in the rooting media can be attributed to reduction of light at the base of the shoots, thus providing an environment conducive to the accumulation of photosensitive auxin or cofactors (Druart et al., 1982). polyvinylpyrrolidone (PVP) has also been included in media to avoid explant browning (Amin and Jaiswal, 1988). Agarwal and Kanwar (2007) reported maximum root development in Morus alba on MS medium supplemented with 0.005 g/l AC. Addition of AC into medium significantly improved the growth of regenerated shoots of Populus trichocarpa (Kang et al., 2009). A high concentration of AC (5%w/v) was optimum for the induction of roots from the regenerated plants of Commiphora wightii (Kant et al., 2010). In Accacia nilotica, the highest number of shoots and their elongation was achieved when 200 mg/l AC was added in MS medium (Dhabhai and Batra, 2010). Highest root development response of regenerated shoots of Morus macroura was observed in half strength MS medium supplemented with 4.0 µM IBA and 0.1% AC (Akram and Aftab, 2012). Glutamine, thiamine and AC were used as additives, which enhance the number of bud break and number of microshoots produced per explant. In presence of additives, microshoots look healthier with dark green leaves and thick stem (Joshi and Dhavan, 2007; Kant et al., 2010; Jeyachandran and Bastin, 2013 and Nandini et al., 2013).

Gelling or solidifying agents are used for preparing semi-solid or solid tissue culture media. Agar-agar is most commonly used at the concentration of 0.8% in culture medium. Use of high concentration of agar makes the medium hard and prevents the diffusion of nutrients into tissues. In static liquid medium, the tissue or cells become submerged and die due to lack of oxygen. Gels provide a support to tissues growing in static conditions. The required concentration of agar should be established systematically by considering specific needs of each case. Yamuna et al. (1993) used 0.6% agar during regeneration of Caphaelis ipecacuanha. Mroginski et al. (2003) added 0.7% agar to solidify the media used for micropropagation of Toona ciliate. Uddin et al. (2005) used 0.5% agar for the solidification of media used for the micropropagation of Peltophorum pterocarum. Normally 0.5 to 1% agar is used in the medium to form a firm gel. A change in agar concentration affects the nutrient availability. A large number of plant species have been successfully culture in vitro on MS medium solidified with agar viz. Tylophora indica (Faisal et al.,2005), Cichorium intybus (Nandagopal and Kumari, 2006), Aristolochia indica (Soniya and Sujitha, 2006), Peristrophe bicalyclata (Sharma and Devi, 2006), Ricinus communis (Ahn et al.,2007),
Dianthus caryophyllus (Pareek and Kathari, 2007), Vitex agnuscastus (Balaraju et al., 2008), Taxus wallichiana (Datta and Jha, 2008), Lillium longiflorum (Tang et al., 2009), Paederia foetida (Alam, 2010), Picrorhiza kurroa (Jan et al., 2010), Alternanthera sessilis (Gnanaraj et al., 2011), Aegle marmelos (Yadav and Singh, 2011), Bacopa monnieri (Rout et al., 2011), Withania somnifera (Rout et al., 2011), Glycyrrhiza glabra (Yadav and Singh, 2012) and Stevia rebaudiana (Rathore et al., 2013). Gelatin at high concentration (10%) can also be used as a gelling agent, but has limited use because it melts at low temperature (25°C). Rahman et al. (1992) reported that 0.6% of agar was optimum for root induction in rose. Other compounds that can be utilized as a gelling agent’s are alginate, phytigel, gelrite and mathacel (Razdan, 2003). In vitro regeneration of Ornithogalum virens was achieved on MS medium solidified with 0.8% bactoagar (Naik and Nayak, 2005). Fatima and Khan (2010) studied the effect of agar 0.8% and gelrite 0.2% concentration on shoot proliferation of Stevia. It was reported that optimum growth of shoots occurred at 0.8% agar concentration. Further, Tiwari (2010) applied 0.6% agar to solidify the media. The shoots become succulent in nature at low concentration 0.6% of agar and at higher concentration 1-1.2%, the rate of shoot bud regeneration declined. The medium containing 3% sucrose was solidified with 0.8% agar (Mehta et al., 2012 and Chakravarthy, 2013).

Plant cells and tissues require optimum pH for growth and development in the culture medium. The pH of culture medium affects the availability of nutrients to the growing explants which influence their growth and multiplication rate. Aswath and Chaudhary (2002) studied the effect of pH on shoot proliferation and reported that the optimum pH for shoot proliferation and shoot development was between 5.6-5.7. Further, Modh et al. (2002) reported that multiplication rate was more adversely affected at lower pH than at higher pH. They obtained best results at 5.6 pH. Most of the researchers have adopted pH range of 5.6 to 5.8 for in vitro propagation of various horticultural crops Amygdalus communis (Gürel and Gülsen, 1998), Fagus sylvatica and F. orientalis (Cuenca and Vieitez, 2000), Ornithogalum virens (Naik and Nayak, 2005), sugarcane (Khan et al., 2006), Gossypium hirsutum (Ozyigit et al., 2007), Pinellia ternata (Peng et al., 2007), wheat (Javed and Ikram, 2008), Phoenix dactylifera (Alkhateeb, 2008), apple rootstock (Bahmani et al., 2009), Morus alba (Balakrishnan et al., 2009), Acorus Calamus (Ahmed et al., 2010), Saussurea esthonica (Gailite et al., 2010) Datura stramonium (Amiri and Kazemitabar, 2011), Gossypium hirsutum (Chakravarthy, 2013), Rumex vesicarius (Nandini et al., 2013), Jatropha Curcas (Gopale and Zunjarrao, 2013).
2.1.3 Culture conditions

The major environmental factors in tissue culture are light and temperature which affects the vascular tissue differentiation. The illumination of cultures is considered in terms of intensity, length of the daily exposure period and the quality. Most of the cultures grow well within a wide range of photoperiod, light intensities and optimal temperature (White and Risser, 1964). In vitro cultures were maintained under controlled conditions of temperature (25±2°C), light (2000-2500 lux) for 16/8 hours light/dark photoperiod provided by fluorescent tubes and 60-70% humidity (Mehta et al., 2012). It appears that the best light exposure period for a given tissue culture is dependent upon the intensity of the illumination employed, and probably other factors. Light intensity has influence on biological effectiveness of the growth regulators added to the growth medium as well as to affect the endogenous hormone balance in the tissues. Murashige (1977) reported optimum root and shoot formation from tobacco callus which involved a 16 hours daily light with intensity of 1000 lux. Gupta et al. (1981) reported multiple shoots production when terminal buds from twenty years old tree of Eucalyptus citridora were cultured on MS medium at 15°C in continuous light followed by culture at 25°C with 16 hours photoperiod. The effect of light and cytokinins interaction on cultured cotyledon explants of radiata pine was studied by Victor et al. (1984). A high rate of multiplication had been achieved on MS medium with long day photoperiod in Ranunculus asiaticus (Pugliesi et al., 1992). A continuous light elicited only a low response (20%) in somatic embryogenesis from leaf explant of Jatropha curcas, 40% in complete darkness and optimum under a photoperiod of 16 hours (Sardana et al., 2000). In Eucalyptus tereticonis, at a slightly higher temperature (30-32°C) a high rate of multiplication has been achieved on MS medium (Das and Mitra, 1990). Calleberg and Johansson (1993) studied that direct regeneration was mostly stimulated when the anther cultured was incubated at 20°C. Rajore et al. (2002) reported multiple shoot formation on MS medium at 25±2°C in 16 hours photoperiod in Jatropha curcas. Formation of multiple shoots at 25±2°C and 16/8 hours light/dark period under light intensity of 3000-4000 lux has been reported in Azadirachta indica (Shekhawat et al., 2002). Azad et al. (2005) maintained the cultures at 25±1°C under an illumination of cool-white fluorescent tubular lamp with a light intensity of 50 µmol m⁻² s⁻¹ for 16 hours photoperiod. Geekiyanage et al. (2006) noticed the effects of photoperiods, light intensity and gibberellic acid (GA₃) on adventitious shoot regeneration from spinach cotyledons and found that the effects of photoperiod and GA₃ on shoot regeneration were significant at high light intensity of 90-100µmol m⁻² s⁻¹. Peng et al. (2007) maintained the cultures at 25±2 °C under lighting with cool white fluorescent tubes at
an intensity of about 2500 lux. Girijashankar (2011) incubated the cultures of *Acacia auriculiformis* under 16 hour photoperiod and 8 hour dark with light intensity of 50 µE/m²/s provided by white fluorescent tube lights and at the temperature 28±2°C. Negi et al. (2011) applied a light regime of 14 hours with 100 µmol m⁻²s⁻¹ light intensity provided by cool-white fluorescent tubes at 25±2°C followed by 10 hour dark period to the cultures of *Cassia auriculata*. The culture vials of *Dendrobium* orchid were maintained in a growth room and allowed to grow at 25±1°C under 16 hours photoperiod illuminated with fluorescent tube of 2000-3000 lux (Parvin et al., 2009). Kowalska et al. (2012) placed the culture tubes at a temperature of 20°C, with a photoperiod of 16 hour illumination at about 30 µmol×m⁻².sec⁻¹ and 8 hour darkness. Cultures were incubated at 22±2°C with a photoperiod of 16 hours light and 8 hours dark at 2000-3000 lux light intensity of cool white fluorescent light (Haq et al., 2013). Further, Sen et al. (2013) maintained the *in vitro* cultures at (25±2°C) and 60%-70% relative humidity, and the light intensity is 3000 lux with a photoperiod of 16 hour light and 8 hour dark.

2.1.4 Plant growth regulators

Regulation of developmental process in plant tissue culture generally requires the addition of plant growth regulators to the medium. Plant growth regulators have a significant influence on shoot regeneration during the initial induction phase (Matt and Jehle, 2005). The growth, differentiation and organogenesis of tissues become feasible only on the addition of one or more plant growth regulators to the medium. The ratio of growth regulators required for root and shoot induction varies considerably within the tissue, which seems directly correlated to the quantum of growth regulators synthesized at endogenous levels within the cells of the explants (Razdan, 2003). The age of the mother plant, the conditions under which it has been growing and the season in which explants taken, are influenced by the level of naturally occurring auxins in them (Cassells, 1979). Skoog and Miller (1957) reported that morphogenesis of *in vitro* cultured tissues as well as plant development were regulated by plant growth regulators especially auxins and cytokinins. As the phyto-hormones are the key compounds in tissue culture studies, hence a good deal of effort is required to determine optimum levels. Skoog and Miller (1957) reported that organogenesis in *Tobacco* culture was governed by the balance of auxin and cytokinin in the tissue culture media. Media with relatively high auxin/cytokinin ratio induces roots and those with low auxin/cytokinin ratio induce shoots and intermediate ratio induces unorganized growth of callus tissue. Goyal and Arya (1979) observed the regeneration in *Posopis cineraria* on MS medium supplemented with different concentration and combinations of Kinetin, IAA, IBA and BAP. Nodal
explants of *Prunus cerasus* responded best on MS medium supplemented with BAP, IBA and GA$_3$ (Curovic and Ruzi, 1987). Muralidharan and Mascarenhas (1987) reported somatic embryogenesis in *Eucalyptus citriodora* on B$_5$ medium supplemented with NAA. Kopp and Natraja (1990) regenerated plantlets of *Tamarindus indica* on MS medium supplemented with 2.0 mg/l BAP. Multiple shoots were obtained from cotyledonary nodes of *Dalbergia latifolia* on MS medium fortified with BAP (Sita and Swamy, 1992). Reddy *et al.* (1998) observed the maximum number of shoots from mature nodal explants of *Gymnema sylvestre*, on the medium containing BAP (5.0 mg/l) and NAA (0.2 mg/l). Ajithkumar and Seeini (1998) also reported that on *Aegle marmelos*, BAP produced longer shoot than Kinetin. Kathiravan and Ignacimuthu (1999) reported that the combination of BAP and Kinetin produced maximum number of shoots in *Canavalia virosa*. Supplementation of BAP (1.5 mg/l) + NAA (0.1 mg/l) have been found to show a good response of shoot proliferation in *Vitex negundo* (Thiruvengadam and Jayabal, 2001). Rathore *et al.* (2004) noticed the multiple shoot formation from the nodal explants of *Syzygium cumini* on MS medium supplemented with BAP. Tyagi *et al.* (2005) reported that the fortification of 2,4-D induced callus mediated embryogenesis in *Capparis decidua*. Gopi and Vatsala (2006) reported the potential of 2,4-D + NAA on callus induction in *Gymnema sylvestre*. Successful in vitro culture reported by applying auxins (2,4-D, NAA, IBA and IAA) and cytokinins (BAP, Kn and TDZ) at various combinations and concentration on various plants viz. *Dianthus caryophyllus* (Pareek and Kothari, 2007), *Epidendrum radicans* (Gaytri and Kavyashree, 2007), *Vitex agnuscastus* (Balaraju *et al.*, 2008), *Taxus wallichiana* (Datta and Jha, 2008), *Pergularia daemia* (Kiranmai *et al.*, 2008), *Lillium longiflorum* (Tang *et al.*, 2009), *Paederia foetida* (Alam, 2010), *Picrorhiza kurroa* (Jan *et al.*, 2010), *Alternanthera sessilis* (Gnanaraj *et al.*, 2011), *Aegle marmelos* (Yadav and Singh, 2011), *Bacopa monnieri* (Rout *et al.*, 2011), *Mentha piperita* (Sujana and Naidu, 2011), *Glycyrrhiza glabra* (Yadav and Singh, 2012), *Stevia rebaudiana* (Rathore *et al.*, 2013), *Fritillaria imperialis* (Rahimi *et al.*, 2013) and *Achyranthes aspera* (Sen *et al.*, 2013).

2.1.5 Organogenesis

Organogenesis is the term which is used for differentiation of cultured tissue into well organized shoot, root and leaf. This adopts two pathways, one when the direct development of shoots takes place from the axillary and lateral buds and another when shoot formation takes place via callus formation. Organogenesis has been reported in different plants involves utilization of leaf segments, axillary buds, shoot tips, nodal segments, cotyledonary nodes, hypocotyls, epicotyls, cotyledons and immature floral buds with inflorescence. However,
organogenesis is greatly influenced by the genotype, physiological state of the explants, age of the explants and in vitro environment, both the light and temperature and the composition of the medium. One of the earliest reports of controlled organogenesis under in vitro was given by White (1939) who obtained shoots from callus of Nicotiana glauca and N. longsdorffii hybrid on a agar-agar solidified medium. Later on number of reports approved depicting the formation of shoots or roots either directly from the explants or indirectly i.e. from the callus. Harikrishnan and Hariharan (1996) reported that direct shoot multiplication was obtained from nodes and leaves on MS medium fortified with a range of concentration of BAP and IAA in Plumbago rosea. In Bacopa monnieri multiple shoots could be induced from leaf segments (Rout et al., 2011). Jan et al. (2010) observed the formation of multiple shoots from nodal segments from in vitro grown seedling of Picrorhiza kurroa. 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. In vitro micropropagation of Moringa pterygosperma from hypocotyls and cotyledonary explants was achieved by Vandana et al. (1995). Abhyankar and Chinchanikar (1996) reported in vitro response of leaf explants of Withania somnifera on MS medium supplemented with IAA, Kn and BAP in various combinations. Patanaik and Debata (1996) developed protocol for in vitro propagation of Hemidesmus indicus from nodal segments. Haque et al. (1997) developed high frequency direct shoot regeneration and plantlet formation from root tips in Allium sativum. Attempts have been made for direct organogenesis from internodal segments of mature Murraya koenigii produced adventitious shoots with different combinations of BAP and Kn (Rajendra and D’Souza, 1998). Reddy (1998) also observed that maximum number of shoots induced on the medium containing BAP (5.0 mg/l) and NAA (0.2 mg/l) from mature nodal explants of Gymnema sylvestre. Jagdishchandra et al. (1999) obtained multiple shoots under in vitro culture of axillary buds of Pisonia alba on MS medium supplemented with BAP and Kn. Philolina and Rao (2000) reported bud break and multiple shoot induction in apical and axillary meristems of Sapindus mukorossi. Maximum shoots were obtained from rhizome explants, cultured on MS medium supplemented with BAP (4.0 mg/l) and IAA (0.5 mg/l) in Acorus calamus (Rani et al., 2003). Chaudhuri and Mukundan (2001) micropropagated Aloe vera using shoot tip explants. Rajore et al. (2002) reported multiple shoots from nodal segments of Jatropha curcas on MS medium fortified with Kn (2.0 mg/l) and IBA (1.5 mg/l). Wadilia chilensis was propagated through axillary bud proliferation from nodal segments
Martin et al., 2003. A protocol for in vitro propagation of Celastrus paniculatus on MS medium supplemented with fructose, Kn and IBA was developed by Maruthi et al. (2004). High rate of multiplication of Maerua oblongifolia was achieved using nodal segments on MS medium (Rathore et al., 2005). Sen et al. (2013) reported maximum culture establishment of Achyranthes aspera L. on 2,4-D, BAP, IBA and NAA. Nodal segments cultured on IBA, 2,4-D and NAA gave satisfactory culture establishment in Calendula officinalis (Victório et al., 2012). A protocol for micropropagation of Pinguicula gigantea from leaf explants has been developed by Saetiew et al. (2011). Mahendra et al. (2006) developed a rapid protocol for high frequency shoot regeneration from nodal explants of Spilanthes paniculata. 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). Reports on callus formation and subsequently plantlet formation from nodal segments in Actinidia chinensis were given by Gui (1979). Khalafalla et al. (2007) raised in vitro plantlets of Vernonia amygdalina from nodal explants. Gharyal and Maheshwary (1990) observed plantlets regeneration on callus culture derived from stem and petiole explants of Albizia lebbeck, Casia fistula and C. siamea. In vitro proliferation was reported in Prosopis juliflora by Nandwani and Ramawat (1991). In vitro organogenesis and somatic embryogenesis in Datura metel was demonstrated by Madhavan and Joseph (2001). A protocol for high frequency shoot regeneration and plant establishment of Tylophora indica from petiole derived callus was developed by Faisal et al. (2005). Likewise, Thomas and Philip (2005) reported high frequency shoot organogenesis from leaf derived callus of a medicinal climber Tylophora indica. Vasantha and Shivanna (2005) observed callus mediated regeneration of Desmodium oojinense by culturing leaf, petiole, node, internode, hypocotyls and cotyledon explants on MS and B5 medium supplemented with 2,4-D or BAP. Optimal callus was developed from petiole explants on MS basal medium supplemented with 2,4-D + TDZ. Callus was efficiently induced when leaf segments were cultured on MS medium supplemented with 2,4-D and Kn. MS medium containing BAP with NAA was reported best for shoot differentiation (Singh et al., 2011 and Pandey and Gupta, 2013). Rahimi et al. (2013) applied NAA and IBA at various concentrations for in vitro regeneration of Fritillaria imperialis.

2.1.6 Root development

The development of a perfect plantlet is incomplete without the regeneration of roots. In vitro root formation can be more synchronous because shoots are uniformly in contact with the medium (Mohammad and Vidaver, 1988). However, the roots produced by this method
are usually thickened and are generally devoid of root hairs. Also, callus may form at the base of the shoots and in case roots originate from these callus cells, vascular connection between root and shoot may be interrupted (Thorpe et al., 1991). No general technique can be used for in vitro rooting because of variation in response obtained (Williams et al., 1985). Gharyal and Maheswari (1990) reported root induction in Albizzia lebbeck, Casia fistula and Cassia siamea on Gamborg’s B5 medium, free of growth regulators as well as on 0.1 mg/l IAA. Regenerated shoots of Prosopis cineraria developed roots by plunging with 100 mg/l IBA for 4 hour and then culturing on ½ MS medium free from growth regulators (Shekhawat et al., 1993). In Moringa pterygosperma, ½ MS medium supplemented with GA3 (0.2 mg/l) produced roots within 7 days, however, better rooting was developed with 0.2 mg/l IBA (Mohan et al., 1995). Sharma and Padhya (1996) observed root induction from regenerated shoots of Crataeva nurvala within 7 days on MS medium fortified with 0.5 µM NAA. Roots were developed on excised shoots when they were transferred to ½ MS medium containing 1.0 mg/l IBA in Murraya koenigii (Bhuyan et al., 1997). Mustafa and Hariharan (1997) noticed best root induction from regenerated shoots of Alpinia galangal on MS medium supplemented with the combination of NAA and IBA. Patra et al. (1998) reported that shoots inoculated on ½ MS medium supplemented with IAA developed roots in Centella asiatica. Root induction took place in Paederia foetida and Centella asiatica implanted on MS medium fortified with BAP + IBA (Singh et al., 1999). MS medium at ¼ strength proved most suitable for root induction in Canavalia virosa (Kathiravan and Ignacimuthu, 1999). A high frequency root development was observed from the regenerated shoots of Morus indica cultured on medium fortified with 1.0 mg/l 2,4-D (Chitra and Padmaja, 1999). Best rooting in Vernonia amygdalina was obtained on MS medium supplemented with 0.5 mg/l IAA (Khalafalla et al., 2007). Yadav and Singh (2012) reported the root development in Glycyrrhiza glabra on ½ MS medium containing IAA (2.0 mg/l). Among the various auxins viz. IAA, IBA and NAA used for in vitro rooting, IBA is the most commonly used auxin for in vitro rooting. Manickam et al. (2000) found that ½ strength MS medium supplemented with IBA gave rise to good roots per shoot in case of Withania somnifera. Saini and Jaiwal (2000) in Pegnaum harmala reported that initiation of roots took place on MS medium containing IBA. Tiwari et al. (2000) achieved 90% root formation in Centella asiatica on MS medium supplemented IBA. In Cardiospermum halicacabum, roots could also be produced in hormone free MS medium (Babber et al., 2001). Supplementation of 2.0 mg/l IBA has been found to be effective for root formation from the excised shoots of Alnus nepalensis (Thakur et al., 2001). In vitro regenerated shoots Acacia sinuata induced roots when transferred to ½
strength MS medium supplemented with IBA (Vengadesan et al., 2002). Better rooting in Artemisia judaica was supported by IBA (Liu et al., 2003). Martin (2003) reported that the shoots of Rotula aquatic developed roots best on ½ MS medium supplemented with NAA. MS medium fortified with IBA induced roots in Phyllanthus amarus (Ghanti et al., 2004). Root induction was observed in Zheneria scabra on MS medium fortified with NAA (Anand and Jeyachandran, 2004). Similar results were obtained on Decalepis hamiltonii (Anitha and Pullaiah, 2002) and Porteresia coarctata (Latha et al., 1998). In Eclipta alba highest root formation was reported on MS medium fortified with IBA (Baskaran and Jayabalan, 2005). Prominent rooting was also observed when shoots of Asparagus adscendens were inoculated on MS medium supplemented with IBA (Saurabh and Subramanian, 2005). Khawar et al. (2005) reported that rooting in Peganum harmala was found on half or full strength MS medium containing IBA. Besides this Chlorophytum arundinaceum showed vigorous rooting on the ½ MS medium supplemented with IBA (Lattoo et al., 2006). Regenerated shoots of Holarrhena antidysenterica were excised and rooted in auxins free basal medium (Mallikarjuna and Rajendrudu, 2007). Vadodaria et al. (2007) found better root formation on medium containing NAA (0.1 mg/l) and 1% sucrose in Glycyrrhiza glabra. IBA has been reported to have a stimulatory effect on root induction in many medicinal plant species including Withania sominifera (Siddique et al., 2004), Centella asiatica (Mohapatra et al., 2008) and ginger (Sultana et al., 2009). Half-strength MS medium containing IBA at an optimum concentration induced rooting in Withania somnifera (Rout et al., 2011). The rooting response in Pongamia pinnata was enhanced on ½ strength MS media supplemented with 0.5 mg/l IBA (Kesari et al., 2012). IAA has also been reported suitable for rooting in several medicinal plants, such as Momordica cymbalaria (Nikam et al., 2009), Celastrus paniculatus (Lal and Singh 2010), Aegle marmelos (Yadav and Singh, 2011a) and Stevia rebaudiana (Javad et al., 2013).

2.1.7 Acclimatization and bio-hardening

The heterotrophic mode of nutrition, poor physiological mechanisms and lack of cuticle on leaves to control water loss, tender the micropropagated plants vulnerable to the transplantation shock. Plants are acclimatized in suitable hardening mixture in pots under controlled conditions of light, temperature and humidity. The plantlets have to become autotrophic in contrast to their heterotrophic state induced in micropropagation culture. The ultimate success of micropropagation depends on the ability to transfer in vitro raised plants to soil with high survival rate. Most of the species grown under in vitro conditions require an acclimatization process in order to ensure that sufficient number of plantlets survive and
grow vigorously when transferred to soil (Hazarika, 2003). The term acclimatization is defined as the climatic adaptation of an organism, especially a plant, which has been moved to a new environment (Conover and Poole, 1984). In conventional acclimatization, the main effort of environmental control in the acclimatization stage is to keep the relative humidity high particularly at an early stage of acclimatization. The high humidity is generally achieved by covering the plantlets with plastic film under shade, together with frequent misting. Shading is necessary firstly, because the strong solar light itself may directly damage the plantlets and secondly, the fluctuating solar light intensity with time leads to fluctuation of temperature and relative humidity and hence an excess water loss from the plantlets (Kozai, 1991). These in vitro formed plantlets are exposed to a unique set of growth regulators, carbon source, high humidity, low light and poor gaseous exchange which may support rapid growth and multiplication but also induce structural and physiological abnormalities in the plants rendering them unfit for survival under in vivo condition. Daniel et al. (1999) nourishes the regenerated plantlets of Naregamia alata in vermiculite with a dilute solution of nutrients. Regenerated plantlets of Litsea cubeba were transplanted into a potting mixture of sand, loam and peat (1:1:1) for hardening (Mao et al., 2000). Sheeja et al. (2000) have reported 43% establishment of Cinnamomum verum plantlets in soilrite. Rooted shoots of Holostemma ada-kodien were transferred directly to small pots filled with sterile soilrite and sand (Martin, 2002). In vitro raised plants of Acacia sinuate were hardened in a growth chamber at 80% humidity (Vengadesan et al., 2003). Joshi and Dhar (2003) achieved hardening and acclimatization of Saussurea obvallata by transplanting the in vitro raised plantlets in plastic pots containing a mixture of autoclaved soil, peatmoss and soilrite (2:1:1). The pots were covered with polythene bags and irrigated at weekly intervals with ½ MS medium to maintain high humidity. Successful acclimatization and field transfer of the in vitro regenerated plantlets have also been reported in Bupleurum disticho-phyllum (Kuruppusamy and Pullaiah, 2007), Eclipta alba (Ray and Bhattacharya, 2008), Balanites aegyptiaca (Siddique and Anis, 2009), Withania somnifera (Rout et al., 2011) and Stevia rebaudiana (Javad et al., 2013). Various types of substrate had been used during acclimatization such as soil and vermiculite mixture (Philomina and Rao, 1999), sterilized soil (Sunaina and Goyal, 2000) and sterilized sand (Thakur et al., 2001). Hardening of plantlets in Jatropha curcas had been achieved by using soil and vermiculite mixture (3:1) by Rajore et al. (2002). Kiranmai et al. (2008) transferred well developed plantlets to pots containing autoclaved peatmoss and sand (3:1) with 50% survival rate. In vitro raised plantlets of Vitex negundo were hardened in pots filled with sterilized vermi-compost and soil mixture (1:3) by
maintaining 85% relative humidity (Sharma et al., 2006). In *Leptadenia reticulate*, hardening had been done by transferring the rooted shoots to peasteurized mixture of soil, sand and cocopit (made up from coconut coir) in equal proportions filled in plastic cups (Parabia et al., 2007). For acclimatization the regenerated plantlets of *Sterculia urens* were transferred to plastic cups containing autoclaved vermiculite (Hussain et al., 2008). Regenerated plantlets of *Oroxyllum indicum* were initially kept in distilled water in flasks covered with beaker for approximately 8 days and finally transferred to soil: sand (1:1) in cups (Gokhale and Bansal, 2009). The rooted plantlets of *Acorus calamus* were transferred into small plastic bags containing sand, soil and compost (Ahmed et al., 2010). Regenerated plants of *Gomortega keule* were transferred to compost and covered with transparent plastic bags for acclimatization (Munoz-Concha et al., 2012). Use of coco peat as hardening medium resulted in maximum survival during hardening phase of *Acacia auriculiformis* (Girijashankar, 2011). The rooted plantlets of *Calendula officinalis* were removed from culture vessels and were transferred to pots containing a mixture of sand and humus (2:1). To maintain humidity, the plants were covered with plastic caps and gradually opened during the two-week acclimatization (Victório et al., 2012). Plantlets with well developed root system were transferred to plastic pots containing sand, soil and vermiculite (1:1:1) Chakravarthy (2013). The major problem of commercial micropropagated plant production is low survival and poor growth while shifting these plantlets to field conditions. Plant loss is associated with the fact that micropropagated plants have functionally less rhizosphere microorganisms such as mycorrhizae. Improved performance of endomycorrhizal plants is usually due to enhanced uptake of nutrients, especially phosphorous (Monticelli et al., 2000). Rai (2001) indicated that AM fungi improves bio-priming of micropropagated plantlets and plays a significant role in ensuring the health of plantlets. Mycorrhizae can act as bio-regulators, bio-fertilizers and bioprotectors. The beneficial interaction between AM fungi and horticultural crops have been well documented (Menge, 1983). AM fungi help the inoculated plants in averting transplantation shock brought about by unfavourable ex vitro environmental conditions in various horticultural crops species. In salt stressed soils, AM fungi are supposed to improve the supply of mineral nutrients to the plants, especially the supply of P, Cu, Mn and Fe (Adivappar, 2001) which otherwise would be precipitated by ions like Ca$^{2+}$, Mg$^{2+}$ and Zn$^{2+}$ (Al-Karaki et al., 2000). Besides improving nutrition, AM fungi improve physiological processes, like water absorption capacity of plants by increasing root hydraulic conductivity and favourably adjusting the osmotic balance and composition of carbohydrates (Rosendahl and Rosendahl, 1991). Thus, they mitigate the adverse effects of excess salt accumulation in
the root (Dixon et al., 1993). Symbiotic association of a plant with AM fungi makes it able to access immobile nutrients in nutrient-poor soils (Marschner and Dell, 1994), biological control of root pathogen (Reddy et al., 2006), drought resistance (Auge, 2000), rehabilitation of degraded land, reclamation and soil fertility, bioremediation (Li et al., 2006) and increase in biomass of plants (Javot et al., 2007b). Leaves with poor or no development of cuticular wax, impaired stomatal mechanism, poor photosynthetic activity, vitrification of shoots, poor vesicular connection, root hair development and pathogenic infection, these problems can be obviated by combining the micropropagation technique with micorrhization during hardening (Singh et al., 2004 and Sivaprasad and Sulochana, 2005).

Bio-hardening is an emerging dimension of micropropagation techniques (Srivastava et al., 2002). Inoculation of compatible AM fungi during the acclimatization period of micropropagated plants has emerged as an alternative strategy for better establishment by improving the plant growth and the acclimatization period can also be shortened (Rai, 2001). The beneficial effects of AM fungal association on the growth performances of several taxa including medicinal plants are well documented (Gianinazzi et al., 1989). Arbuscular mycorrhizal fungi association enhances the plant growth as a result of improved nutrient uptake and consequently leading to better growth performance of plants. In addition, AM fungi also have an influence on the primary and secondary metabolism in the host (Peipp et al., 1997). Mycorrhization of tissue cultured plants is believed to provide advantage to the transplanted propagules in terms of nutrient availability, soil pH, aeration and protection from water stress. Symbiotic association between AM fungi and host plants has been exploited for enhancing survival of micropropagated plants during their weaning or acclimatization phase, such as Feronia limonia (Vyas et al., 2008), Zingiber officinale (Santos et al., 2010), Andrographis paniculata (Tejavathi et al., 2011), Agave tequilana (Ruiz et al., 2011), Aloe vera (Mota-Fernández, 2011) and Spilanthes acmella (Yadav et al., 2012). Puthur et al. (1998) observed cent per cent survival of micropropagated plantlets of Leucaena leucocephala inoculated with Glomus fasciculatum and G. macrocarpum compared to 20 per cent survival in control. They attributed the low rate of survival in non-mycorrhizal micropropagated plants to weak root system, unfavourable nutrition and environmental conditions, which are distinct from in vitro conditions, poorly developed cuticle and non-functional stomata. Micropropagated plants, such as Feronia limonia, Andrographis paniculata, Agave tequilana and Zingiber officinale infected with AM fungi during transplantation from in vitro to in vivo grew faster and higher than the control plants (Santos et al., 2010; Tejavathi et al., 2011 and Ruiz et al., 2011), respectively. Vidal et al. (1992)
observed inoculation of AM fungi at the time of plantlet transfer from *in vitro* to *ex vitro* conditions improved shoot and root growth, enhanced the shoot: root ratio, increased the content of N, P and K in tissues and help plants to tolerate the environmental stress during transplanting. Arbuscular mycorrhizal fungi caused increase in fresh weight, root length and height of mycorrhizal tissue culture derived apple rootstocks (Sbrana *et al.*, 1994). Yano-Melo *et al.* (1999) reported that micropropagated banana plantlets inoculated with AM fungi had significantly greater height, leaf area, fresh weight of roots and shoots, higher photosynthesis and transpiration rates as well as improved uptake of Zn and Mn, leading to enhanced growth of inoculated plantlets. Similarly, Mathur and Vyas (1999) observed improved biomass production, nutrient uptake and plant height of *in vitro* raised *Ziziphus mauritiana* by AM fungi and attributed improved biomass production to improved nutrient uptake and improved photosynthesis. Ramanwong *et al.* (1999) observed that AM fungi increased the various growth parameters like greater height, diameter of shoot and root dry weight and nutrient uptake in teak seedlings. Haripriya and Sriramchandrasekhran (2002) observed increase in growth and yield of chrysanthemum due to AM fungi. Janos *et al.* (2002) observed that inoculation with AM fungi enhanced growth of *Litchi chinensis*. Rashmi and Roy (2003) suggested the positive effect of AM fungi on growth performance of finger millet. Mali *et al.* (2004) observed increase in stem length and plant dry weight when inoculated with AM fungi. Pandey and Tripathi (2004) observed enhancement of plant growth of sugarcane infected with AM fungi. Higher uptake of macro and micro-nutrients is directly associated with the rate of root colonization (Dolcet-Sanjuan *et al.*, 1996). Thus, the work of several workers depicts that plant species forms symbiotic association with AM fungi and it plays a vital role in the growth of plants under natural as well as stress conditions.

Rai (2001) indicated that AM fungi improve bio-priming of micropropagated plantlets and plays a significant role in ensuring the health of plantlets. It has been well established that AM fungi can absorb P, Zn, \(\text{NO}_3^-\) and \(\text{NH}_4^+\) from distances extending up to 25 cm from the roots (well beyond the depletion zone) and transferring them to associated plants (Li *et al.*, 1991). Earlier, Bieleski (1973) proposed that mycorrhizal hyphae extend into soil could increase the effectiveness of absorption of immobile elements by as much as 60 times. Phosphorus is one of the most important macro elements for plant life, it occurs as a part of nucleus, DNA and RNA and as a part of phospholipids, which constitutes plant membrane. Plant growth response to AM fungi are of the result of increased P nutrition (Antunes and
Cordoso, 1991). AM fungi enhance grape vine growth by improving nutrition uptake especially P (Schubert et al., 1988). Arbuscular mycorrhizal fungi inoculation in Wedilia chinensis seedlings increased the concentration of P, K, Zn, Cu, Mg and Fe content (Mathan and Sevan, 2010). Mohandas (1992) observed that papaya plants inoculated with AM fungi (Glomus mosseae and G. fasciculatum) showed improved plant height, dry matter, as well as P, N and Zn nutrition. Menge et al. (1980) found increased N content in avocado leaf by the inoculation of Glomus fasciculatum. Jakobsen (1995) reported the positive effect of AM fungi on Baptista tinctoria due to an improved uptake of P in the plants. Mycorrhizal soybean plants showed higher content of nitrogen at different moisture level when compared to non mycorrhizal control. Mycorrhizal inoculation enhanced the content of N, P and K in plant tissues of micropropagated plantlets of avocado (Vidal et al., 1992). Antunes and Cordoso (1991) found significant increase in K uptake with inoculation of Glomus etunicatum in Rangpur lime. Micronutrients are needed by the plants in small quantities but are very important for proper growth and development as they are the part of various enzymes, pigments and several other biological molecules essential for plant life (Jones et al., 1998). Arbuscular mycorrhizal fungi help the plants in two ways: firstly, they help in the enhanced uptake of different micronutrients (Zn, Cu, Mn and Fe), which are considered to be relatively immobile and secondly, they take up these elements and store them so as to prevent their concentration to reach toxic levels. Arbuscular mycorrhizal fungi were proposed to act as a sink for Cu, Co and Zn (Cooper and Tinker, 1978) and making them highly available and also rendering them as active sink (Marschner and Dell, 1994). Mycorrhizal inoculation was shown to relieve Zn deficiency in peach (Gilmour, 1971). Menge et al. (1982) found increased foliar absorption of Zn with Glomus fasciculatum inoculation in Troyer citrange. Later, Vinayak and Bagyaraj (1990) found that Troyer citrange and Trifoliate orange had more Zn content in shoots with Glomus species. Bavaresco and Fogher (1996) showed that Pseudomonas and Glomus mosseae increased Fe level over untreated plants of grape vine. Higher mortality of in vitro raised plantlets is observed when they are transferred to ex vitro stressful environmental conditions. Several processes are affected or altered during stress such as, stomatal conductance, stomatal opening, CO₂ assimilation, chlorophyll formation, water relation, proline, sugars and phenol accumulation, increased activity of polyphenol oxidase, nitrate reductase etc. The type of stress which plants experience under field conditions could be of different kind e.g. stress induced by pathogen attack, salt induced stress and water stress or moisture deficit stress. Direct transport of several nutrients from soil to plant by mycorrhizal fungi has been demonstrated that the increased absorption provided
by hyphae, increased plant growth (Rhodes and Gerdman, 1979), thereby enabling the plants to sustain their life under unfavourable conditions. Additionally, AM fungi widely exist in the salt affected soils (Juniper and Jbott, 1993) and studies have demonstrated that inoculation with AM fungi improves growth of the plants under the variety of salinity stress conditions (Ruiz-Lozano and Azcon, 2000). The introduction of AM fungi to sites with saline soils may improve early plant tolerance and growth (Jain et al., 1989) and increased protection from salt stress (Rosendahl and Rosendahl, 1991). Arbuscular mycorrhizal fungi inoculation has been found to increase the sugar level in the plants. The higher sugar accumulation favors the plants in maintaining the osmotic balance and preventing dehydration of tissues thereby, helping the plants to grow normal even under stress conditions.

2.2 Salt tolerance

Soil salinity is a widespread problem, restricting plant growth and biomass production especially in arid, semi-arid and tropical areas (Apse et al., 1999). On a global basis, salt affected soils occupy 952.2 Mha of land, constituting nearly seven per cent of total land area or nearly thirty three per cent of the area of potential arable lands of the world (Gupta and Abrol, 1990). Salt-affected soils cover an area of nearly 13.5 Mha in India (Sharma et al., 2004). In India, the problem of soil salinity is fast spreading posing a threat to agriculture in various parts of the country including Haryana, Punjab, Maharashtra, and Karnataka state (Palaniappan, 1986). Modern agriculture largely depends on the use of high cost inputs such as, chemical fertilizers, pesticides, herbicides and hybrid seeds; the application of such high input technologies has undoubtedly increased the production but there is growing concern over the adverse effects of the use of such chemicals on soil productivity, microbial community and environmental quality (Motsara, 2000). In this respect biological processes such as mycorrhizal inoculation to alleviate salt stress and use of moderately salt-tolerant species are better options (Dixon et al., 1993).

Salt tolerance is a complex trait both physiologically as well as genetically. Therefore, attempts to improve the salt tolerance of crops through conventional breeding programmes have met with very limited success (Flowers, 2004). Furthermore, salinity tolerance is a developmentally regulated stage-specific phenomenon; tolerance at one stage of plant development is often not correlated with tolerance at other stages (Foolad, 2004). Plant tissue culture research is a multi-dimensional field that offers exciting prospects for crop productivity and improvement (Jain, 2001). If in vitro cultures are established from explants that did not contain a pre-organized meristem, or if cultures are maintained as callus prior to plant regeneration, the regenerated plants may exhibit variability (Larkin and Scowcroft,
Therefore, plant tissue culture has an important role to play in the production of ornamental or agricultural plants and in the manipulation of plants for improved agronomic performance.

*In vitro* changes in plant tissues induced due to NaCl toxicity would assist in understanding the effect of salinity on plant growth and other endogenous biochemical changes, which further assist in selecting genotypes for successful cultivation under salt affected areas. Changes due to *in vitro* NaCl induced salinity have been studied in grape (Singh *et al.*, 2000 and Khawale *et al.*, 2003) and the results showed that as the level of NaCl was increased there was a linear increase in the level of sugars, total phenols and proline, while the leaf chlorophyll content showed a decline. Foliar mineral contents showed distinct patterns among the salinity tolerant and susceptible genotypes.

Soil salinity is a widespread problem, the growth of higher plants in saline soil depends on salt tolerance of the plant species. One of the main causes of reduced growth might be a reduction in the rate of photosynthesis (Al-Khateeb, 2006 and Koyro, 2006). Photosynthesis is inhibited in the presence of salinity through non-stomatal factors as a reduction in chlorophyll pigments to absorb enough light (James *et al.*, 2002). The decrease in plant growth due to salinity includes a reduction in the leaf area of the plant (Dadkhah, 2012). According to Nikknam and McComb (2000) one of the causes of growth rate reduction under stress was inadequate photosynthesis owing to stomatal closure and consequently limited carbon dioxide uptake. Photosynthesis, as one of the most important physiological processes, provides 90% of the plant dry matter (Steduto *et al.*, 2000). Plants growing in the increasing NaCl concentrations decreased their shoot and root length (Siler *et al.*, 2007). Prajuabmon *et al.* (2009) reported gradual reduction in shoot length, fresh and dry weight of shoots under higher concentration of NaCl. Low concentration of NaCl stress showed positive effect on proliferation while moderate concentrations of NaCl almost checked the proliferation in muskmelon (Carvaja *et al.*, 1998), *Citrus aurantium* (Shiyab *et al.*, 2003), chrysanthemum (Shatnawi *et al.*, 2010). Shibli and Al-Juboory (2002) found that olive shoot growth decreased as NaCl concentration increased. Under stress condition, one of the strategies that plants have adopted is to slow down their growth. This reduction in growth not only helps the plant to save the energy for the defense purpose, but also limits the risk of heritable damage (Hossain *et al.*, 2007). Alizadeh *et al.* (2010) reported gradual reduction in shoot inter-nodal length, fresh and dry weight of shoot and root, number of leaves and leaf area of *Vitis* spp. under salt stress conditions as compared to NaCl free medium. Plant growth parameters such as shoot and root dry matter, number of leaves, and total plant leaf area, all growth characters were
significantly decreased by increasing salinity (Dadkhah, 2012). Salt induced osmotic stress led to a rapid decline in water and the osmotic potential of cells and a decrease both in cell volume and cell expansion (Neumann, 1988). The growth inhibition due to salinity had also been explained by a suppression of nutrient absorption due to uptake of NaCl in competition with nutrient ions (Gracia and Charbaji, 1993). Walker et al. (1981) reported that the decreases in shoot growth of grape vines under saline conditions were related to Cl⁻ accumulation in the plant. The salinity effects on plant might vary depending on the stage of its development. In nature, the extent of salt or drought tolerance often appeared to be inversely related to growth rate. Lack of or low level of hormones could possibly explain reduced growth of leaves and shoot, and decreased fruit set and berry development (Hawker and Walker, 1978). Prisco (1980) was of the opinion that, shoot growth was reduced under salt stressed conditions, similar findings was reported by Munns (2002). Molazem et al. (2011) reported gradual reduction in leaf number, leaf width and length with moderate concentration of salt. Significant reduction in all the leaf-growth parameters as leaf area, leaf dry weight and leaf fresh weight was reported in *Triticum durum* by Fercha (2011).

Shibli et al. (2000) reported gradual reduction in apple shoot fresh weight with increased NaCl concentration. Similar results were obtained in microshoots of chrysanthemum (Shatnawi et al., 2010). Bahmani et al. (2012) also reported gradual reduction in fresh weight as the level of salt was increased. Similar findings were obtained in cotton (Ashraf and Ahmad, 2000), lentil (Misra and Saxena, 2009), maize (Hichem et al., 2009), *Triticum aestivum* (Ashraf et al., 2010), *Salvinia natans* (Jampeetong and Brix, 2009), coriander (Leena et al., 2012), cucumber (Abu-Romman et al., 2012) and *Coriandrum sativum* (Ewase, 2013). Increasing NaCl concentration in the medium caused a significant reduction in fresh weight of both shoots and roots, as well as dry weight (Sasikala and Prazad, 1994). Lerner et al. (1994) observed that the salinity induced a change in the signals of root origin, which changed the hormonal balance of the plant and this further affected root and shoot growth. In addition to the effect of salinity on reducing plant available water, salinity also had a direct toxic effect on plants. In addition specific ions, such as chloride, sodium or boron, exhibited toxic effect on plant roots leading to stunted growth or stopping of growth (Barbour et al., 1998). More specific effects might involve direct toxicity or a variety of nutritional effects (Bernstein and Hayward, 1958). Excess soluble salts in the root zone restricted plant roots to uptake water from the surrounding soil and thus had effectively reduced the plant available water (Hanson et al., 1999). Basically, water was held tighter to the soil in saline
environments and was less available for plant uptake due to osmotic forces. This led to reduced water uptake and increased plant stress. According to Skene (1975) the root growth was severely restricted even in as low as 20 mM concentration of NaCl and it was possible that cytokinin production, known to occur in roots was limited in plants treated with NaCl. Root growth has been found to be more adversely affected than shoot growth by an increasing supply of NaCl (Cano et al., 1998). Further, Abu-Romman et al. (2012) reported reduction in average root length, number of roots, root fresh and dry weights of cucumber at moderate concentration of NaCl. Similar results were observed in grape vine and Citrus macrophylla, in which higher NaCl concentration (85 and 100 mM) inhibit root length, number of roots and bio-mass of roots (Moradi and Zauareh, 2013). It is now known that salt tolerance operates at the cellular level (Mansour et al., 2003). Biochemical aspects of antioxidants and protective pigments have successfully been introduced as markers of environmental stress (Tausz et al., 2003). Understanding biochemical indicators for individual species rather than generic indicators could prove to be quite helpful to understand the physiology of salt tolerance (Ashraf and Harris, 2004).

Reddy and Dass (1978) stated that the adverse effects of salts, on the changes in pigment concentrations in plant, depended upon specific nature of ions, plant species and stage of plant growth and development. In a study by Ashraf and Rasul (1988) on mungbean, at higher concentration of salts the chlorophyll a, b and total chlorophyll contents were reduced significantly and variation between cultivars was evident. They concluded that the specific enzyme which was responsible for synthesis of chlorophyll was suppressed by higher concentrations of salt.

Ramanujalu et al. (1993) observed gradual decrease in the contents of chlorophyll ‘a’ and chlorophyll ‘b’ contents with increase in the salt intensity in mulberry. Among them, relatively higher rate of depletion was found with chlorophyll a than chlorophyll b. Similar results were also reported by Sudhakar et al. (1991) who attributed the results to the increased chlorophyllase activity and also partly due to the interference of salt ions with de novo synthesis of proteins, the structural components of chloroplast. Dhanapackiam and Muhammad Ilyas (2010) observed gradual reduction in chlorophyll a, b and total chlorophyll in Sesbania grandiflora markedly at high saline concentration. Similar findings were also reported in Helianthus annus and Linum usitatissimum by Ahmed et al. (1978) and in Arachis hypogea by Hajar et al. (1993). Decline in chlorophyll content due to increasing salinity has been reported by (Khawale et al., 2003 and Radi et al., 2013). Salinity reduced growth and
chlorophyll content of in vitro raised culture of cherry root stock (Erturk et al., 2007). Similar findings were reported by Elfkeky et al. (2007) in Catharanthus roseus. Plantlets induced from tissue culture under salt stress conditions were found with reduced amount of total chlorophyll in leaves compare to control and recovered plantlets (Josine et al., 2011 and Habibi and Amiri, 2013). Among the biochemical parameters affected by NaCl stress, accumulation of free proline within the tissue is the most significant one (Alizadeh et al., 2010). However, some workers consider an enhanced proline content simply as a stress effect, rather than a cause of stress tolerance (Moftah and Michel, 1987). Hasegawa et al. (2000) reported that proline and glycinebetaine, were the organic solutes that accumulated most commonly under salinity, although other molecules could accumulate to a lesser degree. Proline plays an important role in osmotic adjustment (Cayuela, 1996). This is a nitrogenous compound synthesized mainly in case of stress such as salt stress or water stress. Sairam and Tyagi (2004) observed in organisms ranging from bacteria to higher plants a strong correlation between increased cellular proline levels and the capacity to survive both under water deficit and the effects of high environmental salinity. Proline content served as an organic nitrogen reserve that was utilized during recovery. High proline accumulation was observed in leaves and roots under water stress by Tyagi et al. (1999) in Lathyrus sativus, a hardy grain legume which could withstand drought. Josine et al. (2011) reported that plantlets induced from tissue culture under salt stress conditions were found with increased proline content compare to control. Similar findings were reported in Triticum durum by Fercha (2011). However, the accumulation of proline under stress is species dependent (Nolte and Hanson, 1997). It has been suggested that high proline accumulation induced by NaCl correlates with growth inhibition (Elmaghrabi, 2013 and Radi et al., 2013).

Accumulation of total soluble sugars is a common phenomenon under stress condition (Radi et al., 2013). Garg et al. (2002) reported an increase in total soluble sugars with progressive increase in salinity, which have an important role in osmoregulation (Mohanty et al., 2002 and Martino et al., 2003). Salt stress resulted, decrease in percent soluble sugars but decrease was significantly less in tolerant accessions than the non tolerant ones Lens culinarly (Ashraf and Waheed, 1993) and sunflower (Ashraf and Tufail, 1995). The changes in total soluble sugar contents under salt stress have already been reported for a number of plant species (Khattab, 2007). Dhanapackiam and Muhammad Ilyas (2010) reported an increase in total sugars in the leaves and root of Sesbania grandiflora tends to increase with increasing salinity level. This accumulation has been attributed to impaired carbohydrate utilization (Munns and Termaat, 1986). Ashraf and Tufail (1995) observed that the levels of reduced and
total soluble sugars were the lowest in the absence of salt. Besides, the presence of NaCl even at low doses caused an increase in these levels. In the roots the content of reduced soluble sugars was less than that of shoots both in the controls and in the treaties. An increase in both reducing and nonreducing sugars and polyphenol levels has been reported in leaves of *Bruguiera parviflora* (Parida *et al.*, 2002). In the leaves of tomato the contents of soluble sugars are increased significantly (Khavarinejad and Mostofi, 1998). Ashraf and Tufail (1995) determined the total soluble sugar content in five sunflower accessions differing in salt tolerance; the salt tolerant lines had generally greater soluble sugars than the salt sensitive ones. Sugar content increased in leaves under salt stress as compared to plants grown under normal conditions (Fercha, 2011 and Elmaghrabi, 2013). Similar results were also reported by Garg *et al.* (2002) on rice, Baka *et al.* (2006) on wheat, and Khelil *et al.* (2007) on tomato. Moreover, carbohydrate accumulation plays an important key role in alleviating the salinity stress via osmotic adjustment (Ackerson, 1985). Lewis and Yamamoto (1990) reported that total phenols play a significant role in the regulation of plant metabolic processes and overall plant growth. Leaf phenolics are the most important protective component of plant cells (Ashraf, 2010). The synthesis of phenolics is generally affected in response to different biotic/abiotic stresses including salinity (Parida *et al.*, 2004). Mohamed and Aly (2008) on onion plant and Rady *et al.* (2011) on sunflower genotypes reported increased amount of total phenols under moderate concentration of NaCl. The accumulation of phenolic compounds in response to abiotic stress was reported by Rivero *et al.* (2001) and Radi *et al.* (2013). Various type of plant phenolic has been considered to be the main lines of cell acclimatization against stress in plant.