Review of Literature
REVIEW OF LITERATURE

*In vitro* propagation is applied on a commercial scale for a wide range of ornamental, forest and horticultural species. The independence of seasonal supply, the cloning of plants for which no vegetative method is available, freedom from disease and clonal uniformity - all combine to increase the value of this technique over the conventional methods.

The most important industrial application of tissue culture has been the rapid clonal propagation or multiplication of plants commonly called micropropagation. Plant species that can be propagated through tissue culture comprises of a long list, and the list is rapidly lengthening.

The so called totipotency which states that cells are autonomic and in principle, capable of regenerating into a complete new plant, was the foundation for micropropagation. The history of micropropagation was further dominated by the discovery of plant hormones, auxins and cytokinins. The most important milestones in history of micropropagation are summarised in Table 1.

Table 1 Important discoveries in plant micropropagation

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Event discovered</th>
<th>Reference</th>
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<tbody>
<tr>
<td><em>Sequoia sempervirens</em></td>
<td>Regeneration of whole plants from callus</td>
<td>Ball, 1950</td>
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<tr>
<td>Dahlias</td>
<td>Virus free plants by meristem culture</td>
<td>Morel and Martin, 1952</td>
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<tr>
<td>Orchids</td>
<td>Mericloning</td>
<td>Morel, 1960</td>
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<tr>
<td><em>Citrus sp.</em></td>
<td>Regeneration and organogenesis from nucellus of citrus ovules</td>
<td>Maheshwary and Rangaswamy, 1965</td>
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<tr>
<td>Gerbera</td>
<td>Induction of axillary branching by use of cytokinins from shoot tips</td>
<td>Murashige, 1974</td>
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<tr>
<td><em>Citrus</em></td>
<td>Plants from protoplasts</td>
<td>Vardi <em>et al.</em>, 1975</td>
</tr>
<tr>
<td><em>Rhododendron</em></td>
<td>Breaking of bud dormancy in explants using cytokinins</td>
<td>Pierik and Steegmans, 1975</td>
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Progress made so far in hardwood species using tissue culture technology has been very limited for various reasons like recalcitrancy, browning, contamination, juvenility etc. The first report on propagation of woody species from mature tissues comes from the National Chemical Laboratory, Pune, India. Many important research papers have been published since then (Table 2).

<table>
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<th>Plant species</th>
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<tr>
<td>Citrus sp.</td>
<td>Mitra and Chaturvedi, 1972</td>
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<tr>
<td>Rhododendron</td>
<td>Pierik and Steegmans, 1975</td>
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<tr>
<td>Apple</td>
<td>Huth, 1978</td>
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<tr>
<td>Santalum album</td>
<td>Rao and Bapat, 1978</td>
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<tr>
<td>Betula pendula</td>
<td>Mc Cown and Amos, 1979</td>
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<tr>
<td>Tectona grandis</td>
<td>Gupta et al., 1980</td>
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<tr>
<td>Dalbergia sisoo</td>
<td>Mukhopadhyay and Mohanram, 1981</td>
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<tr>
<td>Alnus crispa</td>
<td>Read et al., 1982</td>
</tr>
<tr>
<td>Eucalyptus globulus</td>
<td>Mascarenhas et al., 1982</td>
</tr>
<tr>
<td>Oil Palm</td>
<td>Jones et al., 1982</td>
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<tr>
<td>Dalbergia latifolia</td>
<td>Rai and Chandra, 1988</td>
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Now-a-days, propagation utilising *in vitro* technique is practised in many forest and fruit trees. In case of economically important woody plants such as apple, grape, cherry, teak, eucalyptus etc., the technique has been commercialised.

**Tea**

Tea tissue culture has gained importance on account of its economic value. *In vitro* propagation has been most intensively investigated specially in the species of great economic value viz., *Camellia sinensis* (L.) O. Kuntze, *C. oleifera* Abei., *C. japonica* L., *C. reticulata* Lindlay, *C. chrysantha* Tuyama, and some of their hybrids.
Camellia has been regenerated in vitro by three methods; induction of adventitious buds (Kato, 1985), somatic embryogenesis, (Nakamura, 1988) and development of axillary buds from shoot tip and nodal explants (Arulpragasam and Lattiff, 1986). However, several constraints have been observed at every stage in the system development of Camellia. Each aspect has been reviewed for a better understanding of tea tissue culture so as to provide an improved protocol.

One of the earliest studies was Lammert's (1958) work on embryo culture, a technique that is very useful for propagation of hybrids in which the embryo may be incompletely developed within the seed. Lammert removed the seed coat and cultured the embryos in the nutrient solution of Tukey (1934). Good results by embryo retrieval were obtained with interspecific hybrids such as C. japonica X C. reticulata, C. japonica X C. cuspidata and C. cuspidata X C. reticulata fully developed plants were obtained within four weeks.

Little research on in vitro culture of Camellia was carried out before 1980, most of which was done on the establishment of callus culture or cell suspension culture for studies on the biosynthesis of phenols that give tea, a dark brown colour (Forrest, 1969; Ogutuga and Northcote, 1970a; Korestkaya and Zaprometov, 1975; Zagoskina and Zaprometov, 1979). However, no reports were available on regeneration of plantlets either by micropropagation or somatic embryogenesis in C. sinensis until late 1980's.

**Culture media**

The mineral salts most widely used for Camellia cultures has been that of Murashige and Skoog (MS) (1964), or some modifications of it. For the shoot multiplication of C. japonica, Samartin et al. (1986) used MS basal medium which
consistently gave the best results in terms of both growth and vigour when compared with the macronutrient formula of Quoirin and Lepoivre (1977), Knop (Tabachnik and Kester, 1977), and Schenk and Hildebrandt (1972). Heller (1953) used the concentration of all macronutrients increased by a factor of 1.25 and the inclusion of one mM ammonium sulphate. MS, nevertheless failed to give satisfactory results for regeneration from adult *C. japonica* cv. Alba Plena material (Vieitez *et al*., 1989a).

In a series of shoot multiplication experiments (Samartin, 1984), the effect of six macronutrient formulae were investigated; H+SO4, MS, half-strength MS (MS 1/2), woody plant medium (WPM) (McCown and Lloyd, 1981); Gresshoff and Doy (GD) (1972) and Anderson (Anderson, 1984). The best multiplication rate and elongation were achieved with WPM and H+SO4 and the least with MS and 1/2 MS. The optimal medium in these experiments was WPM, which is also very efficient for culturing other woody dicotyledonous species (Read *et al*., 1982; McCown and Sellmer, 1987). Although the differences between WPM and H+SO4 as regards multiplication rate and elongation were not significant, the H+SO4 cultures were more chlorotic and less vigorous. The poor performance of MS and MS 1/2 contrasts with Vieitez’s own results (Vieitez *et al*., 1992) with material of juvenile origin and with those obtained by Carlisi and Torres (1986) when they successfully used 1/2 MS to culture *C. japonica* cv. Purple Dawn of adult origin. These apparent contradictions may have an explanation in terms of genotype differences.

In the rooting stage macronutrient formulae have been used at half-strength, as a common practice with both *C. sinensis* (Kato, 1985) and *C. japonica* (Samartin *et al*., 1986). In the latter, 1/2 MS was found to produce significantly
more roots per rooted shoot than undiluted MS medium. The concentration of sucrose was also found to affect rooting, the optimal level being 30 to 50g/l (depending on clone). The important role of sucrose was likewise stressed by Beretta et al. (1987), who found that 30 g/l increased both the number and length of roots.

**Growth hormones**

Growth regulators are usually used at high concentrations in *Camellia* cultures. Growth hormones such as 2 to 4 mg/l of IBA were used by Kato (1985) with 2 to 4 mg/l of BAP to initiate callus from buds of *C. sinensis* which subsequently differentiated. NAA has often been used to induce somatic embryos from cotyledon explants, while 2,4-D said to be essential for callus induction in tea cotyledons, but was ineffective for the regeneration of plantlets (Wu et al., 1981) and detrimental for embryogenesis from *Camellia reticulata* cotyledons (Zhuang and Liang, 1985b). The most widely used cytokinin for somatic embryogenesis is BAP. However, kinetin has also been used to induce the differentiation of plantlets from cotyledon tissues at concentrations of 10 mg/l in *C. sinensis* (Wu, 1976; Wu et al., 1981) and 0.1 to 0.5 mg/l for the hybrid *C. japonica* X *C. chrysanthum* (Wu et al., 1981). BAP at 1 or 2 mg/l has generally been effective for induction of axillary bud growth in both initial explant cultures and subsequent shoot multiplication stages (Creze and Beauchesne, 1980; Samartin et al., 1986; Carlisi and Torres, 1986; Arulpragasam and Lattiff, 1986). Zeatin at 1 to 2 mg/l, together with 2,4-D is reported to be beneficial in the shoot multiplication of *C. japonica* cv. Alba Plena (Vieitez et al., 1989a), *C. japonica* cv. Fimbriata Alba, and *C. reticulata* (San-jose and Vieitez, 1993).
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disinfect buds prior to dissection when very small (0.2 to 0.5 mm) shoot tips are to be used as explants, since these portions of the plant are protected by their scales and final infection is less than 10%.

Sterilizing the material from field plants is more complicated since both bacterial and fungal contamination rates are almost about 90% as seen in case of field grown shoots of *C. sinensis* and *C. japonica* (Haldeman *et al.*, 1987). Haldeman *et al.*, (1987) proposes a 24h of preculture of field grown shoots in liquid medium that were sterilized in the fungicide Benomyl (1g/l) and antibiotic Rifampicin (10 mg/l), in which about 95% of *C. sinensis* shoots were contamination free (Kato, 1986) whereas 4 mg/l of Benomyl and 10 mg/l of Rifampicin gave an average of 85.5% of uncontaminated *C. japonica* shoots with no phytotoxic effects. Frisch and Camper (1987) treated Greenwood stems in a series of stirred baths. The procedure comprised of an initial water rinse, followed by 1.5 to 2 min in 0.1% hydrochloric acid, 10 min in 3.75% sodium hypochlorite (7.5% Chlorax) with three drops of Tween 20 and 10 min in 7.5% calcium chloride.

Shoots forced to develop from buds of a 50-year-old tree by placing the base of collected branchlets in water in a growth chamber with controlled temperature and humidity were used as initial explants by Vieitez *et al.* (1992). The new shoots were collected when grown to a length of 2 to 4 cm long and sterilized by successive immersion in 70% ethanol for 30 sec followed by either 15 min in 15% commercial bleach (having 4% chlorine) or 5 min in 5% calcium hypochlorite. The contamination rate among the shoot tips and nodes was 5%. It is obvious from the above studies that, mother plants have to be essentially
maintained in a clean atmosphere in a glasshouse or phytotron for a better establishment of cultures *in vitro*.

**Micropropagation by neoformation of adventitious buds**

Most efforts of this kind have been made using *C. sinensis*. Wu (1976) and Wu *et al.* (1981) reported successful regeneration of plantlets from callus obtained from cotyledons of *C. sinensis* using medium containing 1 mg/l of IAA and 10 mg/l of kinetin, though for some varieties it was necessary to increase these dosages to 4 and 20 mg/l respectively. However, it is not made clear whether what occurred in the cotyledon callus was somatic embryogenesis or the differentiation of adventitious buds that subsequently root.

Tian-ling (1982) has reported subsequent regeneration of plantlets from a large number of buds that were obtained from callus differentiated from immature cotyledons and embryos of *C. oleifera*. Indirect regeneration of shoot buds from callus initiated from branches or twig explants was achieved using 1 mg/l of NAA and 3 mg/l of BAP by Phukan and Mitra, (1984). Mu-gin and Ping (1983) reported the development of embryoids from cotyledon cultures of tea, however, regeneration was not reported.

Nadamitsu *et al.* (1986) reported the induction of callus and shoot primordia in cotyledon cultures of *C. japonica*. Sood *et al.* (1986) observed direct shoot buds and regeneration of roots in cotyledonary explants. Frisch and Camper (1987) studied the effect of synthetic auxins on callus induction from tea stem callus. Callusing was observed in leaf, stem, cotyledon segments and young anthers but differentiation was not observed. Kato (1985) reported induction of callus from segments of intact stem tissue of tea seedlings, segments stripped off
their epidermal layers, and also from the epidermal layers. Of the callus produced from epidermal layers, 20% buds were differentiated into embryoids, when transferred to a medium containing 0.5 mg/l of IBA and 10 mg/l of BAP. The shoot buds subsequently developed roots on a filter paper bridge in liquid medium supplemented with 0.5 to 1.0 mg/l of IBA, and the plantlets continued to grow successfully in pots.

*Micropropagation by somatic embryogenesis*

Extensive work has been done on the somatic embryogenesis in tea. The *Camellia* seeds have two large cotyledons which appear to have great capacity for morphogenesis by both adventitious buds and somatic embryos, either indirectly via callus as in *C. japonica* (Bennett and Scheibert, 1982) or directly on the cotyledon surface. The technique of somatic embryogenesis has been employed with *C. sinensis* and *C. japonica* (Kato, 1982, 1986; Yan and Ping, 1983; San-Jose and Vieitez, 1993); *C. chrysantha* (Zhuang and Liang, 1985a) and *C. reticulata* (Zhuang and Liang, 1985b; Kato, 1986; Plata and Vieitez, 1990; San-Jose and Vieitez, 1993). For *C. reticulata*, the average embryoid induction rate was about 43% and normal plantlets were obtained when a modified MS medium containing 1mg/l of BAP and 0.2 mg/l of NAA was used (Plata and Vieitez, 1990; San-Jose and Vieitez, 1993). For *C. sasanqua*, the somatic embryogenesis was initiated on the MS medium supplemented with 1 mg/l each of BAP and NAA. The growth regulator test demonstrated that GA3 has an inhibitory effect on formation of embryoids, but GA3 could stimulate shoot growth and root development of the embryoids (Zhuang et al., 1988).
Efficient differentiation of adventitious embryos were first reported by Nakamura (1988), followed by somatic embryos from leaf and stem callus tissue (Plata and Vieitez, 1990) but no regeneration was obtained. Nakamura (1988) also reported the varietal difference in the differentiation of adventitious embryos in stem callus culture in 1988. Das et al., (1990) reported shoot production from callus derived from embryos of zygotic and cotyledon explants. Bano et al. (1991) obtained 70% recovery of somatic embryoids when young cotyledons were used as explants of a south Indian tea variety at CFTRI, India.

According to Kato (1986) the most useful method of micropropagation is the cotyledon slice culture. Somatic embryos of *C. japonica* and less abundantly *C. sinensis* formed directly on swollen parts of the cotyledon surface without an intermediate callus regardless of the presence or absence of the growth regulators used (BAP, IBA and NAA).

Nakamura (1988) brought out successful differentiation of adventitious embryos from the cotyledon cultures of *C. sinensis*. Somatic embryos of *C. japonica* germinated when transferred to a medium containing 1 mg/l of GA$_3$, wherein additional embryos also arose from the hypocotyl during successive subcultures. Whereas, *C. sinensis* embryos germinated in the presence of 10 mg/l BAP and 0.5 mg/l of IBA but not on GA$_3$. Kato (1986) used to obtain plantlets of *C. vietenensis X C. chrysantha* on MS medium supplemented with 3 mg/l of BAP and 1 mg/l of NAA, and the germination of embryos was achieved on GA$_3$ medium with 10% of coconut milk (Nadamitsu et al., 1986).

Somatic embryogenesis and plantlet regeneration were achieved from immature and mature zygotic *C. japonica* embryos cultured on MS medium
without growth regulators or with various combinations of IBA (0 to 2 mg/l) and BAP (1 to 2 mg/l) (Vieitez and Barceila, 1990). This study showed that somatic embryogenesis readily occurs in vitro on the cotyledons, cotyledonary petioles and hypocotyls of the zygotic embryos. Success depends on both physiological maturity of the zygotic embryo and the kind of initial explant used (embryonic axis or cotyledon segments). It is important to emphasize the positive response of the embryonic axes, especially with the immature embryos, 94% of which were embryogenic competent. This improves the efficiency of the embryogenic system described by Kato (1986) in which only cotyledon explants were used. The somatic embryos produced were generally one of the two distinct types (i) seed like embryos, which were yellowish-white, with large cotyledon and (ii) Toad like embryos which were green with leaves. They were genuinely bipolar, developing into whole plantlets with shoot and root, and the process generally occurred in media with a relatively high concentration of BAP. Embryogenic potential was maintained by secondary embryogenesis through successive generation of embryos. Indirect somatic embryogenesis via callus was also evident.

In C. reticulata cv. Mouchnag, somatic embryos were obtained from cotyledon sections and embryonic axes culture (Plata and Vieitez, 1990). Embryonic competence increased with 0.5 to 1 mg/l IBA and decreased with 0.2 to 1 mg/l NAA, though in the cv. Early Crimson, Zhuang and Liang (1985a) induced embryogenesis with 0.2 mg/l NAA and 1 mg/l BAP, but did not consider the effect of IBA. Vieitez et al., (1983) isolated cotyledons and incubated in 1/2 MS medium supplemented with 5 mg/l of GA₃ and 2 mg/l of IAA for regeneration.

Indirect somatic embryogenesis was induced in callus of C. japonica (San Jose and Vieitez, 1993) initiated on cotyledon explants after three months
culturing in media containing IBA and embryogenic capacity being retained by callus subcultured on 0.5 mg/l of IBA and 1 mg/l BAP.

Micropropagation of *Camellia* by somatic embryogenesis from cotyledons or neoformation of buds in callus culture has only been achieved with material of juvenile origin. Since micropropagation by induction of axillary shoots and subsequent transfer to a rooting medium is currently recognised as a valuable method for many kinds of woody species, the potential of bud culture has been explored for *Camellia* too.

*Explants from juvenile origin*

The first attempts to regenerate plants from shoot tips and axillary buds of *Camellia* were reported by Creze and Beauchesne (1980). They used 0.5 mm long meristems with one or two leaf primordia from one-year-old rooted cuttings or more successfully from three to four-year-old seedlings that had not yet flowered. Such explants were cultured on a modified MS medium supplemented with 20 mg/l of adenine, 0.1 mg/l of IAA, 1 mg/l each of kinetin, 2,iP, BA and GA₃, and 10 g/l of polyvinylpyrrolidone. Although the shoot tip cultures were established and elongated, and nodal cultures were observed to produce shoots more rapidly, no multiplication was reported. Rooting and transfer to soil were also not reported.

More work has been done in *C. japonica* seedlings (Samartin, 1984) where seeds were germinated in pots in the glasshouse and shoots from such plants were used as starting material for *in vitro* culture when the plants were three to four months old.
Explants from adult origin

Creze (1980) reported elongation of axillary buds of *C. japonica* when cultured. However, rooting and hardening procedures were not described. Jacquiline and Torres (1986) reported successful *in vitro* propagation of *C. japonica* Purple Dawn in culture. Vieitez *et al.*, (1989a) reported propagation of *C. japonica* var. Alba Plena from mature shoot tip explants. However, in all the cases shoot apex and nodal explants of 1 cm long were aseptically placed on modified basal medium used by Creze and Beauchesne (1980) but without PVP, 2,1 P, and GA3. Cultures were incubated under 16 h photoperiod under cool white fluorescent lamps with day and night temperature of 25 °C and 18 °C, respectively. Shoot tip explants began to develop after four weeks, and on transfer to fresh medium, they produced single shoots whose axillary buds began to grow about six weeks after the transfer. A total of 10 weeks was essential from the time of cutting of explants to the establishment of shoot multiplication stage. Nodal explants formed callus but developed no buds, unlike Creze and Beauchesne’s (1980), a discrepancy that may probably be attributed to the age difference in the starting material and the physiological state of lateral buds.

Micropropagation from shoot tip and nodal explants of *C. sinensis*

Singh (1978) highlighted the advantages of *in vitro* micropropagation over the conventional propagation in tea and shade trees. If propagation of tea and shade trees could be achieved with high degree of efficiency through tissue culture, many benefits of plant genetics and improvement methods can be harnessed in the form of genetically superior planting materials.
Creze (1980) reported the formation of multiple shoots from the shoot tip culture of tea. Apparently, there was no progress on rooting, hardening etc. Subsequently, Phukan and Mitra (1984) obtained regeneration of tea shoots from the nodal explants. Meanwhile shoot tip culture was reported by Meenakumari and Mitra (1984) and growth of buds and callus from explanted organ by Sarwar (1985). Work on micropropagation of tea from axillary buds and shoot tips was initiated by Arulpragasam (1986) in Sri Lanka. However, none of the above reports elaborated the protocol for successful rooting.

Up to mid 1980’s the major work on tea tissue culture was confined to secondary metabolites. Most of the work that had been carried out elsewhere on tea tissue culture has been in connection with plant regeneration from callus and for the investigation of secondary metabolites (Forrest, 1969; Ogutuga and Northcote, 1970).

Arulpragasam and Lattiff (1986) of Sri Lanka reported a method for tea tissue culture from shoot tip explants. Nakamura (1988) reported rapid in vitro multiplication of *C. sinensis*. However, the success rate in both these studies was less than 20%, due to high contamination and browning. Nissaka *et al.* (1986) defined the optimum conditions for culturing the tea shoot tips by means of mathematical design. Seneviratne *et al.* (1988) reported rooting of shoots in culture, although the success rate was minimum. Nakamura (1988) reported the effect of various hormones on growth of shoot buds in tea. But, no rooting was reported. Jain . (1991) induced roots in regenerated shoots of *C. sinensis*, but the rooting was less than 10%. Nakamura (1988) developed a system for multiplication and rooting, for a few Japanese varieties. According to him, the
potential for tissue culture by repeating this technique could be 47000 shoots every month.

Studies on Indian tea cultivars have begun only during 1984 by Phukan and Mitra. First report on propagation of Darjeeling tea by tissue culture was by Jha and Sen (1991). They established cultures from cotyledonary nodes and shoot tips of germinated seedling as well as nodal explants of field grown plants. Shoot multiplication rate ranged from four times in nodal explants to 35 times in cotyledonary nodes after 18 weeks of culture.

Rajasekaran and Kumar (1990), Rajkumar and Ayyappan (1992), and Manivel et al. (1993) worked on some south Indian tea varieties. However, the rooting percentage still remained 10 to 15%. The results obtained by Rajasekaran and Raman (1993) indicated that for different phases of growth, the optimal conditions vary with regard to minerals, as well as the kind and concentration of cytokinins. Initial establishment was better in WPM but for the continued growth, MS was suitable. Rooting was also found to vary depending upon the type of clone ranging from 10 to 50%, the rest eventually died. Polyphenols and contamination were the two factors affecting the cultures to a great extent.

**Browning**

A serious problem associated with the culture of tissues from woody species is the oxidation of phenolic substances leaching from the cut ends of the explants. To tackle the problem, various approaches have been made by those working with tissue culture of woody species as well as tea.
When the problem of browning occurred only during the initial stage of culture, explants were better when cultured in liquid medium containing antioxidant solution for a few days and then transferring to a semi solid medium. In *Tectona grandis* terminal and axillary buds were suspended in 2% sucrose solution and 0.7% soluble PVP to prevent browning (Gupta *et al.*, 1980b). For *Eucalyptus citridora* the explants in a liquid medium were initially incubated in a continuous light for three days and then transferred to fresh media of similar composition and maintained in a shaker for two days. The individual shoots were then excised and transferred to semi solid media for further multiplication (Gupta *et al.*, 1980b). The shoot tip explants of *Psidium* and *Vaccinium angustifolium* were soaked in a solution containing 150 mg/l of citric acid and ascorbic acid prior to surface sterilization (Amin and Jaiswal, 1987). A combination of citric acid (75 mg/l), PVP (5 g/l) and ascorbic acid (50 mg/l) resulted in 80% survival of guava explants (Amin and Jaiswal, 1987). To overcome browning at every subculture incorporation of antioxidant became obligatory (Dhawan, 1991).

In *C. sinensis*, shoot tip and axillary bud cultures were badly affected by browning due to phenols, which was more in shoot tip explants as shoots contain more polyphenols. Rajasekaran and Raman (1993) reported that within hours of inoculation, the media surrounding the explant turned brown due to leaching of polyphenols. When left in situ, the explants themselves subsequently turned brown and eventually blackened succumbing to death (Phukan and Mitra, 1984; Nakamura, 1988; Rajkumar and Ayyappan, 1992). Pretreatment of explants or inclusion of various chemical antioxidants known to mitigate the inhibitory influence of the phenolics did not help in alleviating the problem completely. However, repeated transfer of these explants, two to three times at eight hourly
Rooting

Most reports on tissue culture of *Camellia* lack information on rooting, hardening and subsequent field transfer. It is to be noted that until late 1990’s rooting was a great barrier in micropropagation of tea.

In comparison with other species, *Camellia* requires higher auxin concentrations and longer immersion time for acceptable rooting rates to be achieved (Srikandarajah *et al.*, 1982; San-Jose *et al.*, 1988). Such vigorous rooting treatments are not deleterious to *Camellia*. Druart *et al.* (1982), observed that complete darkness at the beginning of the rooting stage may be beneficial. An initial light period followed by some dark period induced rooting in apple (James, 1983) and raspberry (Welander, 1985). In *Eucalyptus* after repeated subculturing two diverse responses were obtained. In a 20-year-old *E. citridora* the shoots could be rooted only after the third cycle from 35 to 40 % rooting. However, 45-50% rooting was obtained in the fifth and subsequent passages (Gupta *et al.*, 1980a). In some cultivars of apple also, the rooting was improved by repeated subculturing (James, 1983). But in some species rooting frequency decreased with subculturing, perhaps due to their increased levels of endogenous cytokinins. For *Populus deletorius* the rooting frequency of the shoots formed on the mother explant was as high as 80% but later declined to 20% by the end of the fifth subculture.

Earlier work on tea has shown that rooting of regenerated shoots was mostly unsuccessful (Wu, 1976; Wu *et al.*, 1981; Phukan and Mitra, 1984; Sarwar, 1985; Arulpragasam and Lattiff, 1986; Nakamura, 1988).
Stimulatory effect of aluminium on the growth of cultured roots was postulated by Masakitusui et al. (1994). The study showed that growth of tea roots was stimulated by the supply of aluminium and phosphorus together, but was not affected when aluminium and phosphorus were supplied separately.

Kato (1985) reported rooting of shoots regenerated from callus in a medium with IBA but, success rate was less than 5%.

In the initial rooting experiment for *C. japonica* (Samartin et al., 1986), cultures were placed for 11 days in 1/2 MS to which one to 10 mg/l of IBA had been added, after which they were transferred to an IBA free medium. Since rooting rate of only 8 to 16% was achieved, in subsequent experiments, they employed the immersion technique by dipping the cut ends in concentrated solution of auxin.

Tian-Ling (1982) and Kato (1986) induced root formation on a filter paper bridge over liquid medium, whereas Beretta et al., (1987) claimed that agar medium to be superior to liquid medium. In the experiment with cv. Alba Plena, Vieitez et al. (1989b) showed that the type of support (Agar/filter paper bridge) did not significantly affect the rooting percentage or the number of roots per rooted shoot.

Seneviratne et al. (1988) reported rooting of shoots, successfully, produced from cotyledonary explants. The rooting was obtained in medium containing above 3 mg/l of IBA for two cultivars. However, Nakamura (1988) used IBA dip as chronic treatment to induce rooting for 30 to 40 min and obtained 60% rooting.
Matsumoto et al., (1993) reported formation of roots and malformed leaves with IBA 0.5 to 3 mg/l. However, a chronic pulse treatment with IBA (200 to 500 mg/l) has improved the rooting and leaf emergence. A maximum of 90% rooting was obtained when cuttings were treated with 2000 mg/l IBA and the minimum with 200 mg/l IBA.

**Acclimatization**

Very few reports mention conditions and preparation for transfer of Camellia plantlets to soil. For the plantlets obtained by somatic embryogenesis or induction of adventitious buds, Tian-Ling (1982) and Kato (1985) used a mixture of soil and vermiculite for acclimatization. Wu et al. (1981) reported that tea plantlets derived from cotyledon callus grew vigorously in soil, but were morphologically different from naturally grown plants to a considerable extent. Plantlets regenerated in vitro flowered in the following year after their transfer to soil, and subsequently, F₁ hybrids were also obtained from them. Phukan and Mitra (1984) observed genetic variability among plants produced by indirect regeneration via callus tissue, whereas plantlets obtained by axillary bud culture were true-to-type.

Vieitez et al. (1989b) achieved a survival rate of 70 to 90% in C. japonica after transfer of four week-old rooted juvenile or adult plantlets in 1:1 mixture of soil and quartz sand in pots placed under poly tunnel with mist and fog system in the green house. They found that acclimatization can also be done by transferring the plantlets to a tunnel just before roots appear (12 to 14 days after auxin treatment). Roots thus induced were healthy and more efficient.
Other approaches of tea tissue culture

Anther culture: Progress made so far in anther culture has been far less. Doi (1981) obtained roots from cultured anther but shoots were not regenerated. Chen and Leo (1983) cultured tea anthers which were stored at 5 °C for two days. Shiny calli were formed on N6 medium supplemented with 0.5 mg/l 2,4-D, 1 mg/l kinetin, 100 mg/l serine and 800 mg/l glutamine. Transfer of such calli (or prolonged culture in the same medium) to N6 medium containing 2 mg/l zeatin, 20 mg/l adenine and 10 mg/l casein permitted shoot growth. Rooting was obtained in 0.1 mg/l IAA.

Raina and Iyer (1983) obtained multicelled procallus or embryoids in tea anther culture, but no regeneration was obtained. Shimukado et al. (1989) reported formation of embryoids and callus in tea from anthers on modified MS media supplemented with 0.05 mg/l 2,4-D and 0.2 mg/l kinetin. Palni et al. (1991) cultured anthers on MS or N6 medium supplemented with 0.05mg/l kinetin. In some cases anthers were pretreated at 4 °C prior to inoculation. Swelling of anthers and callusing was evident in two to three weeks. Pale yellow callus subsequently turned green. Profuse and dark callus was obtained on N6 medium with 2,4-D (0.5 mg/l) and kinetin (5.0 mg/l). A few celled embryo like structures and tracheids were also observed.

Cell suspension culture: Cell suspension was developed from callus and the formation of multicelled embryoids were obtained. Formation of profuse adventitious (somatic) embryos were seen directly from the cotyledon explants. These embryos were further encapsulated in 2% sodium alginate and made into synthetic seeds (Palni et al., 199*).
Mutation breeding

Except for the few natural triploids and polyploids reported by Janakiammal (1952), the cultivated tea plant is a diploid with chromosome number 2n=30. Despite severe mutilation to which the plant has been subjected by pruning and plucking throughout its long history of cultivation, it is remarkable that cytological behaviour of tea has remained virtually unchanged, although polyploidy is of common occurrence in the ornamental *Camellias* (Janakiammal, 1952). Bezbaruah (1975) has made detailed karyotype analysis of several tea clones belonging to three races of tea cultivars.

Naturally evolved polyploids in tea are very rare. Only a few natural triploids (3n=45) have so far been reported from Japan, North East India and South India (Venkataramani, 1968). The natural tetraploids (4n=60) have also been reported from N.E. India. Triploids in general are more vigorous and hardier than diploids. Simura (1956) found them to be more cold tolerant under conditions prevailing in Japan. Some tetraploids and aneuploids have also been found to possess higher vigour. However, the polyploids are generally poor in cup quality (Bezbaruah, 1975). Tea of acceptable quality and productivity has also been made from south Indian triploid clone ‘Sundaram' B/5/63 or UPASI-3.

Mutagenic chemicals like colchicine, X- and gamma-rays are being tried on tea for the production of triploids. Katsuo (1966) applied 0.2% colchicine to axillary buds on etiolated tea shoots and kept them in the dark. The treated shoots produced a tetraploid and a few chimeras. Sebastiampillai (1976) achieved success by treating the apices of growing shoots *in situ* with colchicine. The terminal buds of shoots carrying four to five expanded leaves were dissected to
expose the meristematic tissues which were then covered with 1% blocks of agar impregnated with 0.2 or 0.5% colchicine in gelatin capsules. The agar blocks with capsules were removed after two to seven days and the shoots were allowed to grow and produce four to five more leaves. In most shoots, only one or two leaves arising just above the point of treatment showed morphological aberrations. Treatment with 0.2% colchicine for not less than five days with 0.5% for a minimum of three days induced tetraploid. Exposure to 0.5% colchicine for six days caused maximum mutation although one of the experimental clones failed to respond even to this treatment, thus displaying differential sensitivity of tea clones to colchicine. Many shoots died but 12 tetraploid plants could be isolated out of 95 treated shoots. However, there is no report on their rooting, hardening or field trials.

Initial attempts at Tocklai Tea Experimental Station, Assam, to induce mutation by treatment of tea shoots with colchicine and ethyl methane sulphonate (EMS) did not meet with success (Anonymous, 1970). However, by resorting to more drastic treatments, Goswami and Sarma (1979) succeeded in inducing mutation. Growing apical buds of tea were kept immersed in 0.5 to 2% solution of colchicine for two to seven days without severing them from the plant. Many treated shoots died and the survival rate decreased with severity of the treatment. A loss of large number of shoots at 6.6%, 13.3% and 20% levels was obtained. From tetraploids of three biclonal varieties of tea used, a differential sensitivity of tea plants to colchicine was clearly displayed.

Amma (1974) succeeded in inducing tetraploidy by irradiating whole plants of the popular Japanese clone Yabukita at 17.9 Kr in a cobalt 60 gamma field. Of the shoots produced by the irradiated plant, one was found to be a tetraploid. The
shoot was longer than those of a source material and it had shorter but thicker internodes, bigger and thicker leaves, heavier flowers with bigger ovary, thicker filaments and larger but less frequent stomata. Fertility and pollen viability of the tetraploids were low. Chemical analysis revealed lower tannin content and higher total nitrogen in the tetraploid than in the diploid. Growth of the tetraploid cutting was also inferior to that of the original diploid.

Induction of somatic mutation by mutagenesis is an important method for improving the specific characteristics of vegetatively propagated crops, particularly tea without altering most of the desired traits of commercial clones. Changes due to different doses of gamma ray, EMS or colchicine were observed in metabolic processes like glycolysis and oxidative phosphorylation and cytochrome oxidase and catalase activities in different plant systems (Sermsiri, 1989). Besides these, it has also been reported that ionising radiations affect auxin metabolism which controls the synthesis of specific enzymes. Effect of gamma rays has shown variations on the activities of invertase, amylase, peroxidase, nitrate reductase, changes in the activities of peroxidase and esterase as reported in Banana (Pedroso, 1990) and Chrysanthemum (Sermsiri, 1989).

However, induction of mutation in tea with the aid of radiation has not met with success anywhere. Attempts made at the Tea Research Stations, to produce mutants by exposing seeds, cuttings and pollen grains to X- and gamma-radiations have not succeeded yet (Anonymous, 199

Despite a very few reports on induction of mutation and in vitro regeneration in Chrysanthemum (Sermsiri, 1989), Banana (Pedroso et al., 199

mutation breeding has not reached to the extent of a theoretical concept, especially
in the tissue culture system. More intensive research is required to be made in this vital area, considering the potentialities in evolving elite cultivars incorporating different desirable traits.