Review Article

Chapter 7

# ASSESSMENT OF PHYSIOLOGICAL, BIOCHEMICAL AND GENETIC FIDELITY WITH A BRIEF REVIEW ON TISSUE CULTURE OF TEA

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## Introduction

Tea [*Camellia sinensis* (L) O. Kuntze] is the oldest non-alcoholic caffeine containing beverage crop, belonging to the genus *Camellia* (family Theaceae). India is the foremost producer, and after Sri Lanka and China, it is the third largest exporter of commercial tea in the world (Anonymous 2000). The ever-increasing demand for higher productivity and better quality of tea has resulted in the need for mass multiplication of elite clones. Tea is propagated largely through seeds or cuttings, but a major concern associated with seed raised populations has been the occurrence of high genetic variability. Although vegetative propagation of elite clones has been used for large scale multiplication of plants with desirable attributes, for example in respect of yield and quality, one of the drawbacks of such clonal plantations, noted over the years in field, is yield variability under adverse/stress conditions. Such plantations are also relatively prone to pathogens in comparison to seed grown plantations (Barua 1989). Tissue culture provides a method of rapid multiplication of selected genotypes (Nakamura 1987; Kato 1989; Arulpragasm 1990; Palni *et al.* 1991; Rajkumar and Ayyappan 1992; Vieitez *et al.* 1992; Sood *et al.* 1993; Dodd 1994; Akula & Dodd 1998; Das 2001; Mondal *et al.* 2004). The conventional breeding and propagation techniques have contributed significantly to the growth of tea industry over the past several decades in a variety of ways including the varietal improvement. However, in view of the limitations of conventional breeding methods coupled with the demand for vertical increase in productivity, at affordable cost of production, application of biotechnology based tools provides attractive alternative approaches (Nakamura 1991; Palni *et al.* 1999; Palni 2001; Mondal *et al.* 2004 and references therein).

While plant tissue culture is essentially used as a method of clonal (true-to-type) propagation, tissue culture induced variation also provides an additional tool for enhancing genetic variability, including the incorporation of desirable traits like high yield. Variation in cultured cells and plants regenerated from these is referred as somaclonal variation (Larkin & Scowcroft 1981; Scowcroft *et al.* 1983; Evans *et al.* 1984; DÁmato 1985; Evans 1986). Under certain conditions plant cells in culture undergo various visible, morphological, biochemical and genetic changes, alteration at the ploidy level, chromosomal aberrations (DÁmato 1985; Buyser *et al.* 1988; Detrez *et al.* 1989; Stelly *et al.* 1989; Ye & Yu 1989) and mutations (Bhatia *et al.* 1985). Besides, changes at the level of DNA, protein and

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enzymes (Berlyn 1982; Cullis 1983; Landsmann & Uhrig 1985; Ball & Seilleur 1986; Brettell *et al.* 1986; Brown *et al.* 1990) have also been reported.

In view of tea being a long lived crop, it is important that well tested and quality planting material should be used for raising plantations. At this juncture tissue culture technology along with advances in the field of genetic engineering is likely to make a major impact in the area of rapid and clonal multiplication of elite clones. Characterization of the existing tea clones/populations on the basis of various parameters, e.g., physiological, biochemical, resistance to biotic and abiotic stresses, yield, etc., and the selection of appropriate tea clones for different agro-climatic zones are important issues that need serious consideration. Temperature is one of the major environmental factors determining the rate of growth as well as the natural geographic distribution of tea (Squire & Callander 1981). Photosynthesis, being one of the first physiological processes to be greatly affected by temperature, is widely used as a tool for rapid selection of plants most suited for different habitats. In case of tea, with some exceptions (Squire 1977; Tanton 1979), studies have indicated that growth of harvestable shoots depends on the photosynthetic rate of maintenance leaves and developing shoots (Manivel & Hussain 1982). The effect of temperature on photosynthetic characteristics of six tea clones, originally from different agro-climatic zones in India, was examined to determine the clonal variation in photosynthesis (Joshi & Palni 1998). The cited study demonstrated that clear cut clonal variations in temperature response of photosynthesis exist and may be used to offer useful suggestions to tea growers. Thus existing variations in the rate of photosynthesis may offer some potential for improvement in tea yield (Joshi & Palni 1998).

Systematic assessment of variability in tea is indeed required to determine the existing genetic diversity and taxonomic relationships between different tea clones. There have been several programmes aimed at improving the genetic potential of tea, both in terms of increased yields and to confer protection against abiotic and biotic stresses. Plant survival, growth and productivity are closely linked with various physiological and biochemical processes. Various morpho-physiological markers used in the past have helped only marginally in the selection of clones for desired agronomic traits as these are liable to be influenced by environmental factors (Kulasegaram 1980; Wachira *et al.* 1995). Thus there is an urgent need for physiological and biochemical characterization and improved understanding of the genetic variation that exists within and between the available cultivated and wild tea accessions. This knowledge is fundamental and will pave the way for undertaking programmes designed to enhance the genetic potential of tea. Therefore, this article intends to review the available information on the assessment of tea clones based on physiological and biochemical parameters, and also to look at the current methods that are in use for examining the genetic fidelity of tissue culture raised tea plants.

### *Tissue culture of tea*

Initial tea plantations across the tea growing regions of the world, as well as in India, are most likely to be seed raised as in the beginning this was the only, and subsequently the preferred method for tea propagation in the past (Barua 1989; Wilson & Clifford 1992; Banerjee 1993, 2001; Jain 1999). Since tea seeds are recalcitrant in nature and very sensitive to desiccation, they lose viability very rapidly as they undergo dehydration with time (Roberts 1973; Chen & Roberts 1980). Therefore,

tea seeds are routinely placed in germination beds, after cracking, as early as possible while still fresh. Tea is also propagated by vegetative means using single node cuttings; it is simple and cuttings can be rooted, in most clones, without any chemical treatment.

A number of reports have appeared on in vitro culture of tea using various plant parts as explants, viz., leaf, node, internode, cotyledon, embryo, root, anther, and pollen, etc. for example on Murashige and Skoog (1962; popularly known as MS) medium. Several reviews have been published on micro propagation of tea and related species (see Introduction) including reports on the production of secondary metabolites in cultured tea cells. Studies by Forrest (1969) and Ogutuga & Northcote (1970) were perhaps the earliest reports on tea tissue culture that were directed towards biosynthesis of caffeine in callus tissues. Subsequently Russian workers (Zaprometov *et al.* 1994) investigated the process of polyphenol metabolism and lignin synthesis in suspension cultures. The first report on the successful regeneration of tea plant was obtained by using cotyledon segments in culture (Wu 1976), followed by reports of plant regeneration from cotyledons and stem callus (Kato 1985, 1986; Sood *et al.* 1993). Arulpragasam & Latiff (1986) developed actively proliferating cultures from tea shoot tips and also reported a regeneration system using cotyledon callus. Production of rooted plants from in vitro produced shoot tips and axillary buds was reported by Nakamura (1987). Plant regeneration has also been achieved by several groups using nodal explants (Phukan & Mitra 1984; Saratchandra *et al.* 1988; Arulpragasam 1990; Nakamura 1991; Jha & Sen 1991; Palni *et al.* 1991; Bag *et al.* 1997; Akula & Dodd 1998).

Anther culture is an effective way to obtain haploids for use in plant improvement programmes. There have been several studies aimed at the production of haploid tea plants using anther culture (Chen & Liao 1983; Raina & Iyer 1983; Shimakado *et al.* 1986; Zhenguang & Huihua 1987). Haploid plants thus obtained can be diplodized to obtain homozygous diploids for use in breeding programmes and for developing inbred lines. Successful production of a homozygous diploid line by anther culture has indeed been reported (Raina & Iyer 1983; Chen & Liao 1983). Homozygosity, if achieved at all loci would greatly improve the process of selection, and for screening mutants. Thus haploidy holds great potential for initiating investigations on plant improvement.

Protoplast fusion and somatic hybridization resulting in the development of interspecific hybrids of tea have been achieved by workers in Japan. The natural infection of tea plants with *Agrobacterium* has been reported (Chen & Chen 1989), and *A. tumefaciens*-mediated transformation, the most widely used technique in woody plants, has also been attemped in tea by several workers (Hooykaas & Schilerpoort 1992; Palni *et al.* 1993; Biao *et al.* 1998; Matsumoto & Fukui 1998, 1999; Luo & Liang 2000; Mondal *et al.* 1999, 2001a, b) using different explant systems, like *in vitro* grown leaves, somatic embryos, etc. However, earlier attempts did not yield encouraging results. The first healthy transgenic plants were produced using somatic embryos as explants (Mondal *et al.* 2001b). Following selection and germination of embryos, the resulting transgenic microshoots were multiplied in vitro and later micrografted on to seedling derived root stocks of the same cultivar; the transgenic tea plants were subsequently hardened in a polyhouse (Mondal *et al.* 2001b). Earlier Zehra *et al.* (1996) have used *A. rhizogenes* (strain A4) to induce hairy roots in the leaves of *in vitro* grown seedlings of tea and obtained fast growing hairy root lines. Konwar *et al.* (1998)

also obtained transformed hairy roots following co-cultivation of in vitro raised tea shoots with *A. rhizogenes*. Although transformed hairy root cultures have been exploited commercially for secondary metabolite production in several plant species, this method has not been further exploited in tea.

Although the first successful report on the regeneration of tea plantlets from the cotyledon derived calli was published by Wu and coworkers (Wu 1976; Wu *et al.* 1981), it was not clarified whether it occurred via somatic embryogenesis or through differentiation of adventitious buds followed by root formation. Kato (1982) and Yan & Ping (1983) reported induction of somatic embryos when mature cotyledon sections were cultured on MS medium supplemented with BAP and IBA. Histological information on the events leading to somatic embryo formation, including a novel pathway of embryo development, as well as report on the formation of secondary embryos have appeared (Palni *et al.* 1991, 1993, 1999). Higher rates of somatic embryogenesis using immature cotyledons have been reported by several workers (Nakamura 1988; Bag *et al.* 1997; Akula & Dodd 1998; Mondal *et al.* 2001c). Somatic embryogenesis has been successfully used for artificial seed production in cultivated tea (Mondal *et al.* 2000) and in some inter-specific hybrid crosses of tea (Nadamitsu *et al.* 1986) where immature somatic embryos were rescued, and cultured before abortion. Highly reproducible and repetitive somatic embryogenesis has been reported where increased rate of multiplication of secondary embryos (24 fold) was achieved using a temporary immersion system (Akula *et al.* 2000). The method involves no culture transfer and does not include plant growth regulators, making it labour, time and cost effective; and the entire process of multiplication, maturation and plant recovery was achieved on the same and single medium within a period of 17 weeks.

The acclimatization (popularly known as hardening) and field performance of micro propagated plants is a particularly important aspect that needs utmost attention. Substantial number of micropropagated plants do not survive after transfer from controlled in vitro conditions to ex vitro environment of the green house and later in the open field. Only 20% survival occurred when tissue culture raised plants were directly transferred to soil, but the survival reportedly improved to 70% when a mixture of peat and soil (1:1) and high humid conditions were used (Banerjee & Agarwal 1990). Among those researchers who obtained plantlets either through the somatic embryogenesis route or by induction of adventitious shoots followed by rooting, Kato (1985) used a mixture of soil and vermiculite (1:1), Bag *et al.* (1997) used soil-sand mixture (3:1) and Akula & Dodd (1998) used mixture of sand, peat and vermiculite (1:2:1) in nursery pots kept under mist in a green house for successful acclimatization. In another study (Rajasekaran & Mohankumar 1992; Mondal *et al.* 1998), micropropagated tea shoots were harvested, subjected to 30 min treatment with IBA, and transferred to Hikkotray containing pre-sterilized sand and cow dung (1:1). These trays were kept in a polytunnel (with intermittent watering for 90 days) inside a polyhouse (90% survival rate). Subsequently the plants were transferred to polythene sleeves containing black virgin soil, and kept for a year in the same polyhouse. This technique has been used for successful hardening of micropropagated tea plants.

Identification and isolation of microorganisms from the tea rhizosphere including a large population of beneficial as well as antagonistic microbes (known to produce antibiotics) were used, for the first time,

for biological hardening of tissue culture raised tea plants (Pandey *et al.* 2000; Bag *et al.* 2001). It was reported that two bacteria, namely *Bacillus subtilis* and *Pseudomonas corrugata* are suitable for hardening of micropropagated tea plants. When tissue culture raised tea plants were treated with antagonistic bacteria during their lab to land transfer, it resulted in highly enhanced survival, i.e. 88-100% as against 36-52% survival in control plants during different seasons. Analyses of rhizoplane and rhizosphere soil indicated that the major biotic factor responsible for mortality following transfer of tissue culture raised tea plants to soil was fungal attack. Bacterial inoculations also resulted in growth promotion of tissue culture as well as seed raised plants of tea (Pandey *et al.* 2000). A number of organisms including antagonistic fungus such as *Trichoderma*, Vesicular Arbuscular Mycorrhizae (VAM) and *Piriformospora indica* have also been found to be suitable for hardening of micropropagated woody plant species (Singh *et al.* 2000).

### *Physiological, biochemical and molecular characterization of tea*

Traditional objective of most tea breeders has been to achieve increased plant yield without compromising the quality. However, in an increasingly competitive market, both increase in yield and betterment in quality of made tea are required to make replanting of old estates economically viable (West *et al.* 1996). Thus, for mass scale production, efficient, reliable and cost effective propagation methods are of pivotal importance; the long term genetic stability of plantations is equally important. Although evaluation of clonal stability is difficult and time consuming, nevertheless ensuring genetic fidelity (stability) is essential, particularly in tissue culture raised populations.

Clonal identification has traditionally been based on morphological descriptors such as plant height and shape, leaf size and shape, young leaf type, fruit shape, etc. However, as in many out-crossing crops, tea is highly heterozygous with most of its morphological, physiological and biochemical descriptors showing continuous variation and high plasticity. Furthermore, most morphological traits are influenced by the environment, plant age and phenology. Wu *et al.* (1981) reported that tea plantlets derived from cotyledon callus grew vigorously in soil, but were considerably different in terms of their morphology from the “normally” or conventionally grown plants. In bamboo (*Bambusa balcooa* and *B. tulda*) the clonal fidelity of regenerants has been determined by Random Amplified Polymorphic DNAs (RAPD) analysis (Das & Pal 2005); the researchers advocated the use of axillary meristem culture for true-to-type or clonal propagation. The regenerants were morphologically indistinguishable under in vitro condition, therefore, RAPD analyses were conducted to sort out this problem, as well as to test the clonal fidelity. In all cases fingerprinting profiles matched exactly with the respective donor (mother) plants, thus confirming genetic homogeneity of in vitro raised plants. In another study (Bhatia *et al.* 2005) genetic analyses of cotyledon derived regenerants of tomato (*Lycopersicon esculentum*) were done using Amplified Fragment Length Polymorphism (AFLP) markers; eighty-five markers were generated using the primer pair M-CAC/E-ACT and M-CTC/ E-ACT. Identical fingerprints were generated for each primer pair for the mother plant and the regenerants. A total of 15 non-parental bands were observed, of which three were shared or common and 12 were

unique. Estimation of genetic similarity coefficient based on RAPD band-sharing data indicated that ten regenerated plants were more than 95% similar to the mother plant, but one was found to be distinctly different. By these results they concluded that tomato is particularly prone to genetic change if produced through callus cultures (Bhatia *et al.* 2005). Furthermore, since objectivity is crucial to accurately do the morphological typing, use of unambiguous descriptors for plant identification and discernment of genetic relationships is essential (Mondal *et al.* 2004).

Physiological and biochemical parameters can also be adopted to characterize tea clones. Plant survival, growth and productivity are reported to be intimately coupled with the aerial environment through processes such as energy and gaseous exchange, e.g. loss of water vapour through transpiration and uptake of carbon dioxide in photosynthesis (Jarvis *et al.* 1988; Stoutjesdijk & Barkman, 1992). The rate of water vapour exchange affects the energy budget and transpiration of a leaf and consequently the physiology of the whole plant (Gates 1975; Berlekamp & Overdieck 1989; Chandra & Dhyani 1997). The chlorophyll 'a' fluorescence technique, a simple, rapid, reproducible and non-destructive method (Mohammed *et al.* 1995) has also been used for assessing the performance of tea plant under a given set of environmental conditions (Joshi & Palni 1998; Vyas *et al.* 1998). Chlorophyll 'a' fluorescence technique was suggested to be used as a screening tool for the selection of tea clones tolerant to low temperature and frost, so that appropriate clones can be recommended for colder regions experiencing frost (Vyas *et al.* 1998, 1999; Joshi *et al.* 2000). Tea clones that were maintained under shade during winter months showed Fv/Fm ratios (measurement of intrinsic efficiency of PS II in the form of a ratio between fluorescence variable-Fv and fluorescence maximum-Fm) closer to 0.80 in comparison to plants kept in the open (Vyas *et al.* 1999). This indicates that shade trees not only protect tea bushes from high irradiance and temperature but also from cold and frost. The reduction in Fv/Fm ratio under field conditions during winter has been reported in other species also, including tea (Somersalo & Krause 1989; Adams & Perkins 1993; Vyas *et al.* 1998). Following in vitro multiplication of *Quercus leucotrichophora* and *Q. glauca* through intact embryos and cotyledonary node and subsequent field transfer, it was found that in vitro raised and hardened plants of both the species were comparable under *ex vitro* cnditions to conventionaly produced plants in terms of gas and water vapour exchange characteristics (Purohit *et al.* 2002). In another study micropropagation and comparison between in vitro propagated plants and seedlings of *Thamnocalamus spathiflorus,* a temperate bamboo, was carried out by Bag *et al.* (2000). These workers compared gas and water vapour exchange rates at different light levels (0, 100, 500, 1000, 1500, 2000 µmol $m^{-2}s^{-1}$) between in vitro raised and seed raised plants of same age and reported that in vitro propagated and hardened plants are morphologically as well as functionally comparable to seed raised plants of the same age. Data on physiological parameters such as gas and water vapour exchange are likely to provide valuable information regarding the suitability of tissue culture raised plants for field plantation.

Biochemical markers have also been used for characterization of different tea germplasm. A chemo-taxonomic method of classifying clones was suggested (Takeo 1983) based on Terpene Index, a ratio between linalools and linalools plus geraniols. Subsequently with the advancement of high performance liquid chromatography, considerable success has been achieved in the identification of

quality indicators (Takeo 1981; Owuor *et al.* 1986). Some of these indicators found much wider use in distinguishing the two main species of tea, i.e. *C. sinensis* and *C. assamica* and their respective clones (Owuor 1989). Hazarika & Mahanta (1984) used quantitative changes in chlorophyll a, chlorophyll b and four carotenoids for characterization of Assam, China and Cambod tea. The total catechin level and the ratio of dihydroxylated to trihydroxylated catechins of green leaves have been used to establish genetic relationship among the 102 tea accessions in Kenya (Magoma *et al.* 2000). Based on the accumulation of different catechins, the tea clones could be separated into 3 major and 5 minor groups, according to their phylogenetic origin. The results on biochemical differentiation indicate that there is potential for broadening the genetic base amongst the existing various types of teas in Kenya, i.e., with Assam, China and Cambod teas (Magoma *et al.* 2000). Although accuracy of detection is higher, accumulation of such chemicals is subjected to post-transcriptional modification, and hence as mentioned by Staub *et al.* (1982) the utility of biochemical markers is somewhat restricted.

Use of isozyme markers was widely used in the past for cultivar identification in plants. Isozymes provide potentially powerful and reliable tools in resolving genetic relatedness/divergence and have been used as molecular markers in genetic, phylogenetic, taxonomic and evolutionary studies (Moss 1982; Richardson *et al.* 1986). In tea, isozymes have been analysed by several workers (Hairong *et al.* 1987; Xu *et al.* 1987; Anderson 1994; Singh & Ravindranath 1994). Among the isozymes, peroxidase and esterase have been studied in several tea cultivars. Some of the other isozymes like tetrazolium oxidase, aspartate aminotransferase and alpha-amylase were also studied among 7 different tea cultivars along with 3 different species (see Mondal *et al.* 2004 and references therein). The banding pattern revealed both quantitative and qualitative variation amongst the different species and their clones; moreover, between these enzymes, tetrazolium oxidase exhibited highest variability. Although isozyme analyses have been used as a marker, in general, isozyme studies in tea were restricted to a few enzymes with inadequate polymorphism (Wachira *et al.* 1995), and thus with the advancement of newer techniques of molecular biology such efforts were shifted to DNA based markers.

Before somatic embryogenesis (or others means of micropropagation) can be utilized for large scale production of tea plants, evaluation of the genetic fidelity of regenerated plants is a must for verification of true-to-type nature of individual regenerants. Tea germplasm has routinely been characterized using morphological (Sealy 1958; Wickremaratne 1981; Amma 1986; Banerjee 1988, 1992), physiological and biochemical descriptors (Takeo 1981, 1983; Nagata & Sakai 1985; Owour *et al.* 1986, 1987; Owour 1989; Joshi & Palni 1998; Vyas *et al.* 1998, 1999; Magoma *et al.* 2000). Although these descriptors are valuable for identification of varietal groups and can reveal, to some extent, inter and intra varietal polymorphism but can not account for the total diversity in the species. Therefore, application of DNA based markers like Random Amplified Polymorphic DNAs (RAPD), Inter Simple Sequence Repeats (ISSR), Single Nucleotide Polymorphism (SNPs), Cleaved Amplified Polymorphic Sequence (CAPS), Simple Sequence Repeats (SSRs), Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), etc. provide more reliable tools for the determination of genetic variability. RAPDs involve the use of single short random

oligonucleotides. The DNA amplification with random primers exposes polymorphism distributed throughout the genome (Williams *et al.* 1990). The use of RAPD markers in genome mapping and gene tagging has been exploited in recent years. ISSR are DNA fragments (100-3000 bp) located between adjacent, oppositely oriented microsatellites, amplified by the PCR using microsatellite core sequences and a few selective nucleotides as a single primer (16-18 bp). About 10-60 fragments from multiple loci are generated simultaneously, separated by gel-electrophoresis, and scored as the presence or absence of fragments of particular size. Because of the multilocus fingerprinting profiles obtained, ISSR analyses can be applied in studies involving genetic identity, parentage and clone, and strain identification (Powell *et al.* 1996; Kaundun & Matsumoto 2002).

RFLPs are genetic differences observable at the DNA level, and characterized by a number of variable length restriction fragments. The analysis involves DNA cleavage by restriction enzymes, electrophoresis of resulting fragments, southern transfer of separated fragments to a membrane support, radioactive labeling of suitable probes, hybridization of probes to membrane supported fragments and detection in the form of a banding pattern on x-ray films. AFLP is a powerful, reliable, stable and rapid method of assay with potential application in genome mapping, DNA finger printing and marker-assisted breeding (Vos *et al.* 1995). This PCR-based technique permits inspection of polymorphism at a large number of loci within a short time and requires very small amounts of DNA. The reproducibility of AFLPs is ensured by using restriction site specific adaptors and adaptor-specific primers with a variable number of selective nucleotides under astringent amplification conditions (Vos *et al.* 1995). The potential use of different molecular markers such as RFLP (Matsumoto *et al.* 1994), RAPD (Lee *et al.* 1995; Wachira *et al.* 1995; Kaundun *et al.* 2000, Kaundun & Park 2002) and AFLP (Paul *et al.* 1997) have been reported in tea. Development of CAPS and their use in tea has also been reported (Kaundun & Matsumoto 2001). Molecular markers highlighted important differences in the partitioning of diversity within and among populations. These markers are useful for characterization, estimation of genetic relatedness and determination of genetic diversity of tea germplasm. In case of tea, the highest level of polymorphism, as determined by the expected heterogygosity ($H_{av}$), was detected with AFLPs. The utility of a given marker system is, however, a balance between the level of polymorphism (information content) and the extent to which the assay can detect multiple polymorphisms (Wachira *et al.* 2001). Powell *et al.* (1996) derived a convenient estimate, i.e. Marker Index (M.I.) of marker utility that is a product of the information content and the multiplex ratio. In case of tea, AFLP assay had a higher M.I. than RAPDs (Wachira *et al.* 2001). Powell *et al.* (1996) also found that AFLP had a higher M.I. than RFLPs, RAPDs and SSRs. The advantage of the cross transferability of heterologous chloroplast and nuclear microsatellite primers was considered to detect polymorphism among 24 tea genotypes (Kaundun & Matsumoto 2002). They used PCR-based marker SSRs for the amplification of DNA. Comparison between RAPD, AFLP and SSR markers in different plant species has revealed that co- dominant SSRs detect the highest level of polymorphism per locus as it surveys the hyper-variable microsatellite regions of the genome; hence they have higher information content (Powell *et al.* 1996; Maughan *et al.* 1996). Nevertheless molecular markers with higher

multiplex ratio, such as AFLP and RAPD may be more useful than SSRs despite their dominant nature (Powell *et al.* 1996; Russell *et al.* 1997). Their multilocus approach allows them to screen a wider region of the genome and to estimate relationships with a high-resolution approach (Teulat *et al.* 2000).

### *Genetic fidelity of tissue culture raised tea plants*

The crucial part of any in vitro propagation system is mass and rapid production of plantlets which are phenotypically uniform and genetically similar to the mother plant, otherwise the advantage of desirable characters of elite/supreme clones will not be achieved. Very limited work has been carried out to assess the genetic fidelity of tissue culture raised tea plants. A number of approaches have been applied for identifying variants among micropropagated plants, for example phenotypic variation (Vuylsteke *et al.* 1988), karyotypic analyses of metaphase chromosomes (Jha *et al.* 1992) and biochemical analysis (Damasco *et al.* 1996). These techniques are not so helpful because of limited number of informative markers and are under the influence of environmental conditions or developmental processes (Rani *et al.* 1995). Moreover, the above methods are not fully suitable for detecting DNA sequence polymorphisms of in vitro raised plants (Mondal *et al.* 2004).

Molecular markers such as RAPD, RFLP, ISSR, SSR, SNP, CAPS, etc. can be used advantageously for assessment of genetic fidelity of tissue culture raised plants. RAPD has been used appropriately for a number of crop species to detect genetic integrity among micropropagated plants (Isabel *et al.* 1993; Rani *et al.* 1995; Rani & Raina 2000). More recently Mondal & Chand (2002) applied RAPD analysis with 50 primers to investigate genetic variability of in vitro raised tea plants of cultivar T-78. Among them 39 primers developed 197 monomorphic bands in all the concerned plants. The primer SC 10-12 produced maximum 8 bands while primer SC 10-57 produced single monomorphic band. It was observed that 24 out of the 221 bands produced by the remaining 11 primers were polymorphic for four micropropagated tea plants. These 24 loci were monomorphic for the remaining plants. The polymorphic amplification products of 11 primers were present in the 4 plants and were compared to remaining 14 plants by conducting a second DNA extraction from respective plants. In a separate experiment, use of these 11 primers indicated monomorphism of the amplified DNA fragments amongst 5 vegetatively propagated plants of the same cultivar, i.e. T-78. As a result they concluded that variation occurred as a result of mutation during micropropagation (Mondal & Chand 2002).

In another study, Devarumath *et al.* (2002) used RAPD, ISSR and RFLP fingerprints to examine genetic integrity of micropropagated plants (produced from nodal explants) of three diploid and triploid elite clones of tea: UPASI-26 representing *Camellia sinensis* (China type) and UPASI-3 and UPASI-27 representing *C. assamica* ssp. *assamica* (Assam-India type). These workers assessed genetic fidelity of these plants by analyzing their nuclear, mitochondrial and chloroplast genomes using multiple molecular DNA markers. A total of 465, 466 and 462 genetic loci were produced with RFLP, RAPD and ISSR fingerprinting in the micropropagated plants and the corresponding mother plant. The micropropagated plants of both the UPASI-3 and UPASI-27 (Assam-India type) clones revealed complete stability in the 462 and 446 genetic loci. In contrast, clone UPASI-26 was different wherein 36 (7.74% of the total) out of 465

genetic loci were found to be polymorphic; this indicates lack of complete genetic fidelity among the regenerants of this clone. The observed polymorphic loci were not restricted to a particular genome. ISSR fingerprinting detected more polymorphic loci than RAPD fingerprinting. No polymorphism was observed in the chloroplast genome of the micropropagated plants of the three tea clones. Among the three clones examined, two clones (UPASI-3 and UPASI-27; Assam-India type) exhibited complete stability during in vitro propagation through enhanced axillary branching culture method, while the China type (UPASI-26) was prone to genomic changes during propagation. These workers concluded that organized meristem cultures are not always genetically true-to-type, and the genomic changes in tea clones are genotype dependent rather than culture condition dependent (Devarumath *et al.* 2002).

The above tests on genetic fidelity of micropropagated tea indicate variation is likely to occur during in vitro culture even when nodal explants or meristematic tissues are used. Hammerschlag (1992) suggested that somaclonal variation among in vitro raised plants can be eliminated or minimized if efforts are made to distinguish between axillary and adventitious shoots that are produced during in vitro propagation and removing the adventitious shoots. Somaclonal variation has been detected in micropropagated plants of a number of species; at the same time studies also report maintenance of genetic integrity among meristem-culture-derived plants (Isabel *et al.* 1993; Rani & Raina 2000; Devarumath *et al.* 2002 and references therein). It is, therefore, important that assessment of genetic fidelity of micropropagated plants is absolutely necessary for successful commercial plant propagation via in vitro methods.

## Discussion

The potential of tissue and organ culture for rapid mass multiplication of elite tea clones and for the improvement of tea quality and production has long been realized. Tissue and cell culture technology along with the advancement in the field of genetic engineering are likely to make a major impact on the tea industry in the areas of rapid and mass clonal propagation of elite clones, disease elimination and incorporation of resistance, production of pure breeding lines, germplasm storage and exchange, interspecific and intergeneric hybridization, development of polyploids and mutants, and the improvement in quality and yield. Plant tissue culture methods can be effectively used in improvement of tea by generating fundamental information on growth, development, physiology, and biochemistry of cells or organs in culture under defined conditions and correlations are likely to emerge which are applicable to whole plants. Suspension cultures and callus tissue have been used to generate information on the biosynthesis of compounds like caffeine, theanine and polyphenols in tea (Ogutuga & Northcote 1970; Koretskaya & Zaprometov 1975; Matsuura & Kakuda 1990). Now a days in vitro multiplication of tea is emerging as an efficient, reliable and convenient system for the mass multiplication of tea which should be exploited commercially.

Improvement in the quality and yield of tea, as in other highly heterozygous, long lived and cross pollinated crops, through conventional breeding method is, however, a difficult and time consuming task. This review focuses on the verification of genetic fidelity of micropropagated plants (true-to-typeness among donor plants and tissue culture regenerants) as well as physiological and biochemical assessment of such plants. At present very few plant species are micropropagated

commercially via the somatic embryogenesis pathway, especially because of clonal fidelity (Cervelli & Senaratna, 1995). For example, Hashmi *et al.* (1997) detected somaclonal variants in peach regenerates initiated from two different embryo callus cultures using RAPD and suggested that genetic changes occurred during tissue culture. In another study RAPD markers were used by Iojkowska & Kawiak (2004) to verify the clonal fidelity of two micropropagated *Drosera* species, *D. anglica* and *D. binata*, which were regenerated by adventitious budding from leaf explants and shoot tips, respectively. Twenty arbitrary decamers were used to screen 15 randomly selected plantlets of each species. No genetic variation was detected among *D. binata* regenerants, whereas a 0.08% polymorphism frequency was estimated for *D. anglica* plantlets. These results indicate that regeneration of plants through shoot-tip culture is a low-risk method for obtaining genetic variability, whereas material regenerated through leaf explants requires further verification.

RAPD analysis was used to estimate genetic diversity and taxonomic relationship in 38 clones belonging to the three tea varieties, *assamica*, *sinensis* and *assamica* ssp *lasiocalyx* (Wachira *et al.* 1995). Extensive genetic variability was detected between species, which was partitioned into, between and within population components. A total of 27 primers were used and 21 generated polymorphic loci. A total of 253 bands were scored, of which 157 (62%) were reproducibly polymorphic. The number of products generated by each primer varied from 5 to 20. The maximum polymorphism of 20 bands was detected by primer SC10-56. The amplified fragments and similarity matrix ranged from 0.3 to 3 kb and 43% to 96%, respectively, among the clones. From these results the researchers concluded that RAPD analysis can discriminate all of the 38 commercial clones, even those which cannot be distinguished on the basis of morphological and phenotypic traits (Wachira *et al.* 1995). Drought tolerance associated DNA marker was developed by Mishra & Sen-Mandi (2004) in tea clones growing in Darjeeling. AFLP fingerprints for 29 Darjeeling-grown tea clones were developed and using eight primer pair combinations in the 29 clones, 677 PCR products, including 469 polymorphic bands were observed. The results of this study indicate that the Darjeeling-grown tea plants taken up in this investigation belong to a narrow gene pool originating through intra as well as inter-specific hybridization. RAPD analysis of DNA of ten short listed (on the basis of field performance for drought tolerance) clones using 11 primers, revealed 180 PCR products of which 131 were polymorphic bands. This shows that the RAPD method is capable of revealing appreciable levels of polymorphism in tea clones (Mishra & Sen-Mandi 2004).

Nguyen *et al.* (2004) used AFLP analysis to evaluate the genetic variation among cultivated chickpea and wild *Cicer* relatives. In total 214 marker loci were assessed, of which 211 were polymorphic (98.6% polymorphic) across the 95 accessions that represented 17 species of *Cicer*. The genetic variation within a species was highest in *C. pinnatifidum* followed by *C. reticulatum* and lowest in *C. macracanthum*. Preliminary studies on cataloging the genetic variation in neem involved scoring for morphological variations. Schmutterer (1995) has described two morphologically distinct varieties in neem, namely *Azadirachta indica* and *A. indica* var *siamensis Valenton* which is an exotic variety from Thailand. These two varieties can also be distinguished on the basis of pollen morphology. Variation in physiological aspects such as rates of net photosynthesis, stomatal characteristics, leaf

area and whole plant phytomass production has also been determined by Kundu & Tigerstedt (1998). Vendrame *et al.* (1999) evaluated the applicability of AFLP analysis for the assessment of the genetic variability in somatic embryos of pecan (*Carya illinoinensis* C. Koch) and made comparisons between and within embryogenic culture lines. AFLP readily detected differences between culture lines, with 368 polymorphic loci identified. Individual culture lines generally produced somatic embryos with similar overall banding patterns. Embryos derived from the same culture line generally grouped together in a phenogram generated by UPGMA (unweighted pair-group method, arithmetic average) analysis.

In conclusion, tea is a crop for which the world demand continues to increase. There is a need for improved techniques of propagation to rapidly supply large number of plants of newly developed varieties. The most important part of any in vitro propagation system is mass multiplication of plantlets which are phenotypically uniform and genetically akin to the mother plant, otherwise the advantage of desirable characters of elite/supreme clones will not be achieved. Concerted efforts are now required for improvement in the quality of tea and mass propagation by using conventional and biotechnological approaches. Estimation of genetic fidelity of micropropagated tea plants is also an important task. If done properly, micropropagation of selected suitable clones is poised to make spectacular contribution to the growth of tea industry.

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