S.L.J. Tea Sci. **66** (1 / 2), 16-26, 2000, *Printed in Sri Lanka*

IN VITRO SHOOT ORGANOGENESIS IN CALLUS DERIVED FROM STEM TISSUE OF TEA (*CAMELLIA SINENSIS* L.)

M.T.K. Gunasekare
(Tea Research Institute of Sri Lanka, Talawakelle, Sri Lanka)
and
P.K. Evans
(Department of Biology, University of Southampton,Southampton SO16 7PX, United Kingdom)

This study demonstrates the possibility of regenerating shoots in vitro from callus obtained from stem tissue of tea (*Camellia sinensis* L.) with a sequential change of growth regulator compositions in culture media. This involves the induction of callus and their proliferation for a certain period under the influence of specific growth regulator composition followed by the regeneration of these proliferated callus into shoots through a gradual reduction of auxin in the medium. *In vitro* shoot organogenesis occurred when callus transferred from MS medium containing 1 mg/l BAP + 1 mg/l NAA to MS medium with 1 mg/l BAP + 0.1 mg/l NAA or growth regulator free MS medium. Best callus proliferation occurred in the presence of NAA at 1 mg/l as compared to IBA at the same concentration. Histological studies were also carried out to identify the precursor cells for callus initiation and to follow the cellular changes during callus dedifferentiation. According to the histological observations cell division leading to callus formation occurred in pith parenchyma cells.

Abbreviations: BAP- 6-benzylaminopurine; IBA – Indole-3-butyric acid; MS – Murashige and Skoog, (1962) medium; NAA- α -naphthaleneacetic acid

INTRODUCTION

Tea (*Camellia sinensis* (L.) O. Kuntze) is the world's foremost beverage and an important plantation crop of great commercial value. At present development of new tea genotypes is mainly done through conventional breeding and selection methods. Theoretically, an elite tea genotype should have a combination of desirable characteristics such as high yield, good quality, resistance to pest and diseases, tolerance to drought, ease of vegetative propagation and regional adaptability. However, recombinants obtained through natural and controlled hybridisation do not always possess many of these desirable features. Hence, tea crop improvement solely through conventional method is frequently hampered and this is mainly due to inherent nature of the tea plant.

The use of explants with pre-formed meristems can preserve the genetic stability of the primary explant material. On the other hand, indirect regeneration or regeneration through an intermediate callus phase may induce genetic variations in the regenerated plants (Bajaj, 1990). Hence, variations that arise from tissue culture provide an additional source of

genetic variants that can be used in crop improvement programmes. It has been known that plant cell and tissue cultures undergo changes of various types, especially in chromosome numbers and ploidy level (Murashige & Nakano, 1966). These *in vitro* induced variations may, therefore, be a promising starting material for plant breeding and selection programmes to recover useful variants. This type of variation is useful since it provides genetic variability within existing cultivars carrying few other alterations and can be exploited more quickly than variations created by classical breeding methods. However, selection of plant or plant lines with useful somaclonal variants could be performed if an efficient *in vitro* regeneration method is available.

Callus cultures can be initiated from variety of plant organs and tissues in a wide range of species. The findings on tissue culture of tea indicate that callus can be obtained from many plant tissues including stem tissue (Wu *et al.*, 1981; Kato 1985; Sarwar, 1985; Frish & Camper, 1987a, 1987b; Siu and Weatherhead, 1995). However, shoot organogenesis via callus seems to be relatively difficult in Camellia species, especially from the vegetative parts (Frish & Camper 1987b; Wu *et al*, 1981). Jha *et al.*, (1992), Bano et al., (1991) and Nakamura (1988) were able to differentiate somatic embryos both directly and indirectly from cotyledon explants. Wu *et al.*, (1981) have successfully obtained plantlets from cotyledon callus. All these work relate to cultures derived from cotyledon and therefore, the material is not true-to-type. Therefore, indirect organogenesis or embryogenesis from callus derived from vegetative parts will be of more importance to make few changes in a known superior genotype for its further improvement in characteristics.

The aim of the present study therefore, was to analyse the effect of various growth regulators on callus proliferation and its differentiation in order to find a suitable culture medium for induction of indirect morphogenesis. Furthermore, histological studies were also performed to observe the cellular changes occurring in stem explants and in subsequent callus cultures raised from stem explants.

MATERIALS AND METHODS

Stem explants were obtained from glasshouse grown one year old seedling plants of tea (*Camellia sinensis* L.). These plants were raised in pots and were maintained at 25 °C with 16h photoperiod using supplementary light when necessary. Primary callus was initiated from stem tissues taken from 3rd to 5th internodes from the apex of the shoot. These were cultured on MS (Murashige & Skoog, 1962) medium supplemented with 2 mg/l BAP and 1mg/l NAA. Five weeks after culture initiation, primary callus was separated from the explant and divided into an inoculum with an average fresh weight of 230±20 mg. These callus pieces were then cultured on MS medium supplemented with various growth regulator compositions. The culture medium was solidified with 0.7% agar. Glass jars (60ml, 45 x 50mm size) filled with 20 ml volume of culture medium were used to culture the callus. All cultures were maintained in a growth room at 25± 2°C under a 12 h photoperiod with a light intensity of 80μE m^{-2} s^{-1}, derived from cool white fluorescent tubes.

Mean weight gain of the callus cultures was determined on a fresh weight basis using 10-15 replicates (samples) per treatment during 3 week sub-culture period.

Weight increase of callus per unit weight was calculated as: $\frac{W1-W0}{W0}$

Where W0 = Initial fresh weight of callus

W1 = Final fresh weight of callus

On differentiation medium the percentage of cultures producing nodules (i.e. structures having precocious compact, smooth surfaced, round or oval shape), number of nodules per callus and percentage of nodules which developed into shoots were also recorded.

Growth of callus on media with various growth regulator composition

Primary callus obtained from stem tissue on MS medium containing 2 mg/l BAP and 1 mg/l NAA was regularly sub-cultured to the following callus proliferation media (PM) at 3 week intervals; 2 mg/l BAP + 1 mg/l NAA (PM1); 1 mg/l BAP + 1 mg/l NAA (PM2); 1 mg/l BAP + 1 mg/l IBA (PM3) and 2 mg/l BAP alone (PM4). Weight gain of callus was recorded during the 3-week sub-culture period at the 4th sub-culture in order to compare the growth of callus on these media.

Effect of growth regulator composition of the medium on differentiation of callus

In an attempt to differentiate callus a parallel experiment was designed in conjunction with the previous experiment. In addition to the culture media used in that experiment, the following media designated as differentiation media (DM), with lower auxin: cytokinin ratios than the proliferation media, were also used for the serial sub-culture of callus: MS medium containing 2 mg/l BAP + 0.5 mg/l NAA (DM1); 1 mg/l BAP + 0.1 mg/l NAA (DM2); 1 mg/l BAP (DM3) and MS medium without growth regulators (DM4).

In one set of experiments, primary callus was sub-cultured directly to DM and sub-culture was done at regular 3-week intervals to the same fresh medium. In the other experiment callus sub-cultured on different PM for 4 months (with regular sub-culture) was then sub-cultured onto various DM. Once callus was sub-cultured on DM sub-culture was continued to the same but fresh culture medium at 3 week intervals. The effect of various media was assessed in terms of percentage of cultures surviving and the number of nodules formed per culture.

Histology

Stem explants from which callus was initiated were fixed for observation of cell division sites. Stem pieces cut into 2-3 mm thickness were fixed immediately in 1% Glutaraldehyde (v/v), 4% Paraformaldehyde (w/v) in 0.05 M Sodium phosphate buffer, pH 7.2 for 15 minutes under vacuum at room temperature. Fixative was replaced with fresh fixative and specimens were fixed overnight at 4°C. Tissue was dehydrated through a grad-

ed etahnol series with 20 min. changes in each step. Specimens were then infiltrated in ethanol and LR white resin (Medium grade, Acrylic resin, London Resin Co. Ltd., England). After thorough infiltration with resin, specimens were embedded in geletain capsules filled with LR white resin and polymerised at 60°C for 24 h. Sections 3 µm thick were cut using rotary microtome (Recichert-Jung, Ultra cut) and were stained with 0.5% Toluidine Blue in 0.5% Borax for 30 seconds prior to microscopic observations.

Preparation of friable callus for microscopic observations were done according to El Maataoui et al., (1990).

RESULTS

Growth of callus on media with different growth regulator composition

Primary callus was often friable and light green to yellow in colour [Fig. 1.(1)]. Weight gain of the callus, based on fresh weight, on different PM is given in Table 1. The best callus proliferation was observed in PM2 and this was significantly higher ($p<0.05$) than the callus growth recorded on the other media. Callus growth was 2.8 fold on this medium during the 3-week culture passage. The lowest callus growth was found in PM4, but this was not significantly different ($p<0.05$) from the callus growth found in PM1 and PM3. However, callus sub-cultured to PM4 showed a necrotic appearance 3 weeks after sub-culture.

Table 1. The growth of callus (fresh weight gain per unit weight of callus) sub-cultured on media with different growth regulator compositions during 3 week culture period

Growth regulator composition (mg/l)	Code	Growth of callus ± S.E
(2) BAP + (1) NAA	PM1	1.2 ± 0.4^{b}
(1) BAP + (1) NAA	PM2	2.8 ± 0.6^{a}
(1) BAP + (1) IBA	PM3	0.9 ± 0.1^{b}
(1) BAP	PM4	0.6 ± 0.2^{b}

Mean values of 3 individual experiments with 10 replicates per treatment in each experiment. Mean values followed by different letters are significantly different at $p<0.05$.

Effect of growth regulator composition of the medium on differentaition of callus

The highest percentage of surviving callus (80%) was found in PM2, which also showed the best callus proliferation in the initial experiment. However, this was not significantly different from the percentage cultures surviving in PM1, DM1 and DM2, which was in the range of 73%-76% (Table 2). In contrast, none of the cultures survived in growth regulator free MS medium as observed 3 weeks after sub-culture, Callus sub-cul-

tured on PM3, PM4 and DM3 showed less than 30% survival. Direct sub-culture of primary callus on any of these media (see Table 2 for growth regulator composition) did not result in any form of differentiation.

Table 2. Percentage survival of primary callus sub-cultured onto media with different growth regulator compositions

Growth regulators (mg/l)	Code	% surviving cultures
(2)BAP + (1)NAA	PM1	75.0
(1)BAP + (1)NAA	PM2	80.0
(1)BAP + (1)IBA	PM3	16.6
(2)BAP	PM4	28.0
(2)BAP + (0.5)NAA	DM1	76.2
(1)BAP + (0.1)NAA	DM2	73.9
(1)BAP	DM3	15.0
Growth regulator free	DM4	0.0

Data were recorded 3 weeks after sub-culture. All cultures were initiated from primary callus. The number of cultures per treatment ranged from 20 –25. Callus proliferation and differentiation media are designated as PM and DM respectively.

Nodule formation was seen to occur in callus when transferred from PM2 to either DM2 or DM4 at a frequency of 35% and 16%, respectively (Table 3). On these media callus become dark green and ceased proliferation but remained alive. Within the mass of callus green nodule-like structures appeared after sub-culturing the callus twice onto the same medium. The nodules formed were round and some were elongate in shape with a smooth surface [Fig. 1.2 (a)]. Callus sub-cultured on any of the other media combinations did not show any morphogenesis or nodule formation even after regular sub-culture onto the same fresh culture medium.

The average number of nodules formed per culture was similar in medium without growth regulators (DM4) as in the medium with 1 mg/l BAP and 0.1 mg/l NAA (DM2) (Table 4). The nodules, which formed among callus cultured on DM2 when separated and cultured on the same medium developed further and produced, shoots [Fig. 1.2 (b) & (c)]. The percentage of nodules, which converted into shoots, was 63.6% in DM2. These shoots were able to form roots on the same medium without any further changes in the growth regulator composition. Although nodule formation occurred on DM4, these did not convert into shoots on the medium.

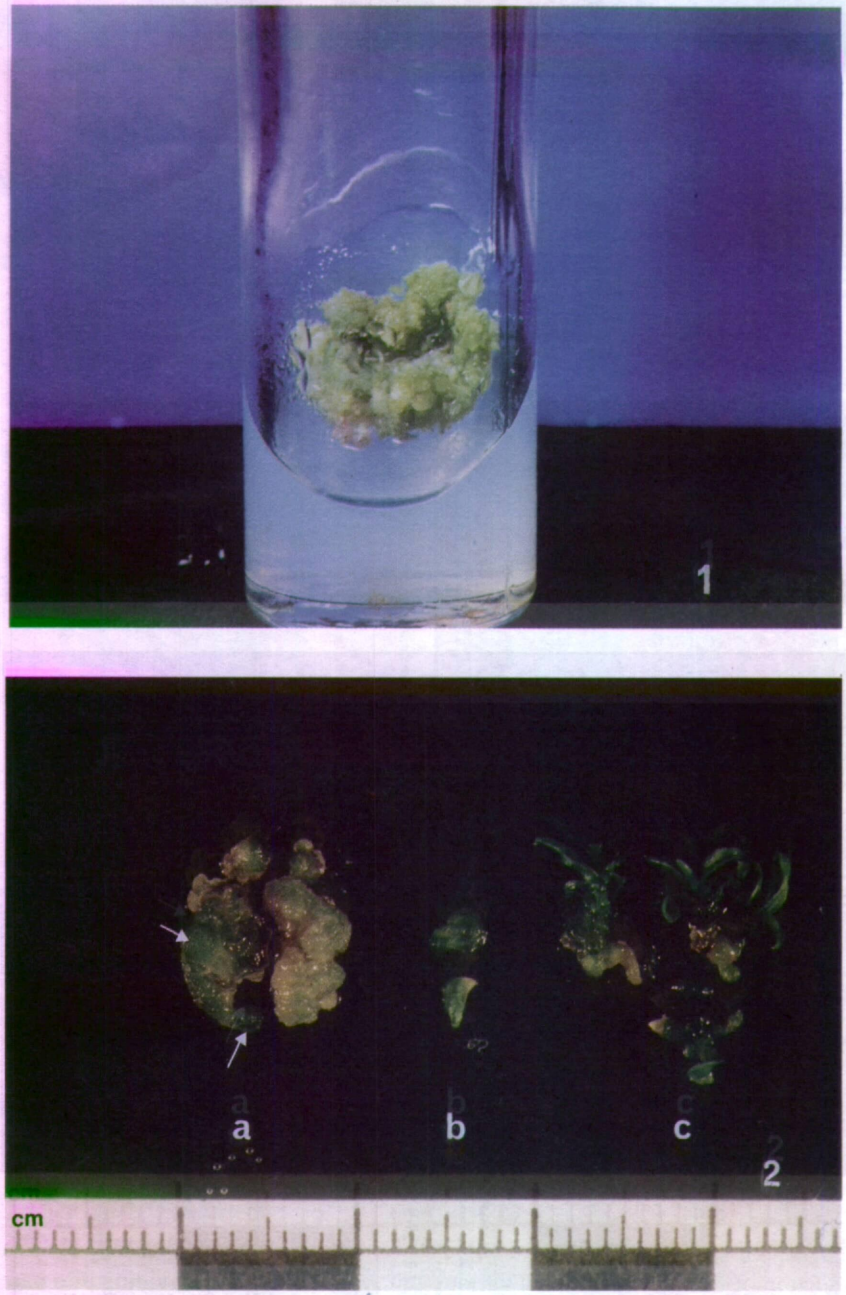

Fig. 1. (1) Growth of callus on callus proliferation medium (MS medium with 1mg/l NAA and 1mg/l BAP) after 4th sub-culture. (2) Shoot organogenesis in stem callus (a). Formation of nodules (arrow) on callus sub-cultured on MS medium with 1mg/l BAP and 0.1mg/l NAA. (b) and (c). Stages of shoot development from nodules formed in callus on the same medium.

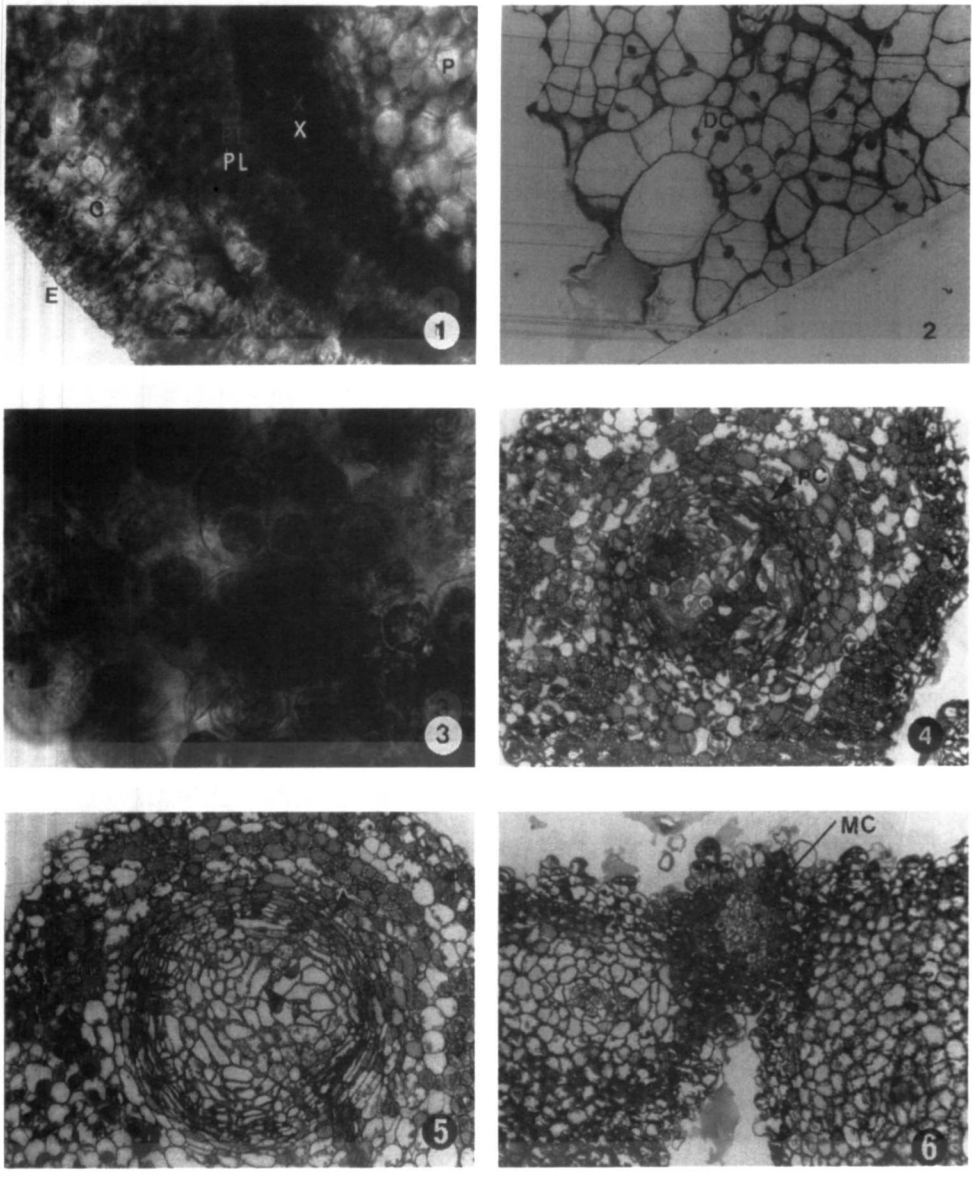

Fig. 2. Cellular changes in stem explants forming morphogenic callus. (1) Hand-cut cross section of fresh stem showing various cell types (x103). (2) Dividing pith parenchyma cells containing prominent nuclei (x203). (3) Squash preparation of friable stem callus showing cells with dense cytoplasm (x403). (4) Formation of cell layer similar to procambium in nodular callus (x103). (5) Formation of vascular elements in nodules (x103). (6) Meristematic cell clusters formed in nodular callus (x103).

E= Epidermis. C= Cortex; PL= Phloem; X = Xylem; P = Pith parenchyma cells; DC = Dividing Cells; PC = Procambium; T = Tracheids; MC = Meristematic Cells.

Table 3. Percentage of callus cultures forming nodules following sub-culture on to callus differentiation medium (DM) from callus proliferation media (PM).

	Callus proliferation medium (PM)			
Differentiation Medium (DM)	(2)BAP+(1)NAA (PM1)	(1)BAP+(1)NAA (PM2)	(1)BAP+(1)IBA (PM3)	(2)BAP (PM4)
(2)BAP+(0.5)NAA (DM1)	0.0	0.0	0.0	NT
(1)BAP+(0.1)NAA (DM2)	0.0	35.0	0.0	0.0
(1)BAP (DM3)	0.0	0.0	NT	NT
Growth reg. free (DM4)	0.0	16.0	0.0	NT

Growth regulator concentrations in mg/l
NT – Not tested
Data as recorded after 6 weeks from sub-culture on to DM. Data based on 20-25 replicates per each treatment combination.

Table 4. Effect of different culture media on number of nodules formed and frequency of nodule conversion into shoots.

Growth regulator Concentration (mg/l)	Average number of nodules per culture ± S.E	% conversion of nodules into shoots
(1) BAP + (0.1) NAA	4.7±0.7	63.6
Growth regulator free	4.0±1.1	0.0

Data based on two individual experiments consisting of 25-30 cultures per each treatment.

Histology

Hand-made cross section of fresh stem showed layers of different cell types; epidermal, cortical parenchyma, xylem, phloem and pith parenchyma [Fig.2(1)]. Sections of stem explants cultured on MS medium supplemented with 1 mg/l NAA and 2 mg/l BAP (callus initiation medium) after 3 weeks culture showed that pith parenchyma cells were activated to divide and formed cells with prominent nuclei [Fig.2(2)]. Squash preparation of friable primary callus showed that the cells had dense cytoplasm but no organisation was shown. During continued sub-culture of callus onto the proliferation medium (1 mg/l NAA + 1 mg/l BAP) callus become highly meristematic [Fig.2(3)]. Thin sections of nodules produced in callus cultured on the MS medium containing 1 mg/l BAP and 0.1 mg/l NAA showed that the callus were gradually forming a cylindrical zone similar to procambium [Fig.2(4)]. At a more developed stage, there was a well-differentiated provascular cylinder with clearly identifiable tracheids [Fig.2(5)]. In some sections meristematic cell groups were seen among the nodular callus [Fig.2(6)]. These nodules eventually formed shoots on the final medium.

DISCUSSION

. Among the various callus proliferation media tested the best callus proliferation was found in MS medium supplemented with 1 mg/l BAP and 1 mg/l NAA. In that medium, during the 3-4 week sub-culture period, growth of callus was significantly higher than that on the medium which was similar in composition to primary callus initiation medium (2 mg/l BAP and 1 mg/l NAA). These two media differed from each other only in terms of their levels of BAP. Therefore, it seems that for better callus proliferation the BAP level needs to be reduced compared to the level used to initiate primary callus. This reduction of BAP concentration may help to achieve the proper balance between auxin and cytokinin, which may support better callus growth although the concentrations of auxin remained unchanged.

When medium supplemented with 1 mg/l BAP in combination with 1 mg/l IBA instead of NAA, was used, it did not promote callus proliferation. Therefore, as an auxin source NAA seems better than IBA to promote callus growth when added at 1 mg/l concentration. This shows that to optimise callus growth the type of auxin may be equally important as the level of auxin used in the medium.

When primary callus was directly sub-cultured on to the whole range of media tested (see Table 2), including different concentrations of auxin and cytokinin, cytokinin alone as well as medium with no growth regulators, no organogenesis was induced. There was no callus differentiation as long as callus was sub-cultured onto the same medium chosen as being best for callus proliferation (1 mg/l BAP + 1 mg/l NAA). During continued sub-culture of callus on to the same proliferation medium callus become highly meristematic and was fast growing with friable texture. On the other hand, callus which was previously on callus proliferation medium (1 mg/lBAP and 1 mg/lNAA) and then sub-cultured to either 1 mg/l BAP and 0.1 mg/l NAA or medium without growth regulators produced nodules. These nodules seem to arise as a result of the removal of growth regulators or reduction in auxin concentration in the subsequent medium. Therefore, it may be that the primary callus needs to be cultured in a relatively high auxin-containing medium to form competent cells before transfer to a medium with a lower auxin level, which encourages the cells to express morphogenesis. Such gradual shifts in exogenously applied growth regulator concentration have proved to be successful for the complete differentiation of plantlets from callus in some other studies (Chang & Hsing, 1980; Mathur, 1993). The reason for the absence of nodule formation in other media tested (i.e. 2 mg/l BAP + 0.5 mg/l NAA; 1 mg/l BAP only), however, is not clear. Perhaps, this may be attributed to the incorrect balance of growth regulators in the culture medium.

In general, it is believed that callus show high potential for morphogenesis in the initial stages but declines gradually with serial sub-culture or they even totally loose their morphogenetic ability (Constable, 1984). However, according to the present study it seems that callus need to be maintained and sub-cultured for a certain period in the presence of relatively high growth regulator levels, especially with a high auxin concentration, to gain a capacity for morphogenesis. This is supported by the work of Noviko, (1972) who found

that organogenesis could not be induced in the early stages of tobacco callus cultures, while after a period of sub-culture the callus acquired organogenic potential.

Even though nodule formation occurred when callus was transferred from PM to medium with no growth regulators, these nodules did not develop any further to form shoots, as observed in growth regulator containing medium (1 mg/l BAP and 0.1 mg/l NAA). This indicates that for successful callus differentiation an exogenous supply of growth regulators may be necessary at a lower level than no growth regulators in the medium.

Although it was possible to obtain shoot organogenesis from stem callus the percentage of callus cultures capable of organogenesis was low (16-35%). This may be due to the differing degree of morphogenic ability shown by the different callus masses, even on a suitable culture medium. Callus cultures may contain both competent and non-competent cells (Street, 1979). Under such circumstances, it is possible to loose the morphogenetically competent cells due to predomination of non-competent cells even under proper culture conditions. Therefore, the low frequency of organogenesis observed among the callus cultures under the present study may partly be due to the heterogeneity of callus originated from the primary explant.

In general, indirect organogenesis via callus seems to be relatively difficult in *Camellia* species especially from the vegetative parts. The present study demonstrates the possibility of differentiation of callus obtained from stem tissue with a sequential change of growth regulator composition in the medium over time during callus sub-culture. This experiment was carried out with the use of material obtained from seedlings and therefore, it may be necessary to test the reproducibility of the callus proliferation and regeneration capacity using clonal material for more consistent protocol for indirect plant regeneration from stem explant of tea.

ACKNOWLEDGEMENT

The financial support received from the Tea Research Institute of Sri Lanka to carry out this work at the University of Southampton, U.K., is gratefully acknowledged.

REFERENCES

BAJAJ, Y.P.S., 1990. Biotechnology in Agriculture and Forestry II: Somaclonal Variations in Crop Improvement. Springer-Verlag Berlin Heidelberg.3-48.

BANO, Z., RAJARATHNAM, S., and MOHANTY, B.D., (1991) Somatic embryogenesis in cotyledon cultures of tea (*Thea sinensis* L.). *J. Hort. Sci.*, 66,.465-470

CHANG, W.C. and HSING, Y.I, (1980) Plant regeneration through somatic embryogenesis in root derived callus ginseng (*Panax ginseng* C.A. Meyer). *Theor. Appl. Genet.*, 57,133-136.

CONSTABLE, F.,(1984). Callus culture: Induction and maintenance. Cell Culture and Somatic Cell Genetics of Plants. 1. I.K. Vasil, ed. Academic Press, London. 27-35.

EL MAATAOUI, M., ESPAGNA, C. and MICHAUX-FERRIERE, N. (1990) Histology of calougenesis and somatic embryogenesis induced in stem fragments of cork oak (*Quercus suber*) cultured *in vitro. Ann. Bot.*, **66**,183-190.

FRISH, C.H. and CAMPER, N.D. (1987a) Callus from *C.sinensis* and *C. japonica* stem tissue. *Camellia* J. **42**, 26-27.

FRISH, C.H. and CAMPER, N.D.,(1987b) Effect of synthetic auxins on callus induction from tea stem tissue. *Plant Cell, Tissue & Organ Culture,* **8**, 207-213

JHA, T.B., JHA, S. and SEN, S.K.(1992) Somatic embryogenesis from immature cotyledon of an elite Darjeeling tea clone. *Plant Sci.*, **84**, 209-213

KATO, M.(1985) Regeneration of plantlets from tea stem callus. *Japan. J. Breed.*, **35**, 317-322.

MATHUR, J. (1993) Somatic embryogenesis from callus cultures of Nardostachys jatamansi. *Plant Cell, Tissue & Organ Culture.*, **33**, 163-169.

MURASHIGE, T. and NAKANO, R.(1996) Tissue Culture as a potential tool in obtaining polyploid plants. *J. Hered.* **57**, 115-118.

MURASHIGE, T. and Skoog, F.(1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant.*, **15**, 473-497.

NAKAMURA, Y. 1988. Effective method of in vitro propagation of tea plant. Recent development in tea production. Proc. Int. Sym. At Taiwan Expt. Station. Feb. 29-March 5, 63-74.

NOVIKO, S.(1972) Progressive change in organ forming capacity of tobacco callus during sub-culture period. *Japan. J. Genet.* **47**, 53-60.

SARWAR, M., 1985. Callus formation from explanted organs of tea. *Sri Lank. J. Tea Sci.* **54**, 18-22.

SIU, L.P. and WEATHERHEAD, M.A. (1995) Tissue culture of the Camellias of Hong Kong. *Botanic Garden Micropropagation News* **2**, 6-8.

STREET, H.E. (1979) Embryogenesis and chemically induced organogenesis. Plant Cell and Tissue Culture Principles and Applications. W.R. Sharp, P.O. Larsen, E.F. Paddockand V. Raghavan, eds. Ohio State University Press. Columbus, 123-154.

WU, C.T., HUANG, W., CHEN, T. and CHEN, S., 1981. A review on the tissue culture of tea plants and on the utilisation of callus derived plantlets. Proc. COSTED Symp. on Tissue Culture of Economically Important Plants. Singapore,104 -106.