J. Phytol. Res. **19**(1) : 23-28, 2006

SOMATIC EMBRYOGENESIS AND PLANT REGENERATION FROM IMMATURE AND MATURE COTYLEDONARY TISSUES OF CULTIVATED DARJEELING TEA [*CAMELLIA SINENSIS* (L). O. KUNTZE]

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Somatic embryogenesis and plantlets regenerations were obtained from immature and mature cotyledonary tissues of cultivated Darjeeling tea (*Camellia sinensis*) variety T_{383}, when cultured on Murashige and Skoog (MS) mineral medium. The somatic embryo induction frequency was influenced when the medium was supplemented with various levels of auxins such as 2,4-D, IAA, NAA and PBOA (1-4mg l^{-1}) in combination with BAP and Kinetin (5 mg l^{-1}) or 10% CM. The highest somatic embryo induction frequency was obtained using PBOA (1mg l^{-1}) + BAP (5mg l^{-1}) or PBOA (1mg l^{-1})+ Kinetin (5 mg l^{-1}) treatments. The most responsive initial explants were immature cotyledonary tissues collected in September, 40% of which produced somatic embryos as against only 18% for mature cotyledonary tissues from mature seeds collected in November. Mature cotyledons not only showed lower rate of somatic embryogenesis but also produced friable callus from the exposed surface of the explants that remained nonembryogenic. The somatic embryos developed, grew to maturity without being subcultured within 7-8 weeks. The embryogenic competence was maintained through repetitive embryogenesis for a period of over 20 months. Embryo conversion rate was only 3% in MS or B_5 medium without growth regulators, while 42% conversion occurred when medium impregnated with GA_3 at 1.5mg l^{-1} or in combination of GA_3 (1.5mg l^{-1}) with IAA (1 mgl^{-1}). Embryo conversion rate had been increased up to 51% when cultured on MS medium with 2 mgl^{-1} Brassin. Secondary somatic embryogenesis from primary embryo had been occurred while explant kept in culture. Somatic embryo derived plants were successfully transferred to potted soil at 60-70% relative humidity under green house conditions.

Keywords: Callus induction and Tea regeneration; *Camellia sinensis* var T_{383}; Cotyledon culture; Somatic embryogenesis.

Introduction

Tea (*Camellia sinensis* [L.] O. Kuntze) plants are perennial and highly heterozygous, requiring many years of selection to incorporate favorable traits. Improvement of tea using cellular and molecular biology technique is difficult, because cellular procedures are not well established[1]. India and SriLanka together exports over 80% in the world tea markets. Darjeeling tea, regarded as 'Champagne of teas' comes from foothills of the Himalayas, due to the richness in tea quality. However, their cultivation, maintenance and conservation are being carried out using conventional breeding techniques. So, it seems inevitable on exploitation of new molecular biological methodologies for the improvement of Darjeeling teas. There are some reports on direct and indirect somatic embryogenesis from immature zygotic embryos for several *Camellia* species which include *Camellia reticulata*[2], *C. japonica*[3,4] and *C. sinensis*[3,5]. Somatic embryogenesis from cotyledons, anthers or leaves has also been documented[3,6-12]. There are very few reports on *in vitro* culture of tea varieties growing in India[13-14] but none in Darjeeling tea varieety T_{383}.

The present study reports on the efficient direct somatic embryogenesis from immature and mature cotyledonary tissues and high rate of conversion of somatic embryos to plantlets in a Darjeeling tea clone, *Camellia sinensis* var T_{383}.

Abbreviations: CM- coconut milk; PBOA-phenylboronic acid; 2,4-D. -2,4-dichlorophenoxyacetic acid; Kin- kinetin; BAP- N6-benzyladenine; GA_3-gibberellic acid; IAA- indole-3-acetic acid.

Materials and Methods

Plant sources : Green capsule and mature seeds were collected from seedling tea bushes of *Camellia sinensis* var. T_{383}, at the Happy Valley Tea Estate, Darjeeling, WB, India. *Camellia sinensis* has high cross-pollinating ability

and the seeds collected from a plant normally have high degree of heterozygosity. Green capsules were collected in September and mature seeds from open mature capsules were collected in November. Mature seeds (13-18mm) were extracted from the mature capsules and floated overnight in water to discern viable from nonviable seed. Floating seeds were discarded whilst those that sank were first rinsed in 5% Tween 80 for 5 minutes, then in 70%(v/v) alcohol for 2 minutes. Surface sterilization was carried out in a 4%(v/v) calcium hypochlorite solution for 15 minutes, followed by five to six rinses in autoclaved double distilled water. The surface sterilized seeds were germinated in half-strength MS basal medium[15], containing 30g/l sucrose and solidified with 0.8% (w/v) Difco-agar. Green cotyledons were harvested from 15-day-old seedlings and their embryonic axes removed. The intact mature de-embryonated cotyledonary endosperm cut into pieces (0.5-1.0cm long) before placing it into somatic embryo induction medium. Green capsule collected in September were sterilized by the same procedure before the immature seeds (9-11mm) were isolated and then each of immature de-embryonated cotyledon were placed into the same medium for somatic embryogenesis.

Culture medium: Both the cotyledonary tissues of immature and mature seeds were placed onto the surface of semisolid, full strength Murashige and Skoog medium. The medium was supplemented with the different plant growth regulators such as BAP, IAA, PBOA, NAA, 2,4-D and Kin (1–5 mgl^{-1}), or 10% CM either singly or in combinations, for induction of somatic embryos. The medium was solidified with 0.8% agar after adjusting to pH 5.8 and sterilized at 121°C for 15-18 min. All the MS media contained Fe-EDTA and micronutrient formula described by Murashige and Skoog plus 0.5mgl^{-1} thiamine-HCl, 0.5mgl^{-1} pyridoxine-HCl, 0.05mgl^{-1} nicotinic acid, 100mgl^{-1} m-inositol, 30gl^{-1} sucrose and 8gl^{-1} Difco-Bacto-Agar. The cultures were maintained for 14 weeks at 25± 2°C under a 12/12 (day/night) photoperiod with light provided by cool-fluorescent tubes at a photon flux density of 52-µmol $m^{-2}s^{-1}$ and 60-65% relative humidity.

For plantlet regeneration, somatic embryos were removed from cotyledons and cultured in MS and Gamborg's B_5[16] basal medium impregnated with various growth regulators like BAP (5mgl^{-1}), IAA (2mgl^{-1}), Brassin (2mgl^{-1}) and GA_3 (1.5mgl^{-1}), either singly or in different combinations, or without growth regulators. All the experiments were repeated three times. Regenerated plants after 6-8 weeks with well-developed roots were transferred into 10cm long layflat polythene sleeves containing a mixture of autoclaved soil and sand in a ratio 3:1. The plants were then transferred to a shaded nursery bed.

Results and Discussion

Induction of somatic embryos:

Seeds collected in September - The immature white cotyledon explants enlarged while in culture, turned greenish in colour and developed translucent outgrowths. After 4 weeks of culture, somatic embryos began to appear on 10-15% of the explants. Then numerous primary somatic embryos developed directly from the adaxial surface of the cotyledons in 40% of the explants (Fig. 1a). During the 4 weeks, various embryonic stages were observed: globular, heart, torpedo. When these structures enlarged they developed into morphologically distinct somatic embryos without subculturing (Fig. 1f). The immature cotyledonary

Table 1. Percentage of somatic embryo induction from immature and mature cotyledons of *C. sinensis* var T_{383}, after 14 weeks of culture on different hormone containing MS basal medium.

Medium (MS) (mgl^{-1})	Immature cotyledons		Mature cotyledons	
	% embryogenic cotyledons[a] ± S.E.	No. of somatic embryos/explants [b] ± S.E.	% embryogenic cotyledons[a] ± S.E.	No. of somatic embryos/explants [b] ± S.E.
MS basal	3.02 ± 1.12	2.2 ± 1.23	3.02 ± 1.02	1.5 ± 1.23
BAP 5	5.09 ± 2.01	3.89 ± 1.67	3.0 ± 1.02	2.0 ± 1.06
2,4-D 4 + IAA 1	12.07 ± 2.01	8.12 ± 2.10	9.50 ± 2.01	9.56 ± 1.56
C M (10%)	15.13 ± 2.03	22.80 ± 2.01	10.12 ± 1.89	10.25 ± 2.34
PBOA 1	30.20 ± 1.02	40.25 ± 1.58	18.00 ± 2.00	22.23 ± 2.06
PBOA 1 + BAP 5	40.40 ± 1.58	45.29 ± 2.24	18.00 ± 2.00	22.23 ± 2.06
PBOA 1 + Kin 5	40.25 ± 2.04	43.55 ± 2.04	17.89 ± 2.35	20.22 ± 1.79

[a] Based on 100 explants per treatment after 14 weeks of culture.

$$\% \text{ embryogenic cotyledons} = \frac{\text{No. of cotyledon explants showing induction of somatic embryos}}{\text{No. of explants cultured}} \times 100$$

[b] Based on 30 explants per treatment ± S.E.

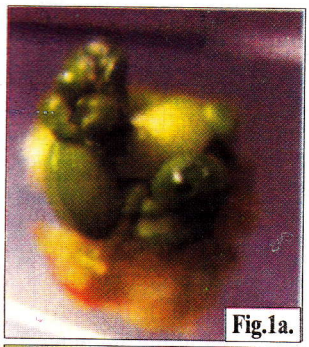
Fig.1a.

Fig.1a. Seed-like somatic embryos with large cotyledons, like those of zygotic embryos. The seed-like embryos developed into whole plant only those were with partially fused cotyledons.


Fig.1b.

Fig.1b. Cup-shaped somatic embryos, which were pale yellow to creamy-greenish in colour.

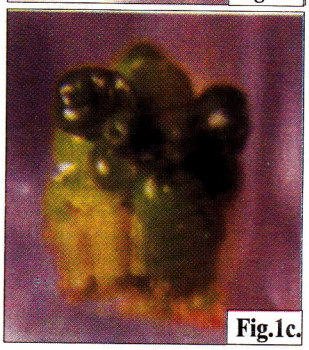
Fig.1c.

Fig.1c. Bud-like somatic embryos, which were green in colour.


Fig.1d.

Fig.1d. Shoots were developed either singly or multiple in number on the somatic embryos.

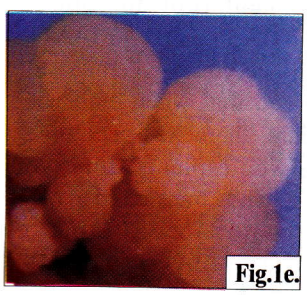
Fig.1e.

Fig.1e. Globular shaped somatic embryo.


Fig.1f.

Fig.1f. Somatic embyros are developing into plantlets through heart and torpedo shaped stages.

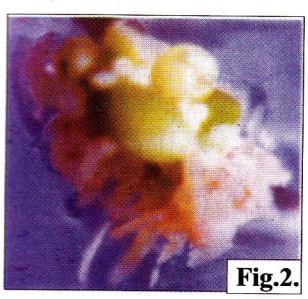
Fig.2.

Fig.2. Somatic embryos were developed along with rhizogenesis when cultured on MS medium in presence of either 2, 4-D (4 mg/1) or IAA (1 mg/1).

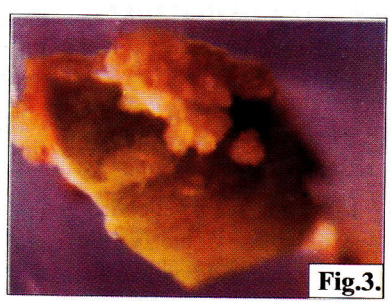
Fig.3.

Fig.3. Non-embryogenic friable callus was developed on the upper surface of the mature cotyledon when cultured on MS medium with 2,4-D (4 mg/1) +IAA (1 mg/1).


Fig.4.

Fig.4. Secondary somatic embryogenesis were obtained in MS/B, medium in presence of BAP (5 mg/1). + IBA (1 mg/1) or IAA (2 mg/1).


Fig.5.

Fig.5. Plantlet regenerated from the somatic embryo in a MS medium containing Brassin (2 mg/1) + GA_3 (1.5 mg/1) and 1% agar. Then the plantlet transferred to potted soil after 6-8 weeks of germenation on the above medium.

Table 2. Effect of hormones and basal medium on embryo conversion frequency in *C. sinensis* var T_{383} after 8 weeks of culture.

Medium (mgl^{-1})	% Embryo conversion ± S.E.
MS basal	3.03 ± 0.68
B_5 basal	3.30 ± 0.99
MS + BAP 5	6.24 ± 1.04
MS + BAP 5 + IAA 2	8.67 ± 1.07
MS + BAP 5 + IBA 1	8.88 ± 2.11
B_5 + BAP 5 + IBA 1	9.12 ± 1.98
B_5 + BAP 5 + IAA 2	23.93 ± 1.08
MS + GA_3 1.5 + IAA 0.5	42.78 ± 1.03
MS + Brassin 2+ GA_3 1.5	51.09 ± 1.78

tissues were on an average more productive than that of mature cotyledons (Table 1.). The embryogenic competence was greatest on culture medium impregnated with plant growth regulators. The number of somatic embryos produced per embryogenic explant ranged from 3 to 45 (Table 1).

Seeds collected in November - Only 18 % of the mature cotyledonary tissues from seeds collected in November were embryogenic. The other 82 % enlarged slightly, and some of them were developed into non-embryogenic callus. Mature cotyledonary explant were green and swollen after two weeks in culture. Somatic embryos continually arose on cotyledonary explants between 5 and 6 weeks. The overall embryogenesis rates were afforded by medium with PBOA 1 mgl^{-1} + BAP 5 mgl^{-1} or PBOA 1 mgl^{-1} (18 %), on the contrary the lowest rates being obtained on medium with BAP 5 mgl^{-1} (3%) or 2,4-D 4 mgl^{-1} + IAA 1mgl^{-1} (13.28 %). The mean number of embryos per embryogenic explant varied greatly from 2 to 22 (Table 1).

Morphology of somatic embryos : Major types of somatic embryos produced were clearly morphological distinct types. These were as follows -

i) 'Seed-like' embryos (Fig. 1a), which were yellowish-white with large cotyledons, like those of the zygotic embryos. They turned green at maturity, and so far the most abandoned in most media. Two cotyledons produced were usually of different size and partially or wholly fused together. Sometimes more than two cotyledons were also formed. They germinate into whole plants in MS medium with 1.5 mgl^{-1} GA_3 + IAA (1 mgl^{-1})(Fig. 2) or MS + 2 mgl^{-1} Brassin.

ii) 'Cup-shaped' embryos, which were pale yellow to creamy-greenish in colour. They emerged as small and rounded structures. The embryo underwent a typical embryogenic pathway as evidend cup-shaped structures. Bipolar nature rarely observed (Fig. 1b).

iii) 'Bud-like' embryos (Fig. 1c), which were green, with cotyledons like true leaves. They were bipolar in nature developing whole plantlets with shoot and root poles, and generally occurred in media with relatively moderate concentrations of PBOA (1 mgl^{-1}) and Kin (5 mgl^{-1}). Germinated well in MS with 2 mgl^{-1} Brassin.

Secondary embryogenesis and germination: When somatic embryos were excised and transferred singly or in embryo-clusters on to embryo germination medium (EGM), either MS or B_5 supplemented with different combinations of BAP (5 mgl^{-1}), IAA (2 mgl^{-1}), IBA (1 mgl^{-1}), Brassin (1 mgl^{-1}) and GA_3 (1.5 mgl^{-1}), there was negligible amount of embryo conversion in some combinations (3-9%). However, secondary somatic embryogenesis had been occurred with in 5-6 weeks of transfer. It has been observed that maximum number of secondary embryogenesis were obtained in MS/B_5 medium in presence of BAP (5 mgl^{-1}) + IBA (1 mgl^{-1}) or IAA (2 mgl^{-1}) (Fig. 4). The secondary somatic embryos were located on the hypocotyl region of the both 'bud-like' and 'seed-like' primary somatic embryos. The embryogenic potential has maintained for over 20 months by successive 6-weekly subcultures. 'Bud-like' embryos had a 42.78% germination rate and subsequent root and shoot development in MS-medium with GA_3 (1.5 mgl^{-1}) + IAA (1 mgl^{-1}), whereas only 12% of 'seed-like' embryos germinated in this medium. It was observed that when embryos transferred to MS or B_5 medium, there was negligible embryo conversion (3%) to whole plants. Embryo conversion rate little bit increased while transferred to MS medium in presence of BAP (5 mgl^{-1}) (6%) or BAP (5 mgl^{-1}) + IAA (2 mgl^{-1}) (8%), or BAP (5 mgl^{-1}) + IBA (1 mgl^{-1}) (9%). The somatic embryo germination frequency was enhanced in both the cases ('bud-like', and 'seed-like') to 51% in MS medium containing Brassin (2 mgl^{-1}) and 1% agar (Table 2). Plantlets regenerated from both the type of embryos ('bud-like', and 'seed-like') were strong enough for transfer to pots after 6-8 weeks on germination medium. The success rate in the acclimatization process was 70% (Fig. 5).

The present results show that somatic embryogenesis readily occurs *in vitro* on both immature and mature cotyledonary tissues of *Camellia sinensis* var. T_{383}. Success depends on both the physiological maturity of the cotyledon and the kind of culture medium used. The fact that the fully-grown but still immature September cotyledonary tissues had the highest rate of somatic embryogenesis than the mature cotyledonary tissues of November. The reduced embryogenic capacity of mature November cotyledon was due to loss of competence associated with maturation. The embryogenic potential of the September cotyledon, is of particular interest with a view to enable the efficient success of embryo conversion for molecular biological works. It is important to emphasize the positive response of the immature cotyledonary tissues with relation to the kind of explants, especially with

September seed cotyledons. The present study improves the efficiency of the somatic embryogenesis system described by Kato[3] in *C. japonica* and *C. sinensis* and by Zhuang & Liang[2] in *C. reticulata* and for *C. sinensis,* in which only cotyledon explants were used. It also enhanced the rate of embryogenesis from immature cotyledons than that of the results reported by Ponsamuel[11] and by Jha[6] in *C. sinensis*, by using novel auxin PBOA (1 mgl^{-1}) alone or in combination with BAP (5 mgl^{-1}) or with Kinetin (5 mgl^{-1}). In a previous study it had been shown that 2,4-D, the auxin most widely used for embryogenesis culture by Evans[17], induced callus but completely inhibited somatic embryogenesis, which is totally in contrary to the present findings. In the present study it has been observed that somatic embryogenesis was quite good in number (13-18%) in culture supplemented with 2,4-D (4 mgl^{-1}) in combination with IAA (1mgl^{-1}) along with rhizogenesis, which was a reverse result of the findings of Jha[6] in *C. sinensis* var T-78 (Fig. 2). Non-embryogenic callus development in some cases of mature cotyledon explants has been occurred here but not found in immature cotyledons (Fig. 3).

In a previous study adventitious embryo formation from cotyledon culture has been reported in a few species of tea (*C. sinensis* L. cultivar Yabukita) by Kato[3], by Wachira[12] in *C. sinensis* and by Vieitez[4] in *C. japonica*. It was obtained either in unsupplemented basal medium[3] or in media supplemented with high cytokinin plus a low auxin or a cytokinin only[3,9]. In the present study somatic embryo was reported without growth regulators, which was not in accordance with the report of Jha[6]. BAP (5 mgl^{-1}) or 10% CM can alone induce the somatic embryo development in 5% and 15% explants respectively. Somatic embryogenesis has been enhanced up to 40% by addition of novel auxin PBOA (1 mgl^{-1}) in combination with 5 mgl^{-1} BAP or Kinetin. Same type of results were reported earlier by Ponsamuel[11] in *C. sinensis*. BAP influenced the morphology of the somatic embryos produced in presence of novel auxin PBOA. Highest rate of differentiation was reported in *C. sinensis* var. Yabukita[9] in presence of 5 mgl^{-1} BAP in MS medium. The developing embryos passed through the typical globular, torpedo, and cotyledon stages in the course of their development to reach maturity. Secondary somatic embryogenesis was noted in the present study when primary somatic embryo transferred to germination medium and maintained the secondary embryogenesis through successive subcultures.

Direct somatic embryo differentiation seems to ensure genetic stability. High rates of largely direct embryogenesis occurred on immature cotyledons of September in comparison to mature cotyledon of November in medium supplemented with BAP and PBOA; suggest that growth regulators increased the number of somatic embryos. Somatic embryo development in *Camellia* did not require two phases of culture procedure, initiation and maturation occurring on the primary medium[18,19]. A different medium was needed for their germination, but sometimes-secondary embryo formation occurred in the germination medium too. The present results seem to indicate the general applicability of the methods described in this paper, to a wide range of Darjeeling tea genotypes, to obtain several cycles of subsequent embryogenesis to be used to produce artificial seeds or to produce genetically modified transgenic tea plants for the improvement of 'Champagne of teas' of Darjeeling Himalayas.

However, there are some reports on embryo conversion methods in Darjeeling tea variety (*Camellia sinensis*) but the frequency was very low[6]. The present study reports the high rate of somatic embryogenesis and enhanced embryo conversion rate in an elite Darjeeling tea variety T_{383}.

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