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RESEARCH ARTICLE

Production of embryogenic callus from leaf explants of *Camellia sinensis* (L.)

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Abstract: An attempt was made to produce embryogenic callus from leaf explants of tea (*Camellia sinensis* L.). The sterilized field-grown leaf explants were cultured on MS media with various combinations of benzyl aminopurine (BAP) and naphthalene acetic acid (NAA) to obtain embryogenic callus. The results showed that the rates of callus initiation (72.2%) and formation (56.9%) were relatively high in the medium containing 2.0 mg/L BAP and 3.0 mg/L NAA (referred as callus medium). To study the development of embryogenic callus morphologically and histologically, *in vitro* leaf segments were cultured on callus medium (0.8% agar) and then transferred at the 8th week to the same medium but solidified with 0.7% agar. Results revealed that leaf segments became slightly swollen at the cut end, subsequently whitish – yellow, friable cell masses were produced with bursting of swollen tissues. The percentage of callus initiation was 93.8% in the 6th week of incubation. 65.8% of explants formed callus covering about half the surface of leaf segment in the 8th week. Cytological examination showed the development of collenchyma cells which were packed within the swollen tissues, and after bursting, parenchyma cells formed gradually as a result of cell division. These parenchyma cells de-differentiated into meristematic and embryogenic cells. A few somatic embryos (3.3%) developed from primary calli (16 weeks after incubation of leaf explants) in the preliminary study. The average number of single embryogenic cells was significantly more in liquid medium than in solid. The protocol developed in this study, will be used to obtain embryogenic callus from leaf explants and also genetic transformation.

Keywords: embryogenic callus, *in vitro* culture, leaves, somatic embryos, tea.

INTRODUCTION

In Sri Lanka, conventional breeding techniques have contributed to improvement of the tea crop within the existing genotypes. *In vitro* plant regeneration through an intermediate callus phase may induce genetic variations in the regenerated plants[1] that could accelerate the production of improved genotypes in *Camellia* spp. A reliable and highly efficient *in vitro* plant regeneration technique is a prerequisite to select the plant lines with useful somatic clonal traits and also for use in genetic transformation.

In tea, occurrence of indirect somatic embryogenesis or shoot organogenesis has been reported from embryonic tissues, particularly cotyledons of zygotic embryos[2-6] and also from non-embryonic tissues such as stem and leaf [7-11]. Indirect organogenesis or embryogenesis from calli obtained from vegetative parts will be of importance to make few changes in a known superior genotype for its further improvement[11]. It had been reported that the low frequency of shoot organogenesis observed in callus cultures may partly be due to the heterogeneity of callus originating from stem tissues. Therefore, success of somatic embryogenesis depends on the type of callus formed on initial explants.

Even though leaves had been used as initial explants in indirect somatic embryogenesis, achievement of significant success in inducing somatic embryos either directly or indirectly from leaf explants (on MS medium with 2, 4 D) has been reported only once[10]. Further it has been reported that only certain genotypes could respond to 2, 4 D. However, detailed studies on the production of somatic embryos indirectly from *in vitro* or field-grown leaves are lacking. Therefore, the present study was undertaken with the aim of developing suitable culture conditions for the production of somatic

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embryoids indirectly from both *in vitro* and field-grown leaves of tea.

METHODS AND MATERIALS

Culture conditions for the callus formation from field grown leaves: Unfurled leaves of tea cultivar, TRI 2043 were collected from field-grown tea bushes (30 year-old) during the rainy season. They were surface sterilized in 70% (v/v) alcohol for 2 - 3 min followed by agitating in a solution of 60% Clorox™ (sodium hypochlorite, 5.25% active ingredient) with 2 - 3 drops of Tween 20 for 30 min. They were then rinsed thoroughly in sterilized distilled water. Leaf segments (5 x 5 mm^2) were dissected and cultured on MS basal media[12] with different combinations of growth regulators to select a suitable medium for the formation of friable calli. The culture media consisted of 3% sucrose with combinations of benzyl aminopurine (BAP) (0.1 – 2.0 mg/L) and naphthalene acetic acid (NAA) (0.1 – 3.0 mg/L) and solidified with 0.8% (w/v) agar (Sigma™). The culture vials (28 mL capacity universal bottles) were then incubated at 22 ± 2 °C under white fluorescent light (16 h photoperiod, 25 μmoles m^{-2} s^{-1}). In each treatment, number of *in vitro* explants that induced callus was recorded weekly. Three independent experiments, each containing 24 explants per treatment were conducted.

After 8 weeks of culture, the leaf explants showing callus initiation were sub - cultured to fresh media with the same composition and maintained for further 8 weeks for callus production. Preliminary investigations on somatic embryogenesis were conducted. The primary calli were transferred to MS medium supplemented with 2.0 mg/L BAP and 0.1 mg/L NAA[13] and observations were made weekly on the induction of embryoids.

Culture conditions for the production of calli from in vitro-grown leaves: Leaf segments (5 x 5 m^2) were excised from *in vitro*-grown micro shoots that were sub-cultured at 12-wk intervals for about two years on MS medium supplemented with BAP (3.0 mg/L) and NAA (0.1 mg/L). The leaf segments were cultured on callus induction medium [MS basal medium with BAP (2.0 mg/L), NAA (3.0 mg/L) and 3% sucrose solidified with 0.8% (w/v) agar] with their abaxial surface in contact with the medium. The culture bottles (125 mL capacity), each consisting of 10 mL of medium and six explants, were incubated under the conditions given in the previous section.Two independent experiments (total explants 276) were conducted. At the 8th wk of incubation, explants with callus covering about half the surface of leaf segment, were transferred to fresh medium with reduced concentration of agar (0.7% w/v) for proliferation of calli. Morphological changes of cultured leaf explants were observed daily. Number of cultured explants that induced callus was recorded weekly. Fresh weight of cultured leaf segments was also measured at 4-wk intervals.

Cytological examinations on the development of embryogenic callus: Parallel experiments were carried out for histological studies on the development of embryogenic callus from *in vitro* cultured leaf segments. The cultured leaf explants were sampled weekly and the explants showing callus initiation were cut into small pieces. They were fixed, embedded in paraffin wax (Sigma™) and sectioned as described by Haris *et al*[14]. The sections were stained with toluidine blue prior to microscopic observation. For friable calli, tiny pieces of calli were placed on the slides and crushed gently with toluidine blue before cytological examination.

A preliminary study on somatic embryogenesis from leaf calli: To initiate somatic embryos from calli, small pieces of primary calli (friable calli obtained 16 wks after culture initiation) were placed on MS medium containing BAP (3.0 mg/L), NAA (0.1 mg/L), 3% sucrose and solidified with 0.7% (w/v) agar. In this medium, the primary calli were maintained for 4 wks and then transferred to another medium for differentiation [half strength of MS basal medium + BAP (1.0 mg/L) + NAA (0.1 mg/L) + 3% sucrose solidified with 0.8% (w/v) agar]. Observations on the induction of somatic embryos were made.

Effect of solid and liquid media on intensity of embryogenic cells: At the 8th wk of incubation, calli with the initial explants were transferred to either solid (0.7% w/v agar) or liquid medium [MS basal medium with BAP (2.0 mg/L), NAA (3.0 mg/L) and 3% sucrose] contained in culture vessels (125 mL capacity), each containing 20 mL of medium. The liquid cultures were placed on a rotary shaker and agitated at 70 rpm for 8 h per day. At the 16th wk, concentrations of single embryogenic cells and embryogenic cell clusters were estimated using a Haemocytometer (x 100 magnification). Callus developed on the solid medium, was collected and 20 mL suspension was prepared prior to assessment. Each treatment consisted of 15 replicates and each replicate had 6 samples. This experiment was repeated once.

Statistical analysis: Wherever possible data were analysed using the SAS software. The percentage data were first subjected to Arcsine transformation before analysis of variance. The significant difference between means was estimated using Duncan's Multiple Range Test at 5% significant level.

RESULTS AND DISCUSSION

Initiation and formation of callus from field-grown leaves

Friable calli were first initiated on cultured leaf segments in the presence of BAP (2.0 mg/L) and NAA (3.0 mg/L) after 3 weeks of incubation. A combination of BAP (2.0 mg/L) and NAA (1.0 mg/L) also induced whitish-yellow friable calli but at a low frequency. A combination of auxin and cytokinin is necessary for good callus formation in tea[15]. The percentages of induction and formation of calli were significantly different among tested media (Table 1). The frequencies of initiation (72.2 %) and formation (56.9 %) of calli from cultured leaf segments were relatively high in MS medium supplemented with BAP (2.0 mg/L) and NAA (3.0 mg/L). The present results indicated that ratio of auxin: cytokinin was critical for inducing callus at a high frequency. It has been shown that NAA seems better than indolebutyric acid (IBA) to promote stem callus growth of tea at 1 mg/L concentration in combination with 1.0 mg/L BAP[11].

Among the tested combination of BAP and NAA, BAP at 2.0 mg/L in combination with NAA at 3.0 mg/L was the most effective treatment in producing the friable calli from leaf explants. At the 16th week of incubation, primary calli formed on the above medium were transferred to differentiation medium containing BAP (2.0 mg/L) and NAA (0.1 mg/L) to induce somatic embryogenesis. Even though smooth, round, prominent structures resembling globular embryos were observed on the surface of calli, they did not develop further upon subsequent transfer to fresh medium.

Initiation and maintenance of embryogenic callus from *in vitro*- grown leaves

After two to three weeks of culture, cut ends of cultured leaf segments became slightly swollen and became yellowish. Subsequently, the swollen tissues burst and callus initiation was fist observed at the cut ends during the 4th week. Most explants curled and formed whitish or greenish yellow friable calli. Friable calli also appeared on veins of leaf segments after 3 weeks. Wounding may not be essential for callus formation in *in vitro* derived explants although it is generally stimulatory for callus formation[16]. The calli formed in the mid rib consisted of compact cell masses. The percentage of explants that induced callus at the 4th and 6th week were 74.3 ± 2.6% and 93.8 ± 1.4% respectively. In the preliminary studies, the frequency of tissue browning increased when leaf explants were cut into small pieces. Therefore, sub-culturing was avoided to reduce the browning effect on explants. Calli derived from stem tissue 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[11].

An efficient callus formation (friable callus covering about half the surface of explants) was observed in 65.8% of leaf explants at the 8th week of culture and callus obtained from each leaf segment was composed of fairly homogeneous cell masses with regard to texture and colour. Out of these explants forming calli (65.8%), greenish or yellowish friable calli were observed in 84.8 % and 73.4% at the 12th week and 16th week of culture but the difference was not significant at $p < 0.05$ ($\div^2$ contingency test). Some of the friable calli gradually became more compact in texture

Table 1: The frequencies of callus initiation and formation on field-grown leaf explants cultured on various combinations of BAP and NAA at the 8th week of culture

Treatment		Callus initiation	Callus formation
BAP (mg/L)	NAA (mg/L)	(%)	(%)
1	0.1	30.6ab	00.0c
1	1	12.5b	12.5b
1	2	55.6a	16.7b
2	1	54.2a	30.6ab
2	3	72.2a	56.9 a
F test		$p < 0.05$	$p < 0.01$

Values represent the means of three independent experiments, each with 24 survived explants. Means with the same letter are not significantly different in each column according to Duncan's Multiple Range test at 5%level.

and green in colour. Mean fresh weight of initial explants (leaf segments 5 x 5 mm^2) was 3.1 ± 0.11 mg. Callus growth was assessed by measuring fresh weight (mg) at 4 weekly intervals. callus growth reaches its stationary phase at 16 weeks of culture (Figure 1) and therefore it is better to transfer the calli into differention medium before 16 weeks. Friable callus was also obtained from leaf lamina where vascular tissue is present in the veinlets.

When calli were maintained in callus medium beyond 16 weeks, they turned friable and yellowish with gradual browning. It has been reported that[17] calli show high potential for morphogenesis in the initial stages but decline gradually with serial subculture. Total loss of morphogenetic ability could also occur. In the present study, after 20 weeks, 2 - 3 embyriods were visible but they did not develop further. No mature embryos could be seen visually, even though calli were maintained for more than 32 weeks on the callus medium.

Histology of embryonic callus

The cells (particularly epidermal cells and mesophyll tissues) near the cut edges of leaf segments began to divide forming circular collenchyma cells (Figure 2A) inside the tiny swellings. Subsequently, they burst and produced calli, which are composed of large, thin walled and highly vacuolated parenchyma cells (Figure 2B). Callus on wounded plants or on a culture medium is made of an amorphous aggregate of loose parenchyma cells that proliferate from the mother cells[18]. The parenchyma cells divided and gave rise to cambium - like structures that were rapidly divided into meristematic cells (Figure 2C, D). These small, thin walled cells had nuclei and were grouped in cell masses. The callus is initiated and multiplied on a medium rich in auxin, which induces the differentiation of localized groups of meristematic cells referred to as embryogenic clumps[19].

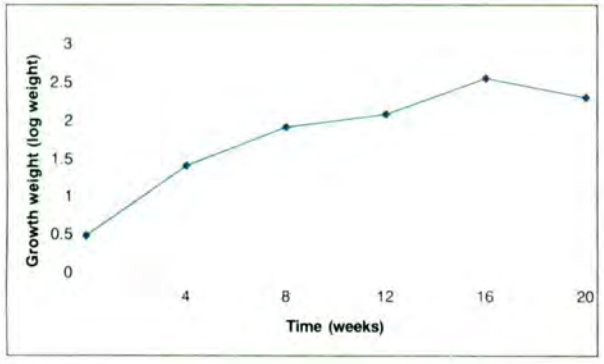

Figure 1: Growth weight of calli formed from *in vitro*-grown leaf segments

Typical embryogenic cells are small with a large nucleus, a very densely stainable nucleolus, dense cytoplasm and small vacuoles[20-23]. After 10 weeks, friable calli consisted of parenchama cell masses, began to exhibit the features of meristematic and embryogenic cells according to light microscopic observations. Cell walls of embryogenic cells were thicker than those of non - embryogenic cells (Figure 2E). Changes in the cell wall composition may be one of the first signs of acquisition of embryogenic competence[24]. Embryogenic cells contained a large nucleus (Figure 2E, ec). Explants cultured on a medium with 2, 4 D for 6 - 8 weeks followed by a similar period in 2, 4 D free medium formed embryogenic callus on tea leaves[10]. In the present study, embryogenic callus was formed in the presence of BAP in combination with NAA. Embryogenic cells differentiated into three to four celled proembryoids after 16 weeks of incubation (Figure 2F, arrowe). Sequential cell division gave rise to somatic embryoids.

A preliminary study on somatic embryogenesis from leaf calli

Somatic embryos (~ 4 mm in length, Figure 3A, B) were developed from embryogenic calli on half MS medium

Table 2: Effect of solid and liquid media on the formation of embryogenic cells

Number of cells/ clusters in 1 mL of sample (x100)	Callus medium		t test
	Solid	Liquid	
Total single cells	33.00 ± 2.22	51.22 ± 4.55	**
Total cell clusters	11.33 ±1.21	13.33 ± 1.11	ns
Single embryogenic cells	06.23 ± 0.62	09.78 ± 1.57	*
Embryogenic cell clusters	05.34 ± 0.61	06.56 ± 0.64	ns

Note: Data were recorded at the 16th week of incubation.
Values represent the mean ± standard errors of two independent experiments; each with 90 samples.
t test: ns - not significant; * - $p < 0.05$, **- $p < 0.01$

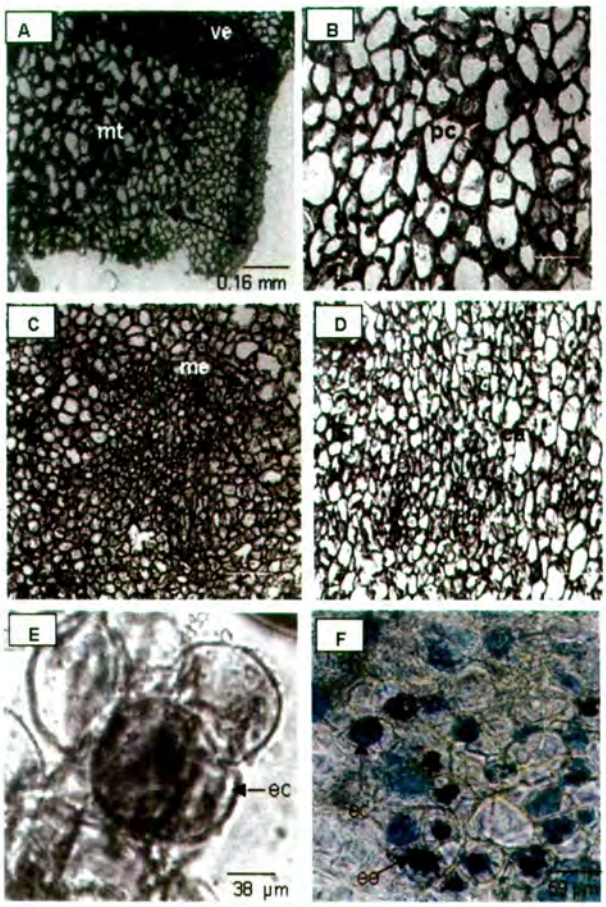

Figure 2: Histology of the development of embryogenic callus from *in vitro*-grown leaves

A: Transverse section of swollen tissue (sl) at the cut end of leaf segment at the 2nd week of culture (mt- mesophyll tissue; cells; ve- vein).

B,C,D: Transverse sections of calli with leaf explants at the 4th, 8th and 12th week of culture respectively. (pc- parenchyma cells; mc- meristematic cells).

E,F: Crush preparation of friable callus obtained from cultured leaf explants at the 16th and 20th week of culture respectively. (ec-embryogenic cell; ee- early embryoid).

with BAP (1.0 mg/L) and NAA (0.1 mg/L). However, primary friable calli turned hard and compact (Figure 3A) and failed to induce more somatic embryos. Therefore, further studies are necessary in order to improve the texture of embryogenic calli to enhance the production of somatic embryos.

Effect of solid and liquid media on intensity of embryogenic cells

Friable calli in liquid medium showed higher embryogenic potential than in solid medium at the 16th week of incubation (Table 2). Single embryogenic cells are round and the size ranged from 35-50 µm in solid and 25-80 µm in liquid medium. The mean number of total single cells and single embryogenic cells in 1.0 mL was significantly higher in liquid than in solid medium. However, there was no significant difference in the mean numbers of cell clusters between the two treatments. Callus cultures may contain both competent and non-competent cells[25]. In general, physical separation of single embryonic and non-embryonic cells is easier in liquid medium. Therefore liquid cultures will be more suitable for the production of single somatic embryos and also for genetic transformation studies.

Morphological and histological studies on the development of embryogenic callus from leaves are important to achieve high production of typical, firm somatic embryos for effective use in tea improvement *via* multiplication and *in vitro* selection as well as germplasm conservation through encapsulation technique under *in vitro* conditions.

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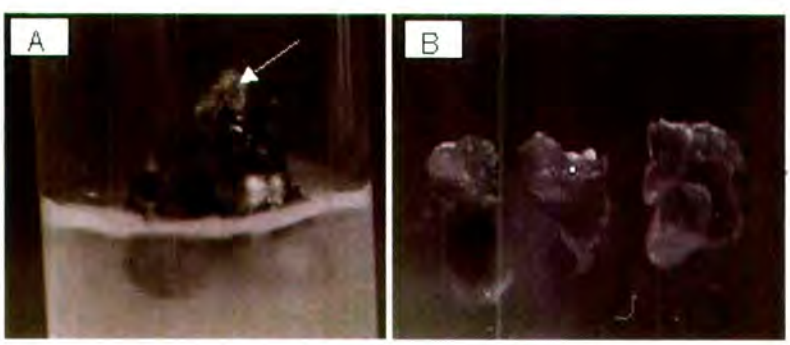

Figure 3: Formation of somatic embryos from leaf derived callus

A: Somatic embryo (arrow) formed from primary calli cultured on differentiation medium.

B: Typical somatic embryos.

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