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Somatic embryogenesis and plant regeneration from the immature cotyledonary tissues of cultivated tea (*Camellia sinensis* (L).O. Kuntze)

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ABSTRACT. Embryogenic callus development, plant regeneration, and plant recovery were achieved from immature cotyledon explants of cultivated tea, when cultured on MS basal medium. The somatic embryo induction frequency was influenced when the medium was supplemented with 1 μM auxin (NAA, NOA, 2,4-D, TPB, and PBOA) in combination with cytokinin (0.5 μM BA, KIN) or 10% CM. The highest somatic embryo induction frequency was obtained using PBOA + BA or PBOA + KIN treatments. All auxins except 2,4-D stimulated rhizogenesis using 0.8% and 1.5% agar concentrations, and differentiation of a characteristic swelling and friable callus from the exposed surface of the explant that remained nonembryogenic. Conversely, the novel auxins TPB and PBOA at 1 μM concentration with 3% or 6% agar, produced somatic embryo induction, while at 0.8% and 1.5% produced nonembryogenic callus. Explants isolated proximal to the zygotic embryonal axis showed a greater somatic embryo induction frequency than did the distal explants. The embryogenic competence was maintained through repetitive embryogenesis for a period of over 18 months. The somatic embryos developed into plantlets when incubated on hormone-free medium. The conversion frequency was increased by 50% in MS medium containing 1 μM Brassin and 0.8% agar. Concentration of agar at 3% and 6% decreased the conversion frequency and promoted anomalous plantlet development. The normal plantlets were treated with 1 μM IAN, 1 μM Brassin and 10 μM Phloroglucinol in liquid MS medium for 15 d, where profuse lateral roots were induced favoring a high rate of plant recovery.

Abbreviations: *BA*=N6-benzyladenine; ***BR (brassin)***=2, 3, 22β, 23β-tetrahydroxy-24β-methyl-β-homo-7oxa-5-cholestan-6-one; *CM*=coconut milk; *2,4-D*=2,4-dichlorophenoxyacetic acid; *IBA*=indole-3-butyric acid; *IPA*=indole-3-propionic acid; *IAN*=indole-3-acetonitrile; *KIN*=kinetin; *MS*=Murashige and Skoog 1962) medium; *NAA*=1-naphthaleneacetic acid; *NOA*=β-napthoxyacetic acid; *PBOA*=phenylboronic acid; *PGR*=plant growth regulators; *TPB*=tetraphenylboron.

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Keywords: Brassin, *Camellia sinensis,* Novel auxins, Phenylboronic acid, Phloroglucinol, Somatic embryogenesis, Tea regeneration, Tetraphenylboron.

INTRODUCTION

Tea [*Camellia sinensis* (L.) O. Kuntze] is the world's foremost beverage plant (Charles 1981) and an important plantation crop of great commercial value. It has been maintained for centuries through conventional vegetative propagation (Eden 1976). *In vitro* clonal propagation of tea offers the advantage of multiplying elite plants in multitude. Selection of plants or plant lines with useful somaclonal traits could be performed if an efficient *in vitro* regeneration method were available. In addition, a suitable *in vitro* regeneration system adaptable to transformation methods would facilitate potential improvement of this crop through biotechnological means. Several reports have been published on the *in vitro* culture of cultivated tea (Wachira and Ogada 1995; Agarwal et al. 1992; Seneviratne et al. 1988; Arulpragassam and Latiff 1986; Kato 1986, 1985; Phukan and Mitra 1984; Mu-qin and Ping 1983; Wu et al. 1981). Other reports on ornamental *Camellia* trees include studies of *Camellia japonica* (Vieitez and Barciela 1990; Kato 1986), *C. vietnamensis, C. chrysantha* (Nadamitsu et al. 1986) and *C. reticulata* (Zhuang and Liang 1985).

Among the published protocols for the genus *Camellia*, regeneration of plants through cotyledon induced somatic embryos is considered to provide a method for rapid propagation with a high plant regeneration frequency. (Vieitez 1995; Kato 1989; Vieitez and Barciela 1990).

Immature cotyledons show a high degree of embryogenic potential in several plant species (Williams and Maheswaran 1986). In *Camellia sinensis,* immature cotyledons have been used to differentiate both direct and indirect somatic embryos (Jha et al. 1992; Bano et al. 1991; Nakamura 1988; Abraham and Raman 1986). Mature cotyledon explants showed lower embryogenic potential than their immature counterparts in *Camellia japonica* (Vieitez and Barciela 1990; Kato 1986), *C. sinensis* (Kato 1986) and *C. reticulata* (Zhuang and Liang 1985). Somatic embryos of *Camellia* were reported to be the only source of obtaining autopolyploids in *Camellia* (Kato 1989).

In *C. sinensis* a high auxin to cytokinin ratio was necessary for somatic embryogenesis (Vieitez 1995). The choice of auxins used in these studies were limited to 2,4-D, IAA, and IBA. Although exogenous cytokinin generally suppresses embryo development and nurtures the growth of the shoot meristem to differentiate shoots *in vitro* (Ammirato 1983), in *C. sinensis* cytokinins promoted somatic embryo induction and development from zygotic embryo and cotyledon explants (Das and Barman 1988; Nakamura 1988).

The aim of the present investigation was to study the effect of various auxins including two novel auxins, tetraphenylboron (TPB) and phenylboronic acid (PBOA), as well as various cytokinins and their combination on *in vitro* embryogenesis using cotyledon explants of UPASI-10, a tea clone cultivated in South India. The novel auxins TPB and PBOA, were substituted for auxin in studies with various plants (*Arachis hypogaea, Coleus parviflorus, Nicotiana plumbaginifolia* and *Cresentia cujete*) and were found to be more efficient in influencing certain types of cell and tissue responses than were 2,4-D and NAA (Ponsamuel 1990). Also, these novel auxins are known to induce somatic embryos in peanut (*Arachis hypogaea*) and country potato (*Coleus parviflorus*) (Ponsamuel and Dayanandan 1994).

MATERIALS AND METHODS

Plant Material. The seeds of UPASI-10 tea clone used in this investigation were obtained from the Tea Research Centre, United Plantation Association of South India, Cinchona, Coimbatore in the State of Tamil Nadu, India. UPASI-10 is a clone selected from the heterogenous tea population grown in Brookfields (Field # 6) in the year 1962. Green, immature capsules were collected when the testa of the seeds were creamish white in color (~ 7 month old seeds). The seeds were stored at 5°C in sealed polythene bags and the samples were routinely tested for viability using the triphenyltetrazolium chloride method (Lakon 1949) to ensure that at least 70% of the seeds were viable. Capsules were disinfested in 70% ethanol for 5 min, followed by a 20% (v/v) solution of commercial bleach treatment (1.8% sodium hypochlorite) for 15 min, and then rinsed 3 times with sterile distilled water. The testa was dissected out and the cotyledons were removed aseptically. The embryonal axis was carefully removed, and 5 mm slices (Fig. 1a) of the cotyledon both proximal and distal to the embryonal zone of the seed were isolated as explants (5 explants per seed). Twenty-five Petri plates each containing 5 explants placed onto 15 ml medium were used per treatment.

Culture Medium and Environment. The culture medium consisted of MS(Murashige and Skoog 1962) mineral nutrients and vitamins with 30g/l sucrose and 0.8% to 6% agar (Himedia Laboratories Pvt Ltd., Bombay, India). Plant growth regulators (PGRs) and combinations of PGRs were added as specified (Tables 1 and 2). BR was the only PGR that was filter sterilized and added to the medium after autoclaving. The medium was adjusted to pH 5.0 prior to the addition of agar and sterilized at 121° C at 103 kPa pressure for 15 min. All the PGRs used in this experiment except BR were obtained from Sigma Chemical Company. BR was a gift from Dr. Werner J. Meudt of the United States Department of Agriculture, Maryland. Cultures were maintained at 28±2°C under cool white fluorescent lamps of 30 μE $m^{-2}s^{-1}$ photon density with a 16 h photoperiod. To evaluate the effect of the absence of light on callus induction and morphogenesis, cultures were maintained in dark at 28±2°C. The tissues were subcultured monthly.

Data collection and statistical analysis. The embryoids induced per cm^2 area of each explant was counted 60 d after culture initiation. A total of 80 out of 125 explants (from 25 replicates of 5 explants per treatment) were selected randomly and analyzed for scoring somatic embryos. Developed plantlets with both shoot and root were counted after 60 d of culture in the conversion medium. Initially 25 (5 Petri plates with 5 embryos per plate) somatic embryos per treatment were cultured on conversion medium. The results obtained from somatic embryo induction and conversion experiments were analyzed by ranking of treatment means to determine whether the results were statistically significant using Duncan's Multiple Range Test (Gomez and Gomez 1976).

Histological investigation. Explants were processed, embedded in paraffin wax and sectioned following standard procedures (Berlyn and Miksche 1976). The sections were stained with Toluidine blue (Sakai 1973) prior to microscopic observation.

RESULTS AND DISCUSSION

Embryogenic callus induction was observed from the immature cotyledon explants (Fig. 1a) 60 d after culture initiation in MS medium containing 3% or 6% agar (Fig. 1b, 1c). Embryogenesis was not observed in the other agar concentrations studied (Table 1). Somatic embryo induction was highly dependent on agar concentration, and PGR treatments generally increased the number of embryos per cm^2 area of the explants (Table 1). In contrast to earlier investigations (Wachira and Ogada 1995; Jha et al. 1992; Bano et al. 1991; Abraham and Raman 1986), explants in the present study produced only nonembryogenic callus in media with 0.8% agar. The apparent discrepancy between previously published reports and the results in this study may have been due to genotypic differences. This supposition is supported by the fact that remarkable differences were observed in the frequencies of somatic embryo induction among cultivars of the genus *Camellia sinensis* (Vieitez 1995). Also, similar differences in *in vitro* response were observed for cotyledon explants of various *Cucumis melo* cultivars using 0.8% agar (Dirks and Buggenum 1989; Moreno et al. 1985; Niedz et al. 1989). However, cotyledon explants of *C. melo* cv. Amarillo Oro regenerated only using 8% agar concentration (Valles and Lasa 1994).

Auxins tested at higher levels (2.5, 5, 7.5, and 10 μM) produced only nonembryogenic callus from cotyledon explants, and at lower levels (0.5 and 0.1 μM) did not show any morphogenetic response (data not shown). At 0.1 μM concentration, the synthetic auxin 2,4-D has been successfully used in tea anther cultures for the regeneration of haploid plantlets (Shimokado et al. 1986). In this investigation, auxin at 1 μM produced optimum response from cotyledon explants. Inhibition of

embryogenesis was observed with IPA, NAA, NOA and 2,4-D compared to the control (Table 1). In contrast, the two novel auxins TPB and PBOA promoted embryogenesis.

Most auxin treatments promoted somatic embryogenesis when used in combination with cytokinins (Table 1). Similar enhancement of somatic embryogenesis was reported earlier when BA was added to the medium (Nakamura 1988; Kato 1986). Somatic embryo induction was high when the explants were cultured in 1 µM PBOA along with 0.5 µM BA or 0.5 µM KIN and 6% agar in the medium. Explants proximal to the zygotic embryonal axis showed a higher number of somatic embryos per cm^2 area of the explant compared to those distal to the embryonal axis (Table 1). Somatic embryo induction is known to be highly dependent on the nature of the explant used (Zimmerman 1993). Various stages of somatic embryos (globular, heart and torpedo) were observed 60 d after culture initiation. The embryos went through a normal maturation process when subcultured in the same medium. Embryogenic potential was maintained through successive secondary embryogenesis for 18 months on MS medium containing 1 µM PBOA with 0.5 µM BAP.

The novel auxins, TPB and PBOA, were previously reported to influence cell and tissue culture responses similar to auxins, including *in vitro* embryogenesis of peanut and country potato (Ponsamuel 1990). The auxin activities of PBOA and TPB in these studies were comparable, and PBOA was more efficient in inducing somatic embryogenesis than were 2,4-D, NAA, and TPB. The same trends were observed in the present study. Although the mode of action of PBOA is unknown, it is a highly active compound and is known to regulate enzyme levels in plants (Ponsamuel 1990). It is proposed that PBOA could be used as a compound

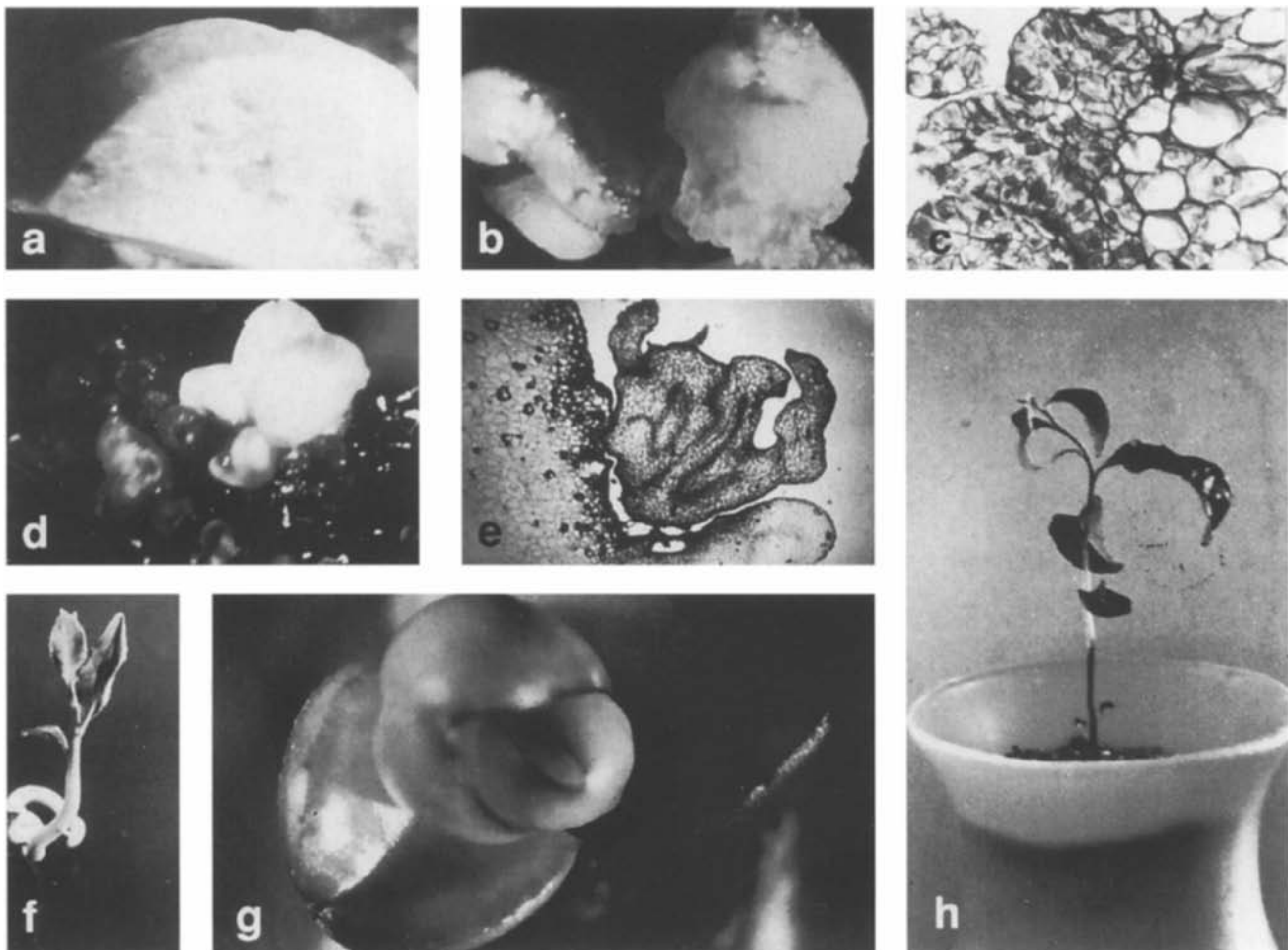

Figure 1. Various stages of plant regeneration via embryogenesis from the immature cotyledonary explants of tea (UPASI-10 clone). (a) Immature cotyledonary explant at the time of culture initiation (X5). (b) Embryogenic callus production from the explant after 25 d of culture in MS basal medium (X3) (c) A section of embryogenic callus produced from the cotyledonary explants 30 d after culture initiation in MS medium containing 1 µmM TPB + 0.5 mM BAP, showing the differentiation of peripheral layers into globular embryoids (X60). (d) Somatic embryos at various stages of *in vitro* development 80 d after culture initiation in medium containing 1 µM PBOA + 0.5 µM Kin (X10). (e) A vertical section of a callus with differentiated somatic embryos, 75 d after culture initiation, showing a bipolar late heart stage embryo. (X20). (f) Normal plantlet developed from a somatic embryo 60 d after culture in the conversion medium (MS medium containing 1 µM BR and 0.8% agar). (g) Anomalous plantlet from somatic embryo in medium containing 6% agar. Note the development of thick, succulent leaves. (h) Established plant in greenhouse 180 d after culture initiation.

of choice for inducing somatic embryogenesis in plants.

Embryos with morphologically distinct cotyledons were obtained 60 d after culture initiation. Some of these somatic embryos attained the late torpedo stage with the characteristic creamish white color 90 d after culture initiation (Fig. 1d). Each responding explant at this stage had at least 4 - 6 embryos at late torpedo stage, along with numerous globular and heart shaped embryos.

Histological analysis of the explants carried out at various stages of *in vitro* development indicated that somatic embryo induction was produced through the development of embryogenic callus, and embryos developed from the peripheral layers of the callus (Fig. 1c). This layer was comprised of meristematic cells with dense cytoplasm and conspicuous nuclei. Longitudinal sectioning of embryos produced from this layer 75 d after

Table 1. Somatic embryo induction from the cotyledon explants of immature tea seeds in response to PGRs and vying agar concentrations 60 d after culture initiation. Number of somatic embryos presented are from 1 cm^2 area of the explants. Mean values from 16 replicates of explants ± SD.

PGR/ PGR comb. tested	Agar Concentration 0.80% CEP	0.80% CED	1.50% CEP	1.50% CED	3% CEP	3% CED	6% CEP	6% CED
Control[a]	NC	NC	S	S	07+0.10l	06.0+0.20l	12.0+0.20j	10.0+0.80k
IPA*	R	R	R	R	S	S	S	S
NAA*	R	R	R	R	S	S	S	S
NOA*	R	R	R	R	S	S	S	S
2,4-D*	NC	NC	NC	NC	NC	NC	NC	NC
TPB*	NC	NC	NC	NC	22.0+0.70i	18.2+0.80i	30.5+0.12g	30.2+0.60g
PBOA*	NC	NC	NC	NC	25.8+0.10n	24.2+0.60h	32.6+0.40g	30.1+0.18g
BAP*	-	-	-	-	S	S	18.2+0.14i	12.2+0.22j
KIN*	-	-	-	-	S	S	22.4+0.20i	14.8+0.18j
CM[1]	-	-	-	-	-	-	14.0+0.70j	10.8+0.11k
IPA+BAP[2]	NC+R	NC+R	NC+R	NC+R	12.0+0.25j	10.0+0.80k	42.1+0.80e	38.2+0.24f
NAA+BAP[2]	NC+R	NC+R	NC+R	NC+R	08.1+0.50l	07.0+0.10l	21.2+1.20i	19.2+0.18i
NOA+BAP[2]	NC+R	NC+R	NC+R	NC+R	10.0+0.12k	11.1+0.82k	26.2+0.80e	22.1+0.70i
2,4-D+BAP[2]	NC	NC	NC	NC	NC	NC	45.2+0.72d	14.8+0.20j
TPB+BAP[2]	NC	NC	NC	NC	28.0+0.40g	26.0+0.80h	53.0+0.60c	40.2+0.90e
PBOA+BAP[2]	NC	NC	NC	NC	35.0+0.50f	34.0+0.60f	65.2+0.90a	59.1+0.21b
IPA+KIN[2]	NC+R	NC+R	NC+R	NC+R	10.0+0.80k	08.0+0.21l	44.6+0.20d	41.2+0.20e
NAA+KIN[2]	NC	NC	NC	NC	19.0+0.20i	12.0+0.40j	28.2+0.12g	24.0+0.12h
NOA+KIN[2]	NC	NC	NC	NC	15.0+0.15j	14.0+0.60j	32.3+0.30g	30.2+0.80g
2,4-D+KIN[2]	NC	NC	NC	NC	NC	NC	20.6+1.20i	15.1+0.15j
TPB+KIN[2]	-	-	NC	NC	30.0+0.12g	27.0+0.16g	58.2+0.81b	39.0+0.90e
PBOA+KIN[2]	NC	NC	NC	NC	39.0+0.90e	35.0+0.13f	62.3+0.40a	56.1+0.10b
IPA+CM	NC+R	NC+R	NC+R	NC+R	18.0+0.50i	08.2+0.20l	39.2+0.30e	35.1+0.12f
NAA+CM	NC+R	NC+R	NC+R	NC+R	14.0+0.60j	12.0+0.80j	20.4+0.20i	20.3+0.10i
NOA+CM	NC+R	NC+R	NC+R	NC+R	13.0+0.80j	14.0+0.60j	25.2+0.80h	21.8+0.14i
2,4-D+CM	NC	NC	NC	NC	NC	NC	18.4+0.40i	15.9+0.12j
TPB+CM	NC	NC	NC	NC	25.0+0.16h	20.0+0.70i	51.2+0.90c	40.3+0.40e
PBOA+CM	NC	NC	NC	NC	32.0+0.80g	32.0+0.60g	56.2+0.12b	42.1+0.30e

R=Rhizogenesis; CM=Coconut milk; NC=Nonembryogenic callus; S=Swelling of tissue; -=No morphogenetic response; CEP=Cotyledon explant proximal to zygotic embryo; CED=Cotyledon explants distal to zygotic embryo; [a]=Explants cultured in basal medium; *=1 μM, [1]=10%, [2]=1 μM auxin + 0.5 μM cytokinin. Identical letter following the values indicate no significant difference according to Duncan's Multiple Range Test ($p<0.05$).

Table 2. Effect of PGRs on somatic embryo conversion (from late torpedo stage obtained 90 d after culture initiation) in *Camellia sinensis*. The embryos were induced from cotyledonary explants in MS medium containing 1 μM auxin ± 0.5 μM cytokinin. Number of plantlets (mean values from 5 replicates of 5 embryoids ± S.D) are shown.

PGRs (1 μM)	Agar concentration (%) 0.8	1.5	3	6
BM[#]	8 ±0.2b	7 ±0.1b	2 ±0.6e	*1 ±0.8f
IAN	5 ±0.8c	4 ±0.8d	2 ±0.8e	-
TPB	4 ±0.4c	2 ±0.2e	1 ±0.2f	-
PBOA	5 ±0.8c	3 ±0.1d	1 ±0.1f	-
GA3	-	-	-	-
Brassin	12 ±0.2a	9 ±0.6b	5 ±0.2c	3 ±0.2d

[#] Hormone free medium. * Anomalous plantlets with short axes and succulent leaves. -= No germination of embryo observed. Identical letters following the values indicate no significant difference according to Duncan's Multiple Range Test ($p<0.05$).

culture initiation showed typical bipolar organization (Fig. 1e). In earlier studies, histological observation of somatic embryogenesis in *C. sinensis* (Nakamura 1988; Kato1986) indicated that embryos developed directly from cotyledons without an intervening callus stage. The somatic embryos at late torpedo stage were isolated and cultured for 60 d in MS medium supplemented with various PGRs and agar concentrations for development and conversion into plantlets (Fig. 1f) (Table 2). MS basal medium with 0.8% agar and 1 µM BR was found to be optimal for embryo conversion (Table 2). Brassinolides have been shown to promote cell division, cell elongation and plant growth at very low concentration and also stimulated a wide variety of responses in bioassay systems for auxin, giberellin and cytokinin (Ikekawa 1991). Brassin, the compound used in this study, is a synthetic analog of the naturally occurring brassinolide (Maugh 1981). Brassinolide has been reported to be present in the leaves of tea (Ikekawa 1991). The positive influence of BR on embryo conversion in the present investigation may be due to the inherent sensitivity of the tissue to this compound. Medium with 6% agar yielded very poor conversion. The plantlets developed in the presence of either 3% or 6% agar were anomalous, in that their extension growth was suppressed and they showed thick succulent leaves without prominent root development (Fig. 1g). These plantlets did not develop further. In *C. japonica*, similar leaf anomaly has been observed in developing somatic embryos (Vieitez 1995).

The development of lateral roots was very poor in all the plantlets developed from the somatic embryos, irrespective of the treatments, and this greatly impeded plant recovery (data not shown). Conversely, the plantlets developed profuse lateral roots and showed shoot extension with 15 d incubation in liquid MS medium containing 1 µM IAN + 1 µM BR + 10 µM phloroglucinol. The plantlets were acclimated in pots containing vermiculite in the greenhouse (Fig. 1h). All acclimated plants were transferred to red acidic soil (pH 5.0) 200 d after culture initiation.

The present study describes a protocol for the regeneration of the UPASI-10 clone of cultivated tea via somatic embryogenesis. Immature cotyledon explants of the UPASI-10 tea clone showed a high degree of embryogenic potential via embryogenic callus proliferation. The somatic embryos readily converted into plantlets and a high frequency of plants were recovered. Also, we report the unique activity of TPB and PBOA, two novel auxins that enhanced the embryo induction rate. Moreover, somatic embryo development was promoted by BR. The repetitive embryogenesis observed in this study may be useful for biolistic gun mediated transformation. This regeneration system could also be employed in the production of somaclonal variants which could be evaluated for commercial use.

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