Research Report

TIME DIVISION CYCLE IN TEA (*CAMELLIA SINENSIS*) CULTURES

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ABSTRACT

*Propagation of tea (Camellia sinensis (L.) O. Kuntze) through tissue culture is gaining momentum worldwide. We have studied the cell division cycle at regular intervals to optimise the medium and time period when maximum mitotic activity is seen. The study also includes effect of medium constituents on the genomic stability since such reports on cell division in "*in vitro*" grown tissues in woody perennials are scanty. Perceptible differences in the mitotic indices were noticed in the cells from root tips, callus and somatic embryos growing under identical conditions. Best response was found in cells of the actively growing root tips. Further, it was seen that there was a very narrow time frame when the cells accounted for maximum divisional activity. Values for mitotic indices varied with the composition of media and growth regulator employed. This information could be helpful for better understanding of the somaclonal variations.*

Keywords: India; tea cell division; tissue culture; mitotic index

INTRODUCTION

Tea is an important commercial crop. It is cross-pollinated and shows high levels of heterogeneity in the progenies. The use of tissue culture methods for mass multiplication has facilitated screening, selection and propagation of useful variants.

In plants, like other organisms, cell division results from the interaction of both intrinsic and extrinsic signals and controls. It is opined that once the cell has passed the G_1 phase, the cell cycle generally unfolds on its own so long as no other cell cycle component is limiting (Chasan, 1995). However, manipulations carried out at cellular level, followed by appropriate selection procedures and subsequent plant regeneration, can lead to variants with desired characteristics like resistance to biotic/ abiotic stresses, improved quality, vigour and yield.

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Cell division plays a crucial role in all phases of plant development, continuing organogenesis (callus tissues, embryoids, shoots/roots etc.) and growth. Responses to a changing environment require precise understanding of cell division and ploidy levels, which may ultimately lead to the understanding of origin of somaclonal variations (Karp and Bright, 1985). Although there are a large number of reports on cytological analysis of *in vivo* grown plants, analysis of *in vitro* grown tissues is rather limited. Das (1992) reported a wide range of chromosomal variations in tea callus. Since physico-chemical factors can be controlled for *in vitro* growth, it would be useful to find their effect on the regulation of cell division cycle. Besides it would also be useful to ascertain the time period when maximum number of cells undergo division.

Chromosomal instability and variation of DNA content of individual cells from callus cultures have been reported in many plant species (Bayliss, 1980; Cavallini and Cionini, 1986). Despite the fact that

chromosomal stability has been recorded in several plant species (Mahfouz et al, 1983; Murashige and Nakano, 1967; Zagorska et al, 1974), factors like composition of nutrient media and hormones/ growth regulators, which have an effect on endo-reduplication, non-disjunction and fragmentation of chromosomes and their variations, have been analysed (Bayliss, 1980; D'Amato et al, 1980; Krikorian et al, 1983). Evans and Reed (1981) examined factors controlling the origin of numerical variants of chromosomes. Polyploidization during callus growth has also been reported in a variety of systems including tea (Kato, 1989). Torrey (1965) suggested that hormonal composition of the medium plays an important role in determining the type of cells of the explant that would undergo division.

Present studies were undertaken with a view to study the role of media constituents (PGRs) and photoperiod on chromosonal behaviour during mitotic division. For this, different *in vitro* grown tea tissues were fixed at regular time intervals round the clock in a bid to find out the time showing optimum number of cells at metaphase (mitotic indices or MI).

MATERIALS AND METHODS

Young developing and mature tea seeds were collected from selected tea bushes of chinary hybrids *(Camellia sinensis* (L.) O. Kuntze) growing in the experimental tea farm at the Institute. The excised cotyledons were initially kept on half strength of Murashige and Skoog medium (1962) for one week before transferring on to various modified MS media (Sood et al, 1993). The subculturing was regularly done at 3 week's intervals. Over 200 combinations and permutations of growth hormones in MS medium were tested for their suitability for induction of somatic embryogenesis, callusing or shoot bud differentiation, but only seven, as listed in Table 1, were found to be the most responsive. The cultures were maintained at 8 hour light and 16 hour dark periods under 46.06 $\mu molsec^{-1}m^{-2}$ photoperiod and at temperature 25±2°C. As the response of the cotyledonary explants differed depending on the media-composition employed (Table 2.), the nodular callus tissue, germinating embryoids and root tips of young plantlets were harvested after every 15 minutes' intervals for 24 hours and pretreated with 0.002M 8-hydroxyquinoline for 3 hours. The materials were then fixed in 1:3 glacial acetic acid/ absolute alcohol mixture and stored in 70% alcohol. Hydrolysis of the tissue was done in 1N HCl at 60°C in an incubator. The time for hydrolysis varied between 6-12 minutes depending upon the nature of the material. Last traces of acid were removed by carefully washing the hydrolysed material. "Feulgen" nuclear reaction was used for staining the chromosomes. Squashes were prepared in a drop or two of aceto-carmine (2%) and, after usual dehydration in aceto-alcohol series (1:3, 1:6,1:9 glacial acetic acid and absolute alcohol), slides were mounted in Euparal (E-Merck, Germany).

Table 1. Murashige and Skoog's medium (1962) supplemented with PGRs that proved to be effective for tea tissue culture.

S. No.	Code		Medium Combinations*
1.	TG_1	=	MS + BAP (0.5) + GA_3 (0.5).
2.	TG_9	=	MS + BAP (2.5) + GA_3 (2.5)
3.	TG_{14}	=	MS + BAP (1.0) + NAA (1.0)
4.	TE_3	=	MS + BAP (0.01) + NAA + (2.5)
5.	TE_{10}	=	MS + BAP (2.5) + NAA (0.1)
6.	TE_{14}	=	MS + BAP (5.0) + NAA (1.0)
7.	BHMS	=	HalfMS + BAP (2.0) + IAA (0.02)
	Sucrose	=	2% : Murashige and Skoog's medium (1962)

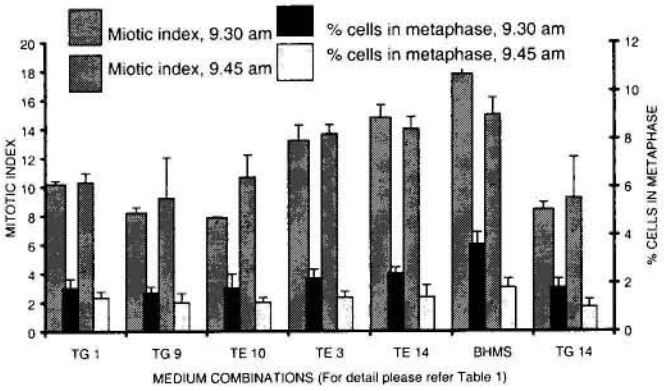
Parentheses represent concentration in mg/l

Table 2. Morphogenetic effect of different growth regulators on cotyledon segment culture in tea.

S.No.	Medium	Code	Direct shoot bud formation			Response	Remarks
			Somatic embryo-genesis	Direct rooting	Callusing		
1.	MS+BAP(0.5)+GA_3(0.5).	TG_1	-	+	-	+	-
2.	MS+BAP(2.5)+GA_3 (2.5)	TG_9	+	++	-	+	-
3.	MS+BAP(1.0)+NAA(1.0)	TG_{14}	+	++	-	++++	Friable callus formed
4.	MS+BAP(0.01)+NAA+(2.5)	TE_3	-	-	++++	++	A large no. of hairy roots.
5.	MS+BAP(2.5)+NAA(0.1)	TE_{10}	-	-	+++	+++	Slow growing nodular pale yellow callus formed.
6.	MS+BAP(5.0)+NAA(1.0)	TE_{14}	-	-	-	++	-
7.	Half MS+BAP(2.0)+IAA(0.02)	BHMS	-	++++	-	-	Most suitable for somatic embryogenesis

MS = Murashige and Skoog's (MS. 1962) mediumand values in parentheses represent concentration in mg/l.
+ = Sign indicates positive response
- = Sign indicates nil response
+ = poor
++ = good
+++ = very good
++++ = excellent response

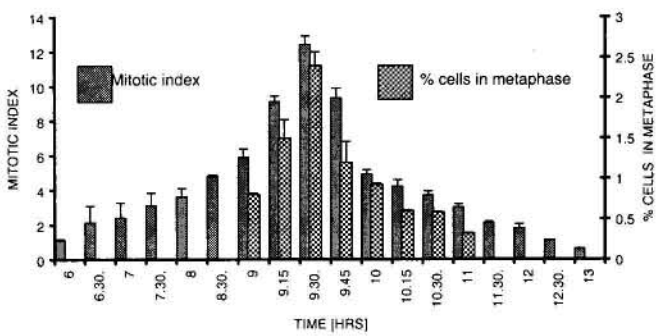
Fig. 1. Mitotic index of tea roots grown in tissue culture



RESULTS

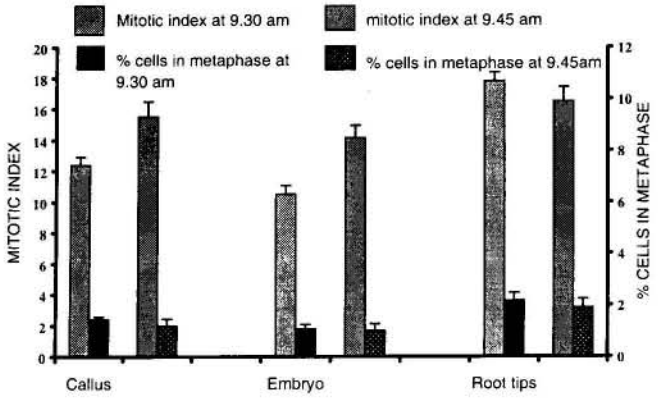
Perceptible differences in the values of mitotic indices and percent cells at metaphase were noticed in different time intervals (Fig. 1) grown under identical conditions, and the cell tissue harvesting was also done at the same time. Roots with well defined metabolically active root tips showed highest percentage of cells at metaphase (3.2) followed by callus cells (2.4), which undergo rapid cell divisions, and then somatic embryos (1.8). It was further observed that these optimal values were discerned during a very narrow time frame of cell fixation in the morning, i.e. between 9.30-9.45 A.M. Similarly, values for MI also showed the same pattern, with a peak of 12.0 at 9.30 A.M., showing a gradual decline on either side of this time period (Fig. 2.) with no cells at metaphase prior to 8.30 A.M. and after 11.30 A.M. Other interesting results were the differences in the MI values in cells from different tissues (Fig. 3). Highest values of MI were found in cells from root tips (15.7), followed by callus

Fig. 2. Mitotic index in tea callus at different time periods



(12.4) and embryos (10.5) as shown in (Fig. 1). Moreover, the chromosome size in tea being quite small, root tips proved to be an excellent material as compared to the compact and nodular structures such as callus as well as somatic embryos.

Fig. 3. Mitotic index of *in vitro* cultured tea tissues at 9.30 and 9.45 am



In a bid to find out whether the medium composition had any effect on MI and percent of cells at metaphase, all the three types of tissues were cultured on different combinations and permutations of MS medium as shown in Table I, and the tissue harvesting was done only from the seven promising combinations where prolific fresh tissue growth was discerned within 3 week's time period. The results of the cells fixed between 9.30-9.45 A.M. are only summarized for the sake of brevity. As is evident from the Table I, incorporation of BAP at concentrations ranging between 0.01-5.0 mg/l was common to all the media combinations presently employed. However, auxins NAA or IAA were also used in certain combinations with/without the presence of GA_3. It was interesting to see that MI values varied with the different media composition (Fig. 1).

Since it is a well-established fact that chromosomal variations tend to increase with duration of the cultures, the samples were collected 3 weeks after sub-culturing on a fresh medium for all types of tissues. For root tips, the shoots obtained through direct regeneration from the cotyledonary surface (Palni et al, 1993) were grown on rooting medium containing either of the auxins IBA or NAA and these uniformly revealed a diploid chromosome number 2n = 30 (X = 15). However, in the callus tissues, some aneuploid cells were also found showing a chromosome range between 2n = 22-42. It is pertinent to mention that different tissues revealed a diploid configuration of 2n = 30, thus indicating genomic conservation of the species as the aneuploid cells with divergent numbers are ultimately eliminated.

DISCUSSSION

Cell division plays a crucial role during all phases of plant development .The complex network controlling growth in eukaryotes is hierarchically organized as reported by Doerner (1994). In *in vitro* systems, the variations like the type and concentration of hormones used in the culture medium definitely affect the cell division rhythm. Such an effect of hormonal composition of culture media on chromosome behaviour is widely reported (Torrey, 1965).

In the present studies, by controlling temperature and photoperiod, it is possible to regulate and synchronize cell cycle in the cultured tissues so that the cells show the highest MI and interphase stage during a very short time frame i.e. 9.30–9.45 A.M. Synchronized cell division in *in vitro* cultures has also been reported by Hirt et al (1992), Nagata et al (1992) and Kodama et al. (1994). Most of the cells (especially from root tips/regenerated plants) showed diploid compliment of 2n = 30. According to Dulieu (1972), the plants regenerated from

superficial layers generally remain diploid and retain the parental chromosome complements. Occurrence of aneuploid cells was also not very uncommon. Such aneuploid cells showed chromosome number ranging between 2n = 22-42 indicating thereby the loss of up to 8-12 chromosomes during cell division in root tip and callus cells.

Earlier Das (1992) had also reported a wide range of chromosomal variations in the tea callus. Merismetic cells with 2n=42, 45 and 60 chromosomes were found in very high frequency along with higher yet undetermined numbers.

According to Lee and Phillips (1988), investigations of cytological variation among cultured cells and regenerated plants have been limited for a number of reasons, mainly being small sized and numerous chromosomes. But, with technological advancements, other species with distinctive chromosomes were thoroughly investigated. Bayliss (1980) re-emphasized the desirability of conducting studies with plant species with easily checked "karyotypes" and encouraged detailed cytological studies with genetic analysis of regenerated plants. Variations in chromosome number and structure have been observed among cultured cells and regenerated plants. Chromosome instability is more frequently associated with disorganized callus growth as opposed to the relative stability of organized cultures derived from meristems.

According to Nuti Ronchi (1990), the assumption that chromosome imbalance does not allow regeneration is not always true. This supports our observations that, despite having found aneuploid cells in all the three types of explants used, growth and differentiation of embryos, shoots or plantlets do not seem to be affected adversely . This is further supported by Nuti Ronchi (1990) that even apparently normal plants may have been carrying chimeral/aberrant cells that might express as somaclonal variation subsequently.

On the other hand, chromosomal stability has also been reported in some plant species by Mahfouz *et. al.*, (1983). Factors like composition of the nutrient media, type and concentration of growth regulators, endo-reduplication, non-disjunction and fragmentation are attributed to such chromosomal aberrations (D'Amato et al, 1980; Dolezel and Novak, 1984). Evans and Sharp (1986) also indicated that higher concentrations of growth regulators could be responsible for chromosomal aberrations in *in vitro* cultures. Such stable aberrations have also been shown to result in somaclonal variants, which in certain cases have proved highly productive than their normal diploid parents.

Bottomley *et al*, (1963) and Gorst *et a.*, (1991) have tested the cell division frequency on *Nicotiana tobacum* var Wisconsin 38 using auxins and cytokinins. They could not find any difference in the results when analogues of cytokinins (BAP) and auxins (NAA) were used unlike what has been presently observed.

CONCLUSIONs

We can say that the cells are amenable to chromosomal aberrations by bringing about changes in the external physico-chemical stimuli. However, there are genomes that show such changes with slight variations in the conditions, but others may be more conservative to such variations. To find stable somaclonal variants is also not very

common as the cells tend to revert back to the normal configuration. And despite some cells showing aneuploidy, it may not affect the process of differentiation or regeneration. Further, the cells from the tissues grown under controlled physico-chemical conditions show synchronization of cell division at a very narrow time frame during a 24 hours cycle, and this time frame may vary with the species.

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