Review of Literature
REVIEW OF LITERATURE

Meristem, shoot tip and bud culture techniques of in vitro propagation are applied for mass propagation of elite germplasm, virus elimination, germplasm conservation, international exchange of germplasm, genetic transformation, molecular farming and phytoremediation (Hu and Wang, 1983; Nehra and Kartha, 1994; Bhojwani and Razdan, 1996; Lynch, 1999, Fischer et al., 2006; Lupotto and Stile, 2006; Meagher et al, 2006). The in vitro methods provide a variety of tools to supplement traditional methods for propagating and preserving endangered and rare plant species (Pence et al, 2006; Ozcan et al., 2006; Gao et al., 2006). These methods are being used in several countries for preservation of plant diversity, conservation of endemic, rare and endangered species and their restoration (Amoroso, 2006; Bunn, 2006; Tandon, 2006; Verma, 2006). The process of in vitro propagation is accomplished in phases and each phase is influenced by several factors. Some factors that contribute significantly to the success of plant micro propagation are explants, culture medium, plant growth regulators, genotype and culture environment (Hu and Wang, 1983). Considerable work has been done on in vitro propagation of some species of the Genus Curcuma, Dioscorea, Pueraria and a considerable number of other medicinal plant species. In vitro propagation is now practiced at commercial scale using standard protocols. The present review describes some of these aspects briefly.

2.1 Commercialization of micro propagation

Micro propagation is the true to type propagation of selected provenances using in vitro culture techniques (Debergh and Read, 1991). It has been applied successfully to the production of more than 1000 species. Many of these are ornamental, but the technique is also applied to food crops and trees. Commercial
Micropropagation of plants is used for the production of large number of copies of selected provenances. The operational steps in micropropagation are labor-intensive and repetitive that escalates production cost. The relatively high production cost of *in vitro* micropropagation compared with the conventional methods of propagation are compensated for by the several benefits: (a) rapid propagation because of short propagation cycle (b) large volume propagation of high value ornamentals like orchids shrubs and trees (c) easy storage and transport of large number of plants (d) plant production independent of season (e) quick supply of plants with the change in demand (Evans *et al*., 2003).

### 2.2 World and Indian scenario

Commercial application of plant tissue culture started in USA with micropropagation of orchids in 1970s. Global market of 15 billion US dollars was estimated for tissue culture products (Govil and Gupta, 2006). During the last 30 years, tissue culture based plant propagation has emerged as one of the leading global agro-technologies. Between 1986 and 1993 the worldwide production of tissue cultured plants increased 50%. In 1993, the production was 663 million plants. By 1997, production had risen to 800 million plants. During 1990-1994, the micropropagation industries declined in Europe, mainly due to production shifting to developing countries, but since then because of the demand for high quality and number, production in European countries has increased. Since 1995, production has increased by 14% in the Asian countries, mainly due to the market entry of China, while the increase in the South and Central America was from production in Cuba. More recently some companies from Israel, the USA and UK have shifted their production requirements to Costa Rica and India.

Commercial tissue culture started in India when AVT, Kerala was established in 1987 for large-scale production of cardamom. The second unit of plant tissue culture was established by the Indo-American Hybrid Seed at Bangalore in 1988. Now, there are more than 75 companies in this field ([http://agri.mah.nic.in/agri/hort/html/hightech/tissueculture.html](http://agri.mah.nic.in/agri/hort/html/hightech/tissueculture.html)). The consumption
of tissue culture plants in India for 2002 - 2003 had been approximately 44 million units with banana constituting 41%, followed by sugarcane 31%, ornamental plants 14%, spices 6%, medicinal plants 4% and others 4%. The demand for tissue culture plants is expected to increase at the rate of 25 - 30 percent per year. The overall market of tissue culture plants is expected of 144 million plants during the year 2007 – 2008 (Swarup, 2004).

2.3 Quality control and certification:

Tissue culture is a useful approach for generating virus free planting material. In order to minimize the risk of inadvertent micro propagation of virus infected plants and introduction of somaclonal variability, a tissue culture raised plant need to be thoroughly indexed for freedom from viruses and checked for quality (Swarup, 2004). Considering the aforesaid fact under the section 8 of the Seeds Act, 1966 (54 of 1966), Ministry of Agriculture has authorized Department of Biotechnology (DBT), Ministry of Science and Technology to work as Certification Agency. Accordingly, DBT has launched National Certification System for Tissue Culture Raised Plants (NCS-TCP) in 2006. Biotech Consortium India Limited (BCIL), New Delhi is the Accreditation Unit of NCS-TCP and assisting the Certification Agency in Accreditation of Test Laboratories and Certification of Tissue Culture Production Facilities. Advance Centre of Plant Virology, IARI, New Delhi and Centre for DNA Fingerprinting and Diagnosis (CDFD), Hyderabad are the referral centers for virus indexing and testing of genetic fidelity, respectively. Eight Accredited Test Laboratories are situated across the country and will certify the tissue culture raised plants of the recognized production facilities (www.dbtncstcp.nic.in). The scenario of plant tissue culture is changing fast in India. So far, the emphasis of commercial micro propagation was limited to cash crops, ornamental plants and forest trees. The standardized protocols that have proven commercial viability are available for these species and are being improved further. It is expected that many new species of diverse nature would attract the
attention of entrepreneurs and would get the place in commercial plant tissue culture laboratories.

2.4 Medicinal Plants:

Humans have exploited the medicinal properties of plants since the dawn of history, and even today more than one quarter of our drugs are either derived from plants or contain plant extracts (Raskin et al., 2002). In vitro propagation of medicinal plants is an important research area. Several medicinal species have been successfully regenerated in vitro. Chakraborty and Roy (2006) developed an efficient procedure for rapid and large-scale propagation of the elite exotic, fruit yielding, medicinal tree, Cyphomandra betacea through in vitro axillary bud multiplication. In vitro plant propagation has been achieved in Azadirachta indica (Shekhawat et al., 2002; Quraishi et al., 2004), Bacopa monnieri (Sharma et al., 2007), Balanites aegyptica (Gour, et al., 2007), Gagnepainia godefroyi and G. thoreliana (Prathanturarug et al., 2007), Gloriosa superba (Mukhopadhyay et al., 2006), Gymnema sylvestre (Komalavalli and Rao, 2000) Hemidesmus indicus (Sreekumar et al., 2000), Holostremma adakodien (Martin, 2002), Ocimum sanctum (Girija et al., 2006), Panax quinquefolius (Zhou and Brown, 2006), Pedalium murex (Saravanan, 2007), Peristrophe bicalyculata (Sharma and Devi, 2006), Rauwolfia serpentina (Tomar and Tiwari, 2006), Ruta graveolens (Faisal et al., 2006), Tylophora indica (Faisal and Anis, 2003), Vitex negundo (Sharma et al., 2006; Sahoo and Chand, 1998), etc.

2.5 Routes and stages of micro propagation

There are three major routes of micro propagation namely shoot bud proliferation, organogenesis and somatic embryogenesis. The organogenesis and somatic embryogenesis usually involve intermediate callus, therefore, the frequency of somaclonal variation is usually high in the plants regenerated through these two methods. The shoot buds are preformed meristems that elongate to produce shoots and new buds appear at nodes. Plants regenerated through this route are expected to
vary less than those obtained from callus (Drew and Smith, 1990). Murashige (1974) developed the concept of developmental stages: stage I: explant establishment, stage II: multiplication of the propagule, and stage III: rooting and hardening. Debergh and Maene (1981) introduced the stage 0, making micropropagation a five-stage process (Bhojwani and Razdan, 1996): stage 0: preparative stage, stage 1: initiation of cultures, stage 2: multiplication, stage 3: rooting of shoots, and stage 4: transplantation. This concept emphasizes that a single medium or environment is not sufficient for in vitro multiplication and complete regeneration of plants. Transferring the propagules through a series of specifically designed chemical and physical environments at each developmental stage holds the key to success.

2.5.1 Stage 0: Preparative stage

The preparative stage is sometimes essential to get quality explants for establishment of culture in stage 1. In this stage, the mother plants are grown under suitable environmental conditions to reduce the chance of contamination during explants establishment. Keeping the woody and bulbous plants under the suitable temperature treatment help in breaking bud dormancy and provide more responsive buds (Bhojwani and Razdan, 1996). Balachandran et al. (1990) planted rhizomes of Curcuma aeruginosa, C. caesia and C. domestica (synonym C. longa) in sand to obtain new sprouts. Clean rhizome pieces with sprouted buds served as explants. Senawi (1985) obtained responding cultures of Theobroma cacao only from the stock plants grown under the glass house condition. Niedz and Bausher (2002) observed that explants of Citrus sinensis from field grown plants were less responsive to disinfestation treatment than those collected from greenhouse grown plants. Rathore et al. (2006) maintained Pueraria tuberosa plants in green house to obtain fresh sprouts suitable for initiating cultures from nodal explants.
2.5.2 Stage I: Initiation of culture

Shoot tip and shoot bud cultures are the most successful tissue culture techniques in recent years as an alternative means of asexual propagation of economically important plants. Morel (1965) was the pioneer in applying shoot tip culture in cloning of the orchids. The ability of explants to survive, multiply and regenerate is a consequence of a wide variety of factors such as the origin of plant material, history of explants, physiological status, endogenous hormone concentrations and general culture conditions, i.e. mineral salts, carbohydrate, light and temperature.

2.5.2.1 Plant Material:

The size of the explants determines the survival of the culture. In general, the larger the explants better the chance for survival. Kartha and Gamborg (1975) demonstrated that only explants of cassava exceeding 0.2 mm in length formed complete plants. The large explants such as shoot tips and buds instead of the minute meristems should be selected for micro propagation. However, the smaller explants have the advantage of greater chances of virus elimination (Nehra and Kartha, 1994). Nadgauda et al. (1978) reported that rhizome bud explants of Curcuma longa less than 2 mm in length showed very poor growth or died in the culture medium. They found that 2-5 mm size was optimum for proper growth. Meenakshi et al. (2001) also found that explants of 2.5 and 3.0 mm size were optimum for shoot induction of Curcuma longa.

Roest and Bokelmann (1981) reported that in carnation nodal segment culture, the percentage of shoot development between explants taken from the top and the base of the shoot was 88.6 and 69.8 respectively. Since there is only one terminal bud per shoot, many workers also use axillary buds. Mittal et al. (1989) reported development of plantlets from axillary buds of Acacia auriculiformis, a leguminous tree. Lauzer et al. (1992) reported that the size of the nodal explants
was critical; segments shorter than 0.5 cm were less suitable for micro propagation of Dioscorea abyssinica and D. mangenotiana.

The nodal explants were employed to initiate cultures for in vitro propagation of some Dioscorea species such as D. zingiberensis (Chen et al., 2003), D. alata (Narula et al., 2003), D. cayenensis and D. trifida (Mitchell et al., 1995), D. alata (John et al., 1993), D. abyssinica and D. mangenotiana (Lauzer et al., 1992), D. alata and D. abyssinica Jean and Cappadocia (1991), D. bulbifera (Forsyth and Van Staden, 1982) and D. alata and D. rotundata (Mantell et al., 1978). Borthakur and Singh (2002) reported direct plantlet regeneration from male inflorescence of D. floribunda. Kohmura et al. (1995) used immature leaves for micro propagation of D. opposita.

Young vegetative buds of sprouted rhizome were used as explants for the micro propagation of Curcuma species such as C. longa (Nadguda et al., 1978; Balachandran et al., 1990; Meenakshi et al., 2001; Shirgurukar et al., 2001; Sunitabala et al., 2001; Salvi et al., 2002; Ali et al., 2004; Rahman et al., 2004; Prathanturarug et al., 2005), C. amada (Prakash et al., 2004; Nayak, 2002), C. aromaticum (Nayak, 2000), C. aeruginosa, C. aromatica, C. brog, C. caesia, C. malabarica, C. raktakanta, C. soloensis and C. sp (unidentified) (Tyagi et al., 2004), C. caesia and C. zedoaria (Bharalce et al., 2005) and C. zedoaria (Loc et al., 2005). Salvi et al. (2000) reported direct regeneration of shoots from immature inflorescence cultures of C. longa.

2.5.2.2 Time of the year

The time of the year that explants are collected from stock plants has an influence on axillary shoot outgrowth. This may be related to the differences in the physiological condition of stock plants grown under natural environmental condition. Mellor and Stace-Smith (1969) found that, for the most of the varieties of potato, meristems excised right after dormancy, i.e. in spring and early summer, root more readily than those taken later in the year. Quraishi et al. (1996) reported that the best period for initiation of shoot culture of Cleistanthus collinus was April
June. At this time of the year explants produced significantly high shoot number and maximum length than during other time of the year. Quraishi et al. (1996) further reported that contamination of the cultures was also dependent on the season; it was maximum during July to September and minimum during January to June. Seasonal variation in axillary shoot proliferation was observed in *Artocarpus heterophyllus* (Amin and Jaiswal, 1993) and *Eucalyptus tereticornis* (Das and Mitra, 1990).

### 2.5.2.3 Juvenile and mature sources

A young developmental stage had often been found to be optimum for shoot regeneration. The physiological status of explants is affected by the age of the donor plant, which is directly related to regenerability of the explants. The young and meristematic tissues in many cases got success in raising regenerative cultures whereas mature explants failed to show such response. Wernicke and Brettell (1980) reported that the two youngest leaves and the basal part of third leaf of *Sorghum bicolor* had regeneration capacity. Five days-old seedlings of *Brassica juncea* produced more regenerative cotyledons (Sharma et al., 1990). The most severe difficulties with the use of vegetative propagules in forestry are associated with the phase change and the maturation of meristems (Libby, 1974). Jones et al., (1985) reported that the shoot tip explants of dwarf apple were established readily when obtained from newly grafted shoot rather than taken directly from orchard trees. Vieitez et al. (1985) did not observe influence of source material on *in vitro* establishment and multiplication of *Quercus robur*, but achieved 83% rooting with explants from juvenile seedlings and 63% with the mature explants. Quraishi et al., (1997) reported that the cultures derived from terminal branches of 50 years old tree explants of *Lagerstroemia parviflora* died after third transfer, whereas micro shoots obtained from seedlings and basal sprouts of another 50 year old tree explants showed 10% rooting on MS medium supplemented with 4.9 μM IBA.
2.5.2.4 Surface disinfection:

The explants obtained from plants grown in vitro do not require any treatment for surface disinfection. However, the explants from greenhouse plants, field grown plants and subterranean parts of the plants need to be surface sterilized before inoculation. The most commonly used surface disinfectant is sodium hypochlorite (NaClO), which is often used as 5-6% commercial bleach. Plant segments are usually soaked in a 10-15% solution of such bleach for 15 - 30 min. Other commonly used surface disinfectants include calcium hypochlorite and mercuric chloride. One to three rinses with sterile distilled water are necessary to remove the disinfectants before the final excision of the explants (Bhojwani and Razdan, 1996). Zok et al. (1998) reported reduction of microbial contamination in yams from 80% to 4% through double surface sterilization using calcium hypochlorite at 8%. Among the several chemicals, HgCl₂ is considered as a potent surface-sterilizing agent; however, its residual inhibitory effect is also greater than the other sterilizing agents commonly used in plant tissue culture (Bhojwani and Razdan, 1996). As the rhizome surface of Curcuma species is rugged and covered with scaly leaves so use of a strong disinfectant like HgCl₂ is essential. Rahman et al. (2004) established 90% of the contamination free culture of Curcuma longa rhizome buds treated for 14 minutes with 0.1% HgCl₂.

2.5.2.5 Medium:

Plant tissue culture medium usually contains inorganic salts, trace elements, carbon source, vitamins, growth regulators and a gelling agent. A wide range of nutrient formulations were developed to fulfill the nutrient requirements of the plant tissue under in vitro conditions. Most commonly used medium in the laboratories are those of Murashige and Skoog (1962); White (1963); Linsmaier and Skoog (1965); Gamborg et al., (1968), Schenk and Hildebrandt (1972); Woody Plant Medium (Lloyd and McCown, 1980) etc. Prakash et al. (2003) studied the influence of different adjuvants viz activated charcoal, casein hydrolysate, coconut water, polyvinylpyrrolidone and triiodobenzoic acid on the shoot production.
potential of the nodal explants derived from in vitro raised male and female jojoba (Simmondsia chinensis) shoots and found difference in morphogenic behaviour of male and female shoots placed on various adjuvants.

2.5.2.6 Carbon source:

Sucrose is one of the most commonly used carbon source in the plant tissue culture media. In the medium it is essential for various metabolic activities. Higher concentrations of sucrose had been utilized to induce embryogenesis (Lu et al., 1983). Different types of sugars in culture media had shown increased level of plant regeneration (Jain et al., 1997). For example, Tissue cultures of Malus pumila grow well in sorbitol as with sucrose or glucose (Chong and Thaper, 1973). Tissue cultures of Sequoia (Ball, 1955) and maize endosperm (Straus and La Rue, 1954) can metabolize starch as the sole carbon source. Brown et al. (1980) replaced sucrose in tobacco callus medium with mannitol without effecting callus growth.

2.5.2.7 Gelling agent:

Semisolid medium is prepared with the addition of gelling agent, which provides support to the tissue and also maintains the level of oxygen, which is generally reduced in the liquid medium. One of the important characteristics of gelling agent is that it can withstand sterilization by autoclaving and the medium turns semisolid when it becomes cool. Most commonly used gelling agents in plant tissue culture medium are agar, agarose, gelrite etc. (Bhojwani and Razdan, 1996). There are many reports indicating the influence of gelling agents on culture characteristics such as somatic embryo formation (Tremblay and Tremblay, 1991), shoot multiplication (Podwyszynska and Olszewski, 1995) and hyperhydricity (Franck et al., 1998).

2.5.2.8 Growth regulators:

Although shoots grown in vitro may synthesize a small quantity of cytokinin (Koda and Okazawa, 1980), roots are the principal sites of cytokinin biosynthesis.
Meristem, shoot tip and bud explants do not contain sufficient endogenous cytokinin to support growth and development. Thus 85% of the explants establishment media were supplemented with a cytokinin. There are three cytokinins frequently used: Kinetin (Kin), 6-benzyladenine (BA) and 2-isopentenyladenine (2-ip). BA is most effective for meristem, shoot tip and bud cultures, followed by KIN. 2-ip has been used less frequently (Nair et al., 1979). About 15% explants can be established without cytokinin. It is likely that a sufficient quantity of endogenous hormone is already present in the explants.

Chen et al. (2003) established nodal cutting of Dioscorea zingiberensis on half strength MS basal medium supplemented with 20.0 g/l sucrose, 8.0 g/l agar, 4.4 μM BAP and 1.1 μM NAA. Narula et al. (2003) reported the best medium for regeneration of D. bulbifera was MS supplemented with 0.5 μM IAA, 20.0 μM Kn, 500.0 mg/l casein hydrolysate and 20% activated charcoal. Borthakur and Singh (2002) used MS basal medium for initiation of culture using male inflorescence explants of D. floribunda. The basal medium was further supplemented with Kinetin, BAP, NAA, IAA either alone or in combination at various concentrations. By the sixth week 80% of the cultures containing 13.94 μM Kn showed development and emergence of green vegetative buds directly from the axils of the bracts. Zok et al. (1998) found suitable media for establishment of yam was MS supplemented with 2 mg/l Kn and 2 mg/l IAA. Kohmura et al. (1995) observed multiple bud formation in D. opposita after 2-3 months of inoculation of immature leaves on MS medium supplemented with 8.9 μM BAP. Mitchell et al. (1995) studied the effect of various factors such as inorganic ammonium, growth regulator supplements, propagule type and subculture period affecting in vitro establishment of D. cayenensis and D. trifida. MS medium supplemented with 800 mg/l ammonium nitrate and 0.5 mg/l BAP was found optimum for explants establishment. Inagaki et al. (1985) established nodal segment of D. opposita on MS medium supplemented with 20g/l sucrose, BA and NAA.
Prathanturarug et al. (2005) established bud culture of Curcuma longa on MS liquid medium supplemented with 7264 μM thidiazuron (TDZ). Then multiple shoots were transferred to MS gelled medium without growth regulator for 8 weeks. The regeneration rate up to 11.4 ± 1.7 shoots/ explants was recorded. Loc et al. (2005) induced shoot culture from rhizome of Curcuma zedoaria on MS medium containing 20.0 g/l sucrose and 5.0 g/l agar, supplemented with 20 % (v/v) coconut water (CW) and BAP concentrations from 0.5 to 5.0 mg/l. Ali et al. (2004) established bud culture of C. longa on MS medium containing 1.0 mg/l BAP and observed that shoot initiation started within 5 days after inoculation. Meenakshi et al. (2001) established culture of C. longa in the MS medium supplemented with 1.0 mg/l BAP, 0.1 mg/l GA and 0.1 mg/l NAA. The same medium was employed for shoot multiplication. Nadgauda et al. (1978) cultured young vegetative buds of C. longa (cvs. Duggirala and Tekkurpata) on modified Smith’s medium supplemented with coconut milk, kinetin, BA and inositol or modified MS medium supplemented with coconut milk, Kinetin and BA resulted in good growth and plantlet formation. Rathore et al. (2006) reported culture establishment of Pueraria tuberosa on MS medium supplemented with 2 mg/l BAP. Li and Zhang (2006) reported callus induction of Pueraria lobata from leaf explants on Gamborg B5 medium supplemented with 1.0 mg/l 2,4-D, 1.0 mg/l NAA, 0.5 mg/l kinetin and 30.0 g/l sucrose. They observed better cell growth and higher yield of puerarin when medium was supplemented with coconut milk and casein hydrolysate. Matkowski (2004) induced callus from roots, leaves and stem segment and determined isoflavonoid content by the chromatographic methods. He reported highest content of isoflavonoids in root callus, followed by leaf and stem callus. Yu and Li (1999) successfully induced callus from leaf and stem explants of Pueraria lobata and found that mature leaf is the best one for callus formation on MS medium supplemented with 1.0 mg/l NAA and 3.0 mg/l BAP. They reported that MS medium supplemented with 2.0 mg/l BA, 4.24 g/l AgNO₃ and 0.5 mg/l 4-PU was suitable medium for bud formation. Callus and cell culture of Pueraria lobata was
reported by several workers (Li and Zhang, 2006), (Liu and Li, 2002), Hakamatsuka et al. (1989) and Takeya and Itokawa (1982).

2.5.2.9 Phenolic exudation:

If the plant species are rich in polyphenolic compounds then after tissue injury of such explants during dissection, such compounds will be oxidized by polyphenoloxidases and the tissue will turn brown or black. The oxidation products are known to inhibit enzyme activity, kill the explants and darken the tissue and culture media. Such phenomena impose a serious block on the establishment of primary cultures. Some of the procedures used by various workers to combat this problem are (1) adding antioxidants to culture medium; (2) presoaking explants in antioxidants before inoculating into culture medium; (3) incubating the initial period of primary cultures in reduced light or darkness; and (4) frequently transferring explants into fresh medium whenever browning of the medium is observed.

Melo et al. (1998) studied the effect of four types of antioxidants on the control of explant and culture medium oxidation during in vitro cultivation of D. cayenensis. They used MS medium with few modifications, supplemented with or without (control) ascorbic acid, cysteine, polyvinylpyrrolidone (PVP) and activated charcoal. They reported higher shoot average number on medium supplemented with 100 mg/l PVP and the use of 150 mg/l ascorbic acid was more efficient in the control of medium darkening. The treatments with cysteine, ascorbic acid and activated charcoal showed decreased shoot average number. Bhat & Chandel (1991) revealed a novel technique to overcome browning during tissue culture of D. alata. They observed that browning did not affect growth of roots and shoots when explants were cultured in large volume of medium, but in a small volume it was lethal. Sealing the cut ends with paraffin wax was found to control browning by preventing exudation. This simple technique permitted establishment of cultures in a small volume of medium. Rathore et al. (2006) incorporated 100.0 mg/l ascorbic
acid, 25.0 mg/l PVP and 0.02% activated charcoal in the MS medium to prevent leaching during tissue culture of *Pueraria tuberosa*.

**2.5.3 Stage 2: Propagule proliferation:**

The main objective of this stage is to produce the maximum number of useful propagule units. In axillary shoot proliferation cytokinin is utilized to overcome the apical dominance of shoots and to enhance the branching of lateral buds from leaf axils. In general, it appears that BA is the most effective cytokinin for stimulating axillary shoot proliferation, followed by in decreasing order KIN and 2ip (Bhojwani, 1980; Yang et al. 1981). Exogenous auxins do not promote axillary shoot proliferation; however, culture growth may improve by their presence. One of the possible roles of auxin in multiplication medium is to nullify the suppressive effect of high cytokinin concentration on axillary shoot elongation and restore normal shoot growth (Lundergan and Janick, 1980). Too high a concentration of auxin may not only inhibit axillary bud branching (Hasegawa, 1980) but also induce callus formation.

Lakshmi Sita *et al.* (1976) concluded that Kinetin is the effective cytokinin for nodal cultures of *D. floribunda*, while Mantell *et al.* (1980) found BAP to be preferable to Kinetin in apical shoot tip of *D. alata*. Borthakur and Singh (2002) used modified MS medium (MMS) for shoot multiplication of *D. floribunda*. Thiamine at 10.0 mg/l was added to MS basal medium in place of the MS standard three vitamins. This MMS was further fortified with 13.94 μM Kn to get multiple shoots. This hormone concentration was the similar to culture establishment stage except modification in vitamin concentration. In this medium maximum of five shoots per shoot explant were obtained within 8 week of culture. Forsyth and Van Staden (1982) reported propagule proliferation of *D. bulbifera* on MS medium supplemented with 5 mg/l Kinetin. When Kinetin was substituted with BAP at only 1 mg/l, 9 shoots formed on each node. Mantell *et al.* (1978) reported rapid clonal multiplication of *D. alata* and *D. rotundata* on the MS medium without any growth regulators.
Bharalee et al. (2005) reported in vitro propagation of *Curcuma caesia* and *Curcuma zedoaria*. They reported the best medium for shoot multiplication was MS medium supplemented with 4 mg/l BAP and 1.5 mg/l NAA for *C. caesia* and MS medium supplemented with 1 mg/l BAP and 0.5 mg/l NAA for *C. zedoaria*. Shirgurkar et al. (2006) reported efficient callus formation of *Curcuma longa* when shoot tips along with leaf base was inoculated on MS medium supplemented with 27 μM NAA, 3% sucrose and 0.2% gelrite. They used callus for gene transfer and regeneration of plantlets. Loc et al. (2005) sub-cultured excised shoot of *Curcuma zedoaria* on MS medium supplemented with 20% (v/v) CW and different concentrations of BAP and Kinetin, either alone or in combination with IBA or NAA. They reported that best medium for shoot multiplication was MS supplemented with 20% (v/v) CW, 3 mg/l BA and 0.5 mg/l IBA resulted in 5.6 shoots per explants after 30 days of culture. Ali et al. (2004) induced multiple shoots of *C. longa* on MS medium containing 1.0 mg/l BAP and 0.25 mg/l Kinetin. Tyagi et al. (2004) reported in vitro plant regeneration and provenance conservation of eight wild species of *Curcuma*. These phenomena were found provenance dependent and influenced significantly by type and concentration of cytokinins used. BAP was found superior to other cytokinins tested for plantlet regeneration and 2 ip for conservation. Rahman et al. (2004) reported that the best medium for shoot proliferation for *C. longa* was MS supplemented with 2 mg/l BAP. In this treatment, number of shoots per culture was 14.5 ± 1.82 and average length of shoots per culture was 6.2 ± 0.04 cm.

Salvi et al. (2002) observed that liquid media was more favorable than agar medium for shoot multiplication of turmeric. Among the different carbohydrates tested, they found that sucrose, fructose, glucose, sugar cubes, maltose and market sugar were equally effective for shoot multiplication and xylose, lactose and soluble starch were inhibitory. Sunitibala et al. (2001) found the best medium for propagule proliferation of *C. longa* was MS medium supplemented with BAP 2 mg/l and NAA 1.0 mg/l. Rout et al. (1995) studied the metabolic changes during in vitro multiplication of *C. longa*. In vitro propagation
was achieved on MS medium supplemented with 4.0 mg/l BAP, 1.0 mg/l IAA and 100-150 mg/l adenine sulfate and 3% sucrose. The biochemical changes occurring during shoot elongation, multiplication and rooting were determined. Balachandran et al. (1990) reported in vitro clonal multiplication of Curcuma species and concluded that for shoot multiplication, a concentration of 3.0 mg/l BAP was found to be optimum for all the species. Yasuda et al. (1988) reported plantlet formation of C. zedoaria from stem tip on MS medium containing 3 mg/l BAP and 1 mg/l NAA. Nadgauda et al. (1978) obtained shoot multiplication of C. longa by subculturing the sterile shoots produced on to the same medium. The multiplication rate increased after the initial few subcultures. Plantlets could be produced throughout the year without any dormancy, which occurs in nature. Rathore et al. (2006) reported propagule proliferation of Pueraria tuberosa on MS medium supplemented with 1.0 mg/l BAP, 0.5 mg/l Kinetin and 0.5 mg/l IAA.

2.5.3.1 Proliferation rate:
Since multiplication is the major economic criterion for successful commercial tissue culture propagation, the proliferation rate determines the feasibility of in vitro propagation of a given species. The chemical composition of culture medium and the physiological state of the plant is of major importance. Numerous examples indicated that the recalcitrant state of many species can be gradually modified through serial sub-culturing. Litz and Conover (1978) experienced an increase in shoot multiplication rate with the increased number of subculture. A proliferation rate of seven fold was reached after nine subcultures. Incubating the plant material in a liquid shaking system for a brief period also appears to result in a modification of the physiological state of certain plant species and speeds up shoot proliferation. However, the liquid shaking system is not necessarily beneficial to all plant species (Kusey et al., 1980). Mantell et al. (1978) reported that one excised segment of D. alata or D. rotundata would produce a complete plant in 3-5 weeks. Each small plant could be split into 3-5 separate nodal segments and inoculated into fresh medium. After 14 - 20 day cycle, it would be
possible to obtain 65,000 plantlets from single node within six months. Zok et al. (1998) concluded that one hundred thousand planting sets of yam could be produced per year from one tuber. Rout et al. (1995) reported that frequency of shoot multiplication of Curcuma longa was increased 4-fold at every 4 weeks interval by sub culturing it in the same medium. Naik et al. (2003) compared the axillary shoot elongation, formation of multiple shoots and rooting of shoots from the nodal segments of Gmelina arborea from the seedlings from six provenances, over several subcultures and they observed a subculture-dependent decrease in multiple shoot formation and root induction.

2.5.3.2 Liquid culture:

Liquid media are ideal in micro propagation for reducing per unit plant production cost and for automation (Deberg, 1988; Aitken-Christie, 1991). Temporary immersion generally improves plant material quality. It results in increased shoot vigour and in the frequency of morphologically normal somatic embryos. Hyperhydricity, which seriously affects cultures in liquid medium, can be eliminated or controlled with these culture systems. Etienne and Berthouly (2002) demonstrated successful regeneration of Solanum tuberosum microtubers and Coffee arabica somatic embryos produced in temporary immersion bioreactors. Lorenzo et al. (1998) found that temporary immersion reduced costs by 46% compared with the standard procedure on semi solid medium. This gain not only resulted in labour reduction but also reduced the space required. Escalona et al. (1999) used temporary immersion to propagate pineapple shoots that resulted in 100-fold increase in the number of shoots during the four-month period following culture establishment and this protocol ultimately reduced the production costs per pineapple by 20% when compared to the conventional method in liquid medium.

Plant tissues from numerous species showed better performance in liquid medium rather on semi solid medium. For example, Hammerschlag (1982) reported production of large numbers of shoots in Prunus persica. Aitken-Christie and Jones (1987) showed that better shoot growth was obtained for Pinus radiata by
replenishing liquid nutrient medium in contrast to monthly transfers on semi solid medium. More somatic embryos were produced in *Triticum aestivum* (Jones and Petolino, 1988) and *Gossypium hirsutum* Gawel and Robacker, 1990). Aitken – Christie and Jones (1987) defined automating organogenesis as regular and complete harvesting of shoots or somatic embryos for acclimatization and plant conservation can be produced in the same container for a longer period. Han et al. (2004) reported the proliferation of shoots of *Lilium longiflorum* was achieved very efficiently on medium with 2.2 μm BA and 2.9 μm IAA. The addition of liquid medium in the same vessels was performed to form normal bulblets, after shoots were proliferated in the above medium for 8 weeks. The addition of liquid medium stimulated formation and growth of bulblets remarkably, compared with no addition of liquid medium. Rizwi et al. (2007) reported higher shoot growth and multiplication of *Chlorophytum borivilianum* in liquid culture medium as compared to solid medium. They also reported that the use of liquid culture medium resulted up to 92.31% reduction in single shoot production cost compared to solid medium.

2.5.4 Stage 3: Root regeneration:

The purpose of this stage is de novo regeneration of adventitious roots from shoot obtained in proliferation stage. There are three phases involved in rhizogenesis: (a) induction, (b) initiation and (c) elongation. Root initiation depends on a low cytokinin to a high auxin ratio. Since the developing young shoots are a rich source of auxin production, the addition of exogenous auxin to rooting media becomes unnecessary in many species (Haswgawa, 1980; Lee et al., 1977). Basal Media such as MS, B5 are rich in salt concentration. Sometimes roots are unable to initiate in such high salt concentration regardless of the types of hormone present. When the salt concentration in the medium is lowered to one half, one third or one fourth of the standard strength, rooting becomes abundant. Although lower salt concentration in a medium may be beneficial to root induction, it sometimes results in poor top growth. Sometimes medium supplemented with activated
charcoal improves root regeneration and development by absorbing toxic substance in the medium.

Chen et al. (2003) reported that rooting of *D. zingiberensis* micro shoots within 20 days of inoculation on MS medium containing 4.9 μM IBA. They reported that more than 85% of the regenerated plantlets survived and grew vigorously 1 month after transplantation in vermiculite. Borthakur and Singh (2002) reported rooting of *D. floribunda* shoot, simultaneously in the shoot proliferation stage on modified MS supplemented with 13.94 μM Kinetin. They also reported root induction in MS medium supplemented with 10.74 μM NAA or 11.41 μM IAA. Grubishich et al. (1991) reported that IAA facilitates rooting in *D. caucasica* and *D. balcanica*. Malaurie et al. (1995) reported significant decrease in rooting and appearance of callus in *Dioscorea* species with the higher concentration of NAA.

Some workers reported spontaneous rooting in *Curcuma* species during multiplication stage such as Prathanturarug et al. (2005), Nayak (2000) and Balachandran et al. (1990). Nadgauda et al. (1978) reported that few roots were formed in *C. longa* during explant establishment stage giving rise to complete plantlet, but to ensure a healthy root system these plantlets were transferred to liquid White's medium and supported on a filter paper platform. Mello et al. (2001) reported root induction in *C. zedoaria* in MS medium without any growth regulators, within 40 days of incubation. Bharalee et al. (2005) observed 9.2 ± 0.15 roots per explant in *C. caesia* and 8.9 ± 0.09 roots per explants in *C. zedoaria* when MS media was supplemented with 0.5 mg/l IAA. Ali et al. (2004) reported 100% rooting when individual micro-shoots of *C. longa* were transferred to MS medium supplemented with 1.0 mg/l NAA. Loc et al. (2005) induced rooting of well developed shoots (30–40 mm in length) of *C. zedoaria* on MS medium containing 20 g/l sucrose and 8 g/l agar, supplemented with 20 % (v/v) CW and 2 mg/l NAA. Rahman et al. (2004) reported root induction in *C. longa* when in vitro proliferated shoots were transferred to half strength MS medium supplemented with 0.1-1.0 mg/l either of NAA, IBA or IAA. They observed that the IBA at concentration of 0.2 mg/l was the most effective for proper rooting, in which 100% shoots rooted
within six weeks of culture. Meenakshi et al. (2001) reported development of healthy roots in C. longa when shoots were transferred to MS medium supplemented with 0.3 mg/l NAA. Salvi et al. (2000) reported root induction in C. longa in the MS medium supplemented with 0.1 mg/l NAA. Rout et al. (1995) reported that the isolated shoots of C. longa were rooted on half MS medium supplemented with 0.25-0.5 mg/l IBA or IAA and 2% sucrose. Balachandran et al. (1990) found 13.32 μM BAP alone to be sufficient for shoot multiplication and rhizome buds of Curcuma species produced shoots and roots simultaneously, and in 4 weeks complete plantlets could be produced. There was no effect of increasing concentration of BAP on in vitro root production. Rathore et al. (2006) reported rooting of Pueraria tuberosa on ½ MS medium supplemented with 0.02% activated charcoal and 2.0 mg/l IBA. Yu and Li (1999) reported that MS medium supplemented with 1.0 mg/l NAA was effective for root induction in P. lobata.

2.5.5 Stage 4: Acclimatization to ex vitro conditions

Acclimatization involves adaptation of plantlets from heterotrophic to autotrophic conditions, and exposure to increase in light intensity, decrease in humidity, diurnal temperature changes and plant pathogens (Gowen, 1995). Comparatively, in vitro plants have less developed cuticle, limited stomata activity, limited mesophyll development and many intra cellular cavities as compared with ex vitro plants. Stem and root anatomies also differ and are not adapted to function under ex vitro conditions (Preece and Sutter, 1991). Gradual hardening to ex vitro conditions is therefore essential. Most important factors at this stage are control of irradiation and water supply (Gowen, 1995). After removal from the culture flasks the roots are rinsed to remove agar residues that might encourage microbial attack. Roots are shortened, since longer ones break and interfere with planting. The containers for acclimatization are trays or root trainers. The potting mixture is aerated with relatively high water-holding capacity (Gowen, 1995). Daniells and Smith (1991) recommended peat: washed sand mixture in 1:2 ratio, for
acclimatization of banana. Acclimatized plants are transferred to nursery and then planted in the field to evaluate their performance.

2.5.6 Field trial of micro propagated plants

Salvi et al. (2002) reported field evaluation of turmeric. They found 95% survival of the micro propagated plants in sterilized soil in paper cups and all of them survived in the field. The micro propagated plants showed a significant increase in shoot length, number of tillers, number and length of leaves, number of fingers and total fresh rhizome weight per plant when compared with conventionally propagated plants. Schneck and Ewald (2001) reported field trial of hybrid larch (Larix × eurolepsis Henry). They recorded the growth and performance (stem form), five years after planting in the field. Micro propagated clones showed a good performance (3.9 m average height) compared to seedling plants (3.1 m average height). Agnihotri et al. (2006) reported field evaluation of in vitro propagated ‘Maggar’ bamboo (Dendrocalamus hamiltonii). They compared TC raised plants with mother plants, in respect of gas and water vapour exchange rates at constant temperature (25 °C) and light intensity (1000 µmol m⁻² s⁻¹). They found that overall photosynthetic characteristics of TC raised bamboo were comparable to their corresponding mother plants.

2.6 In vitro micro rhizome and micro tuber formation:

Micro tuber formation has been observed in many genera (Hu and Wang, 1983) and the procedure has become very important in the propagation of potato (Solanum tuberosum). In a similar fashion, the micro propagation of elite cultivars and the induction of micro tubers could provide an excellent means for both storage and rapid, inexpensive delivery of clonally propagated Dioscorea varieties to the field (Ng, 1988). Uduebo (1971) first reported formation of tubers from nodal cuttings of D. bulbifera on medium supplemented with low levels of IAA. Nodal segments of both D. bulbifera and D. alata produced tubers directly in culture (Ammirato, 1976, 1982). In D. bulbifera, a single shoot followed by a root, formed
tuber on MS medium without any supplement. In the presence of NAA, shoot growth was repressed with formation of tuber. In the case of D. alata, there was tuber, shoot and root growth on the MS medium and tuber formation and callusing with NAA in the medium. Ammirato (1982) reported that both D. alata and D. bulbifera, after growing for 4-5 months in continuous light, developed numerous aerial tubers. They ranged in weight from 3 to 1486 mg and from 2 to 32 mm in length. When planted in soil, tubers larger than 90 mg and 8 mm in length sprouted and produced normal plants and tubers. Xue (2006a) reported that the optimal media to induce micro tuber from stem of D. opposita was MS supplemented with 0.5 mg/l BAP, 1.0 mg/l NAA, 0.5 mg/l PP333 and 3% sucrose. The more suitable carbohydrate was 3% sucrose or 5% glucose. Li et al. (2006) reported micro tuber formation of D. opposita on MS medium supplemented with 2,4-D 0.5mg/l and NAA 0.5 mg/l after 2-3 months of inoculation. Jasik and Mantell (2000) studied the effects of jasmonic acid and its methyl ester on in vitro micro tuberisation of food yam species D. alata, D. cayenensis and D. rotundata. Micro tuberisation was supported in all three species by adding either jasmonic acid or methylester to the medium. Borthakur and Singh (2002) reported in vitro tuberization in D. floribunda. They found that 60% of the cultures produced one to three tubers at the base of rooted shoots. A combination of 40-50 g/l sucrose and 2.68 μM NAA added to the medium induced tubers in 20% of the cultures. Alizadeh et al. (1998) studied individual effects of sucrose, plant growth regulators and basal salt media formulation on in vitro microtuber induction in steroid yam D. composite. Sucrose at 8% (w/v) was the single most significant medium constituent for microtuber induction. BAP was found inhibitory for tuber induction, while NAA and IBA showed promotive effect. John et al. (1993) investigated the effects of plant growth regulators and light intensity on microtuber induction in D. alata. They concluded that abscisic acid (1 μM stimulated and cytokinins (2.5 μM) inhibited microtuber development. Lauzer et al. (1992) reported micro-tuber induction of D. abyssinica and D. mangenotiana. They observed induction of large size micro tubers in the 8 hr day length in both the species. In D. abyssinica microtubers were induced on
media containing 20, 40, 60 and 80 g/l sucrose, whereas in *D. mangenotiana* only 40 and 60 g/l sucrose favoured tuberization. Jean and Cappadocia (1991) reported the effects of light and ammonium ion concentrations on micro tuberization of *D. alata* and *D. abyssinica*. In both species, large number of micro tubers was obtained under 16h and 24h photoperiods, whereas large micro tubers were produced at 8 h photoperiod. In *D. alata* further increase in microtuber size was observed in a culture medium where ammonium nitrate was omitted. Mantell and Hugo (1989) studied the effects of photoperiod, medium strength, inorganic ammonium, sucrose and cytokinin on microtuber development of *D. alata* and *D. bulbifera*. They concluded that ammonium ion inhibited microtuber induction in *D. alata* but not in *D. bulbifera*. Microtubers of *D. alata* were only formed on shoot cultures if these were incubated under 8 h light. Most microtuberization in *D. alata* shoot cultures occurred in full strength MS medium supplemented with 2% sucrose, 2.5 μM Kinetin held under 8 h photoperiods at 25 °C, whereas most micro tuberization in *D. bulbifera* shoot cultures occurred on full strength MS medium supplemented with 4% sucrose, 2.5 μM Kinetin held under 8 h photoperiods at 25°C. Ng (1988) reported in vitro tuberization in white yam *D. rotundata* on MS revised medium supplemented with various concentration of sucrose, 20 mg/l L-cysteine, 0.5 mg/l Kinetin and 0.7 % agar. The frequency of tuberization was affected by the daylength, which is optimal at 12 h and 16 h of light depending on the sucrose concentration. Inagaki et al (1985) reported in vitro bulbils formation in *D. oppositata*. They observed that BA inhibited Bulbils formation, whereas NAA and ABA stimulated bulbils formation. Half MS medium gave the highest percentage of bulbils formation. Sengupta et al (1984) reported tuberization of *D. floribunda*. When they maintained shoots of *D. floribunda* in half MS medium and 0.5 mg/l NAA, 70% of the plantlets formed aerial tubers at nodes.

Shirurgurkar et al. (2001) described the factors affecting in vitro micro rhizome production in *Curcuma longa*. Half strength MS basal medium supplemented with 80 g/l sucrose was found to be optimal for micro rhizome production. Cytokinin BAP had an inhibitory effect on micro rhizome production.
Micro rhizome production depended on the size of the multiple shoots used. Micro rhizomes produced were of a wide range in size (0.1 g - 2.0 g) and readily regenerated when isolated and cultured in vitro on culture initiation medium. Sunitibala et al. (2001) observed in vitro rhizome formation of C. longa in MS media containing NAA 0.1 mg/l, Kn 1.0 mg/l and 6 or 8% sucrose under 8 hr photoperiod. Nayak (2002) reported in vitro production of micro rhizomes of C. amada. She multiplied the propagules in the MS medium containing 3.0 mg/l BAP and 0.5 mg/l NAA to obtain maximum number of shoots. Micro rhizomes were induced at the base of in vitro grown shoots when cultured in MS liquid medium with varying concentration of BA and sucrose under different duration of photoperiod. Optimum response was obtained in the medium supplemented with BAP 5 mg/l, sucrose 80 g/l and incubated under 16 hrs photoperiods. Nayak (2000) reported micro rhizome formation of C. aromatica in MS basal medium supplemented with 5.0 mg/l BAP, 60.0 g/l sucrose at 8 hrs photoperiod within 30 days of incubation. Xue (2006b) reported that formation of micro rhizome of Zingiber officinale in vitro was regulated and controlled by endogenous hormones.

2.7 Some aspects of micro propagation

2.7.1 Hyper-hydration: It causes morphological, physiological and metabolic derangements of plants during in vitro culture. The shoots show poor growth and necrosis before they die. It occurs during intensive multiplication stage, but not during rooting (Gasper, 1991). Mainly hyper-hydration occurs due to the physical and chemical nature of the culture medium and the environment around the cultured plant material. It occurs more in liquid medium than solid medium. Gelling agent is also one of the causes of hyper-hydration. Compared to agar, gelrite or phytigel cause more hyper-hydration (Zimmerman et al., 1991). Generally high concentration of cytokinin, media rich in mineral salts such as MS medium (Paques and Boxus, 1987) and high relative humidity (Debergh and Maene, 1984) promote hyper-hydration. Hyper-hydration can be reduced by increasing the concentration of...
agar (Debergh et al., 1981), improving aeration of the container (Rossetto et al., 1992), overlaying the medium with paraffin (Wardle et al., 1983), using desiccant such as CaSO$_4$ (Ziv et al., 1983) and silica gel (Wardle et al., 1983), lowering or replacing cytokinin by another (Debergh et al., 1981), Lowering concentration of salt from MS medium (Zimmerman et al., 1991), diluting or replacing with some other medium (Vieitez et al., 1985) etc.

2.7.2 Somaclonal variation: The main objective of micro propagation is to obtain true to type plants. Sometimes variants are produced in culture, which may be undesired off type plants. The permanently of off type plants are called somaclonal variants, which are heritable and are an expression of pre existing variation in the source of plants or are due to the de novo variation via undetermined genetic mechanisms (Larkin and Scowcroft, 1981). The presence and absence of variation depends upon the source of explants and method of regeneration or on the source of regenerants (Larkin et al., 1989).

Zhao et al. (2005) found variation in morphological traits and increased disease susceptibility in micro propagated plants of rhubarb (*Rheum rhaponticum*). They reported that micro propagated plants produced significantly more leaves than conventional plants under the same growth period, with a more bushy growth habit. A higher incidence of disease (petiole spotting) was also observed in micro propagated plants. They arrived at a conclusion that micro propagated plants had substantially higher incidence of somaclonal variation and regenerants were not suitable to establish an economic plants comparable to the conventional plants.

Appearance of off-types is another problem associated with micro propagated plants. The induction of such abnormalities are influenced by the choice of explants, composition of culture medium, length of time, provenance material etc. Oil palm is one of the examples, which shows such type of abnormalities in micro propagated plants (Duval et al., 1995). Feminization is one of the common abnormalities seen in this crop plant (Duval et al., 1995). The occurrence of off-type plant in *in vitro* propagated banana has been reported from different places in
the world (Gowen, 1995). The frequency of off-type has been reported to vary from 1 to 50 percent (Daniells, 1988; Stover, 1987a; Hwang and Kao, 1987; Arias and Valverde, 1987) Development with dwarf phenotypes is the common abnormality seen in this case (Stover, 1987b).

DNA markers are useful tools to study variation among individuals. In several studies, they have been used to confirm true to type plants or otherwise among in vitro regenerated plants. Several DNA markers such as AFLP (Amplified fragment length polymorphism), RFLP (Restriction fragment length polymorphism), RAPD (Randomly amplified polymorphic DNA), SSCP (Single stranded conformation polymorphism), SSR (Simple sequence repeats) etc are available. However, RAPD is the most commonly used technique as it is simple and less expensive. Salvi et al., (2002) reported RAPD analysis of regenerated turmeric plants in which they did not find any polymorphism. Prakash et al., (2004) had reported the random amplified polymorphic DNA (RAPD) analysis of the regenerants for clonal fidelity assessment of Curcuma amada Roxb. Tyagi et al. (2007) assessed the genetic stability of Curcuma longa L. using RAPD markers. Rout et al. (2001) reported RAPD analysis Paulownia tomentosa plants raised directly from the meristems. The regenerated plants were genetically similar with the mother plants.

2.7.3 Contamination: Contamination is one of the major problems faced during micro propagation of plants. Microorganisms contaminate the cultures. Slow growing bacteria may remain latent at the initial stage that may be carried out with the explants and show up in the later stage. Some times the culture containers spread contamination due to the fungal spores, present in air with high concentration that may be carried with the worker’s body hair or by the vectors such as mites and thrips (Deberg et al., 1990). Spore concentration in the lab can be controlled by regular vaporization of formaldehyde or a fungicide such as thiobendazole. Thrips can be controlled by treating daily with Bagon TM or treating twice within 5 days with 10% dichlorvos (Deberg et al., 1990).
2.7.4 Browning of medium: Browning or Blackening of the culture medium or the explant is one of the problems faced generally in culturing the adult tissues from woody species and some other species. During the excision of explant the plant face an injury which ultimate response with leaching of phenolic compounds that readily oxidize and produces quinones resulting discolouration of the medium/explant. The phenolic compounds can be phytotoxic which may cause necrosis and ultimately death of the explant (Preece and Compton, 1991). Adoption of some measures is necessary to prevent or reduce browning of medium/explants. When browning persists at each subculture then the addition of some antioxidants such as cysteine, ascorbic acid or citric acid to the culture medium is recommended (Skirvin and Chu, 1979), immersing the explants in a solution of antioxidants for 24 hrs (Ziv and Halvey, 1983), addition of Polyvinylpyrrolidone (PVP) in the culture medium which has capacity to absorb phenolic compounds (Gupta et al., 1980), quick transfer of explants to the fresh medium 2-3 times at short intervals (Preece and Compton, 1991), keeping the cultures initially in the dark may also help to reduce browning (Adams et al., 1979). Activated charcoal is often included in medium due to its absorbing power. It is used to remove toxic metabolites and growth regulators from cultures. Charcoal forms a black suspension, which may conceal infections and make the examinations of cultures difficult, it can however enhance rooting by reducing illumination. Polyvinylpyrrolidone (PVP) can serve a similar purpose but is soluble, giving a transparent solution.

2.7.5 Cost of production: Tissue culture technique is generally more expensive than the other forms of clonal propagation through cuttings or seeds (Pierik, 1991). This technique is highly laborious and requires manual handling at 3-4 stages. Therefore micro propagation is recommended only when the conventional methods do not work or the product is of high value. Since labour charges account for 60-70% of the cost of production of tissue culture plants in developed countries there is considerable interest in automating the process partly or completely (Aitken-
(Christia et al., 1995). In developing countries the labour charge is very cheap which
helped in the establishment of several commercial tissue culture labs. Due to his
reason the developed countries has found an alternative way to reduce the cost of
tissue culture propagated plants is to sub-contract in vitro multiplication of their
materials in the developped countries.

2.3 Application of micro propagation

The FAO Committee on Agriculture has perceived plant tissue culture as a
major technology for the developing countries for the production of disease-free,
high quality planting material and its commercial applications in floriculture and
forestry (FAO, 1999b). *In vitro* clonal propagation is only way to produce millions
of plantlets in one year. Multiplication can continue throughout the season
irrespective of season. *In vitro* conservation and propagation of endangered plant
species is one of the important application of micro propagation technique (Fay,
1992). Earlier tissue culture field was the research area for the plant physiologist
and botanist. Its commercial application was realized through orchid multiplication.
Nowadays a large number of horticultural, plantation and forest species are being
multiplied on commercial level. Many new species are being introduced to meet the
demand of the farmers. *Curcuma angustifolia, Dioscorea hispida and Pueraria
rubrosa* are the important plant species in terms of usage as the food and medicine.
These species have high potential to become valuable cultivated crops. They may
be cooked as delicious food from tikhur and baichandi or may be used in industries
as industrial starch. The present work was planned and executed to achieve *in vitro*
propagation of these three valuable plant species.