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Establishment of dedifferentiated callus of haploid origin from unfertilized ovaries of tea (*Camellia sinensis* (L.) O. Kuntze) as a potential source of total phenolics and antioxidant activity

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Abstract This is the first report of induction of haploid callus with significant antioxidant activity from unpollinated ovary cultures of tea. Out of the five cultivars tested, TV18 gave the highest percentage of callus induction. Within 1 wk of induction, ovules swelled to almost double their original size, and white, friable callus emerged. A high cytokinin/auxin ratio, provided by 8.5 μM benzyl adenine and 4.5 μM 2,4-dichlorophenxyacetic acid, and high-temperature treatment (33°C) for 10 d in the dark promoted maximum callus induction. Callus was maintained on MS medium containing 22.2 μM benzyl adenine and 9.8 μM indolebutyric acid (callus line RM 1) in the light at 25°C. Well-developed tracheids were formed within 4 wk in callus subcultured on MS medium containing 1.8 μM thidiazuron and 5.0 μM 2,3,5-triiodobenzoic acid (line RM 2). Flow cytometric analysis revealed that most cells were haploid. Both RM 1 and RM 2 produced phenolic compounds with significant antioxidant capacity. Phenolic content showed a positive linear correlation with antioxidant activity. The total phenolic content of RM 1 was 3.47±0.21 gallic acid equivalents (GAE) mg/g dry weight and that of RM 2 was 2.39±0.12 GAE mg/g dry weight. Antioxidant activity was measured using IC_{50}, a measure of inhibitory concentration; a lower IC_{50} value reflects greater antioxidant activity. The IC_{50} value of RM 1 was 2,530 μg/ml and that of RM 2 was 3,170 μg/ml. The results suggested that the phenolic compounds contributed significantly to the antioxidant capacity of the *in vitro* cell lines.

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Introduction

Camellia sinensis (L.) O. Kuntze (Theaceae), commonly referred to as tea, is an evergreen, perennial, cross-pollinating plant. It grows naturally as tall as 15 m; however, it is usually trimmed to below 2 m when cultivated for its leaves (Mondal *et al.* 2005). The cultivated taxa of *C. sinensis* comprise three main natural hybrids: *C. sinensis* (L.) O. Kuntze, or China type; *Camellia assamica* (Masters), or Assam type; and *Camellia assamica* ssp. *lasiocalyx* (Planchon ex Watt.), or Cambod or Southern type (Wight 1962).

Tea is one of the most highly consumed drinks throughout the world, second only to water (Heiss 2008). The Chinese were the first to use tea, initially as a medicinal drink and later as a beverage (Eden 1958). Tea is an important socio-economic crop that plays a major role in the economy of India. The tea industry in India contributes significantly towards the national and state economy by uplifting the foreign exchange pool and providing employment to a multitude of people. India ranks first as a producer, consumer, and exporter of tea, although it represents only about 16.4% of the total tea-growing area of the world (Mondal *et al.* 2004).

Tea consumption is associated with various health-promoting properties. Several studies over the past decade have demonstrated that tea is more than a mere stimulant and may owe its special medicinal properties to its high polyphenol content (Astill *et al.* 2001). Polyphenols account for about 25–35% of the total dry weight of fresh tea leaves; two-thirds of the polyphenol content is contributed by catechins (Saravanan *et al.* 2005). The antioxidant activity of

tea has been studied extensively, and numerous studies have been performed to assess the effects of tea polyphenols on the mutagenicity of carcinogens (Weisburger 1996). However, most studies on the antioxidant activity of tea have been limited to field-grown plants and diploid callus tissue of somatic origin (Shibasaki-Kitakawa *et al.* 2003; Saravanan *et al.* 2005; Chan *et al.* 2007; Yang *et al.* 2007; Khalaf *et al.* 2008). Haploid callus can be very important from a biochemical point of view because recessive mutations, which do not express themselves in the presence of their dominant alleles in heterozygous, cross-pollinating trees, can be easily expressed in haploid callus and plantlets. These mutations may prove to be beneficial for consistent yield of desired secondary metabolites, independent of seasonal and geographical variations. Haploids can be obtained by triggering callus development from either the young pollen grains/microspores in anther/microspore culture or the unfertilized cells of the female gametophyte (embryo sac) in floret or ovary/ovule culture to undergo sporophytic development (Bhojwani and Razdan 1996; Chaturvedi *et al*. 2003).

The objective of the present study was to develop a reproducible protocol for production of haploid cell lines from unfertilized ovaries of tea. To achieve this, ovary slice sections were cultured *in vitro*, the most effective media formulations were optimized, and the effects of genotype, embryo sac stage, sucrose concentration, and temperature pretreatments coupled with dark incubation were evaluated. Flow cytometric analysis was carried out to determine the ploidy of the callus. Total phenolic content and antioxidant activity of the established callus lines were also assessed because these features are important for the commercial application of this system.

Materials and Methods

Plant material and experimental design. Unopened 4–10 mm flower buds from five different cultivars of tea (TV1, TV7, TV18, TV19, and 317/1) were used to initiate *in vitro* ovary slice cultures. The source plants were approximately 45 yr old, growing in field in the germplasm collection center at Tocklai Experimental Station (Tea Research Association) Jorhat, Assam, India, and tissues were collected between December and February. The study was divided into two experiments, which were each repeated in 2008, 2009, and 2010. The first experiment dealt with optimization of media, evaluation of genotypic competence, and appropriate ovule developmental stage for induction of gynogenic callus *via* ovary slice cultures. Based on these results, the second experiment was carried out to assess the effect of temperature and dark pretreatments and sucrose concentration on gynogenic callus induction on the media compositions giving the greatest response. Finally, the ploidy level of the callus tissue was analyzed along with simultaneous determination of total phenolic content and antioxidant activity of the ovary-derived *in vitro* cell lines.

Establishment of aseptic cultures. Unfertilized flower buds were harvested early in the morning and surface sterilized with 0.1% $HgCl_2$ (Merck, India) solution for 7 min, followed by rinsing with sterile distilled water at least three times. Unfertilized ovaries of three different sizes (4, 6, and 8 mm) were harvested for the study. The 4- and 6-mm buds were collected 3 to 4 d before anthesis, and the 8-mm buds were collected 1–2 d before anthesis. The entire procedure was carried out in a laminar-air-flow cabinet. The ovaries were carefully removed and dissected into three transverse sections under a stereo microscope (Nikon, Japan) using presterilized Petri dishes, forceps, and fine needles. A total of six ovary slices of 1-mm thickness, obtained from two ovaries, were cultured on 60×15–mm presterilized, disposable Petri dishes (Tarsons, India) containing 10 ml MS (Murashige and Skoog 1962) medium supplemented with growth regulators, as discussed below.

Culture medium. For callus induction, MS medium was supplemented with varying concentrations of the auxins [2,4-dichlorophenoxyacetic acid (2,4-D) and α-naphthalene acetic acid (NAA)] and cytokinins [N^6-benzyladenine (BA) and N^6-furfurylaminopurine (kinetin)], either alone or in combination with additives such as L-glutamine and L-serine. All growth regulators and additives were purchased from Sigma Aldrich Co. (St. Louis, MO). Sucrose and glucose (Merck, Mumbai, India) concentrations in the medium ranged from 30 to 90 g/l, and all media were solidified with 0.8% agar (Himedia, Mumbai, India). The pH of the medium was adjusted to 5.8 with 0.1 N NaOH or 0.1 N HCl before autoclaving at 1.06 kg cm^2 pressure and 121°C for 15 min. Out of 16 media compositions assessed, only three were capable of initiating callus response in the cultured unfertilized ovary slice sections (Table 1).

Table 1. Compositions of media producing callus induction from unfertilized ovary slice cultures of *Camellia sinensis* (L.) O. Kuntze

	Medium A	Medium B	Medium C
Basal medium	MS	MS	MS
Sucrose (g/l)	90	–	30
Glucose (g/l)	–	30	–
BA (μM)	5.0	–	8.5
Kinetin (μM)	–	5.0	–
2,4-D (μM)	1.0	5.0	4.5
NAA (μM)	1.0	–	–
Glutamine (g/l)	–	0.8	–
Serine (g/l)	–	0.2	–

Culture conditions. The cultures on each medium were subjected to pretreatment conditions such as cold shock (4°C) and heat shock (33°C) under dark incubation; 25°C dark incubation served as a control in all the experiments. At the same time, the effect of duration of temperature shocks of 5, 10, and 15 d was also evaluated. To perform these experiments, ovary slice sections were cultured on the optimized induction media identified as described above (see "Results and Discussion"; Table 1). After treatment, the cultures were maintained continuously at 25°C±2°C with 50–60% relative humidity under 16-h photoperiod irradiance (1,000–2,000 lx) provided by cool daylight fluorescent tubes (Philips TL 40W). The cultures were examined periodically, and morphological changes were assessed visually. The results were expressed as the percentage of responding cultures.

Histological analysis. Plant materials were fixed in FAA (5:5:90 *v*/*v*/*v* formaldehyde/acetic acid/70% ethanol) for 48 h and then preserved in 70% ethanol. The material was passed through a tertiary butyl alcohol series for dehydration, infiltrated with paraffin wax (Merck, Darmstadt, Germany), and embedded in pure paraffin wax. The paraffin blocks were mounted on wooden stubs, and 8-μm-thick sections were cut using a manual rotary microtome (Leica, Germany). The sections were mounted on microscope slides, dewaxed and double-stained with safranin (1%) and astra blue (1%), and examined under the light microscope (Nikon, Tokyo, Japan).

Flow cytometric analysis. Well-developed callus obtained 2 m after culture initiation was used for ploidy analysis. Extraction and analysis of nuclei were carried out by finely chopping callus placed in 1 ml ice-cold woody plant buffer according to the protocol of Loureiro *et al.* (2007) with slight modifications. The modified woody plant buffer was prepared by mixing 0.2 M Tris×HCl, 4 mM $MgCl_2 \times 6H_2O$, 2 mM EDTA-$Na_2 \times 2H_2O$, 86 mM NaCl, 10 mM sodium metabisulfite, 1% Triton X-100 (*v*/*v*) (all from Merck, Mumbai, India), and 2% polyvinlypyrrolidone (PVP)-10 (*w*/*v*) (Sigma). The pH of the buffer was adjusted to 7.5; then, the buffer was filtered through 0.22-μm polyvinylidene fluoride membrane filter (Pall Corporation, Mumbai, India) and stored at 4°C. The suspension containing the nuclei was mixed by pipetting up and down several times and then filtered through a 30-μm nylon mesh (Millipore, Mumbai, India). The nuclear suspension was stained with 50 μg/ml propidium iodide (Sigma). At the same time, 50 μg/ml RNAse (Sigma) was added to the nuclei (Dolezel *et al.* 2007). The ploidy level was determined using a FACS Calibur cytometer (Becton-Dickinson, Franklin Lakes, NJ). All measurements were carried out in triplicate using fresh *C. sinensis* leaves as an external standard. Using instrument gain (photomultiplier voltage and amplitude gain), the position of peak G_1 nuclei of the reference sample was established at channel 200 on a 1,024-channel scale, after which the instrument settings were kept constant and the unknown samples were run under the same parameters. The mean channel number of the unknown sample G_1 peak was determined, and the DNA ploidy was calculated according to the following relationship:

$$\text{Sample ploidy(integer)} = \text{reference ploidy} \times (\text{mean position of the } G_1 \text{ sample peak} / \text{mean position of the } G_1 \text{ reference peak.})$$

Estimation of total phenolics and free-radical scavenging assay. For preparation of methanolic extracts, the air-dried callus tissue was soaked in methanol (200 ml) for 48 h, after which the tissue was sonicated in an ultrasonic processor (Sonics & Materials Inc., Newtown, CT) for 40 min at 30% amplitude (pulser, 5 s on/off). After filtration, the methanolic extract was centrifuged at 10,000 rpm for 10 min. The supernatant was pooled, filtered, and dried in a rotary vacuum evaporator (Buchi Rotavapor R-200, Tokyo, Japan) at 40°C.

The total phenol content was determined spectrophotometrically according to the Folin–Ciocalteau colorimetric method (Slinkard and Singleton 1977) using gallic acid (Sigma) as a standard polyphenol. Folin–Ciocalteau reagent and methanol were purchased from Merck, India. All reagents used were of analytical grade. The extract (20 μl) was mixed with 1.58 ml water and 100 μl Folin–Ciocalteau reagent. After 3 min of incubation, 300 μl of 20% Na_2CO_3 (Merck, India) was added. The reaction mixture was incubated at 40°C for 30 min, and the absorbance was measured at 765 nm using a spectrophotometer (Labomed Inc, Culver City, CA). The concentration of total phenolics was expressed as gallic acid equivalents (GAE) (mg/g dry weight).

The free-radical scavenging activity of the different cell lines of *C. sinensis* was analyzed using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Braca *et al.* 2001; Saha *et al.* 2004; Boskou *et al.* 2006). Ascorbic acid, DPPH, gallic acid, and vanillic acid were purchased from Sigma. The antioxidant assay is based on the measurement of the loss of DPPH free-radical color by the change in absorbance at 517 nm caused by the reaction of DPPH with the tested samples. Diluted working solutions of the test extracts were prepared in methanol. Stock concentrations (1 mg/ml) of gallic acid, ascorbic acid, and vanillic acid were used as standards. For the reaction mixture, 1 ml of DPPH solution was mixed with 1 ml sample solution, the solution was incubated in the dark for 30 min, and the optical density was measured at 517 nm. The reaction was measured by an untraviolet–visible double-beam spectrophotometer (Labomed). The determinations were performed

in triplicate. The percent inhibition of DPPH was calculated from the decrease in absorbance according to the following relationship:

$$\%\,\text{inhibition} = \left[\left(A_{\text{Blank}} - A_{\text{Sample}}\right)/A_{\text{Blank}}\right] \times 100$$

where A_{Blank} is the absorbance of the control reaction (methanol–water with DPPH), and A_{Sample} is the absorbance of the tested sample. The antioxidant activity of the extracts was expressed as IC_{50}, which is defined as the concentration in micrograms per milliliter of extract that inhibits the formation of DPPH radicals by 50%. Linear regression analysis was used to calculate IC_{50} values.

Statistical analysis. To evaluate the effect of temperature and dark pretreatments on gynogenic callus induction, a completely randomized experimental design was followed. All data were analyzed using SPSS 16.0 software and Microsoft Excel. Data were analyzed by one-way ANOVA, followed by the separation of mean values using a post hoc Duncan's multiple range test (DMRT). Differences for which $p \leq 0.05$ were considered significant. For ANOVA, the data were divided into three experiments (three replicates) conducted over 3 yr (three flowering seasons) in 2008, 2009, and 2010. Each year, the mean of the data generated from four Petri dishes was taken to represent each treatment. There were 72 observations in total (6 ovary sections × 4 dishes × 3 experiments) for each treatment. The final response is expressed as the percentage of responding ovary sections. For the biochemical studies, the readings are either represented as percentage values or taken in triplicate and presented as mean±SD.

Results and Discussion

Effect of genotype on gynogenesis. All five tea cultivars tested (TV1, TV7, TV18, TV19, and 317/1) were responsive to ovary culture on media A, B, and C (Table 1) under dark incubation; however, the response varied greatly among both cultivars and media types (Fig. 1). This is consistent with other reports of differences in gynogenic response among genotypes and species (Chen *et al.* 2011). Based on the two-way ANOVA analysis, genotype had a significant effect ($p \leq 0.05$) on *in vitro* gynogenic callus induction but medium did not (Table 2). Out of all the cultivars selected for the study, TV18 gave the highest and most consistent results in terms of callus formation either from intact ovules or from inside ovules that had burst open. The least responsive genotype was TV7. In subsequent experiments, evaluation of factors that affected gynogenic response was carried out with TV18 alone.

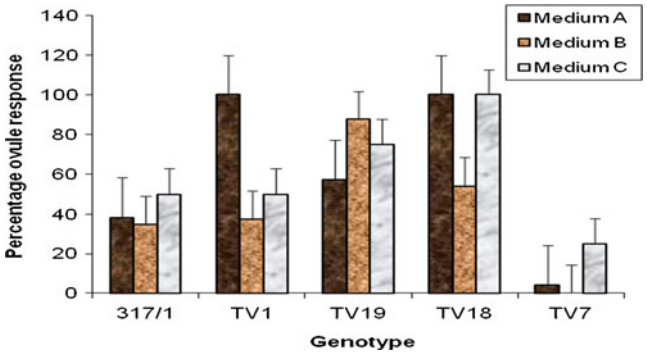

Figure 1. Effect of genotype and medium composition on ovule response under dark incubation. Media compositions are given in Table 1.

Effect of ovule developmental stage. In the present study, buds of three different sizes were assessed: 4, 6, and 8 mm. The 4- and 6-mm buds corresponded to 3–4 d before anthesis, and no response was observed when these ovules were cultured. Only the ovules from 8-mm buds, collected 1–2 d before anthesis, responded by formation of callus on all three media compositions (Table 1) and under all treatment conditions. The histological analysis of flower buds collected 1–2 d before anthesis and fixed at the time of culture revealed the presence of a mature embryo sac with a mature, receptive egg cell at the micropylar end (Fig. 2*a–c*). The egg cell is slightly larger than the synergid and can be easily differentiated as it appears regular in form and structure, with a large nucleolus, whereas synergid nuclei are normally smaller, denser, and irregular in form and contain small nucleoli or none at all (Tsou 1997). The presence of a distinct synergid and antipodal cells was also observed in the embryo sac of the tea ovules. Although it has been reported that the embryo sac of *C. sinensis* is 8-nucleate, not all of the cells were visible on the same plane in the same microtome section. This may be because the egg cell is placed chalazally to the synergid cells, as has been reported in angiosperms (Wu 1962; Kapil and Sethi 1963; Watson and Dallwitz 1992; Tsou 1997). Moreover, the synergid cells are similar in size to the egg cell and are placed in such a manner that they surround the egg cell, where they providing nutrition to the egg and direct the sperm cells to the specific site for fertilization to occur (Russell 1993). After 2 wk of ovary culture, the antipodal cells had degenerated, the synergid cells had shrunk, and the egg cell had enlarged and become multicelled (Fig. 2*d*).

Optimization of growth regulator combinations for in vitro callus induction. Out of 16 media combinations tested, only the three media compositions (Table 1) induced gynogenic response in the cultured ovary slice sections. MS medium devoid of any growth regulator did not induce any response. All three media compositions induced initial swelling of

Table 2. Two-way ANOVA showing the effects of genotype and medium composition on *in vitro* gynogenesis in tea

Source of variation	SS	*df*	MS	*F*	*p* value
Genotype	6,649.735	4	1,662.434	5.174003	0.023467
Medium	946.7552	2	473.3776	1.473296	0.285262
Residual	2,570.441	8	321.3051		
Total	10,166.93	14			

SS sum of squares, *df* degrees of freedom, *MS* mean square

ovules and subsequent callus initiation, but the frequency of gynogenic callus induction varied with the type and concentration of growth regulators used. Medium C, which contained 8.5 µM BA and 4.5 µM 2,4-D, gave the best results among the three media, as it promoted 62.5% callus induction from the ovules under 25°C dark incubation (Table 3). This is in agreement with other reports in which a combination of auxin and cytokinin promoted gynogenesis in *Allium* spp. (Alan *et al.* 2003), *Eragrostis tef* (Gugsa *et al.* 2006), *Vitis labruscana* (Nakajima *et al.* 2000), *Guizotia abyssinica* (Bhat and Murthy 2007), and *Morus alba* (Thomas *et al.* 1999).

Gynogenic callus induction was characterized by swelling of ovules within 1 wk of culture and emergence of callus tissue from within the ovules after 4 wk (Fig. 3*c–f*). The callus tissue was white, light brown to light green, and initially friable (Fig. 3*g, h*), but gradually turned green and nodulated when transferred to light conditions (Fig. 3*i*).

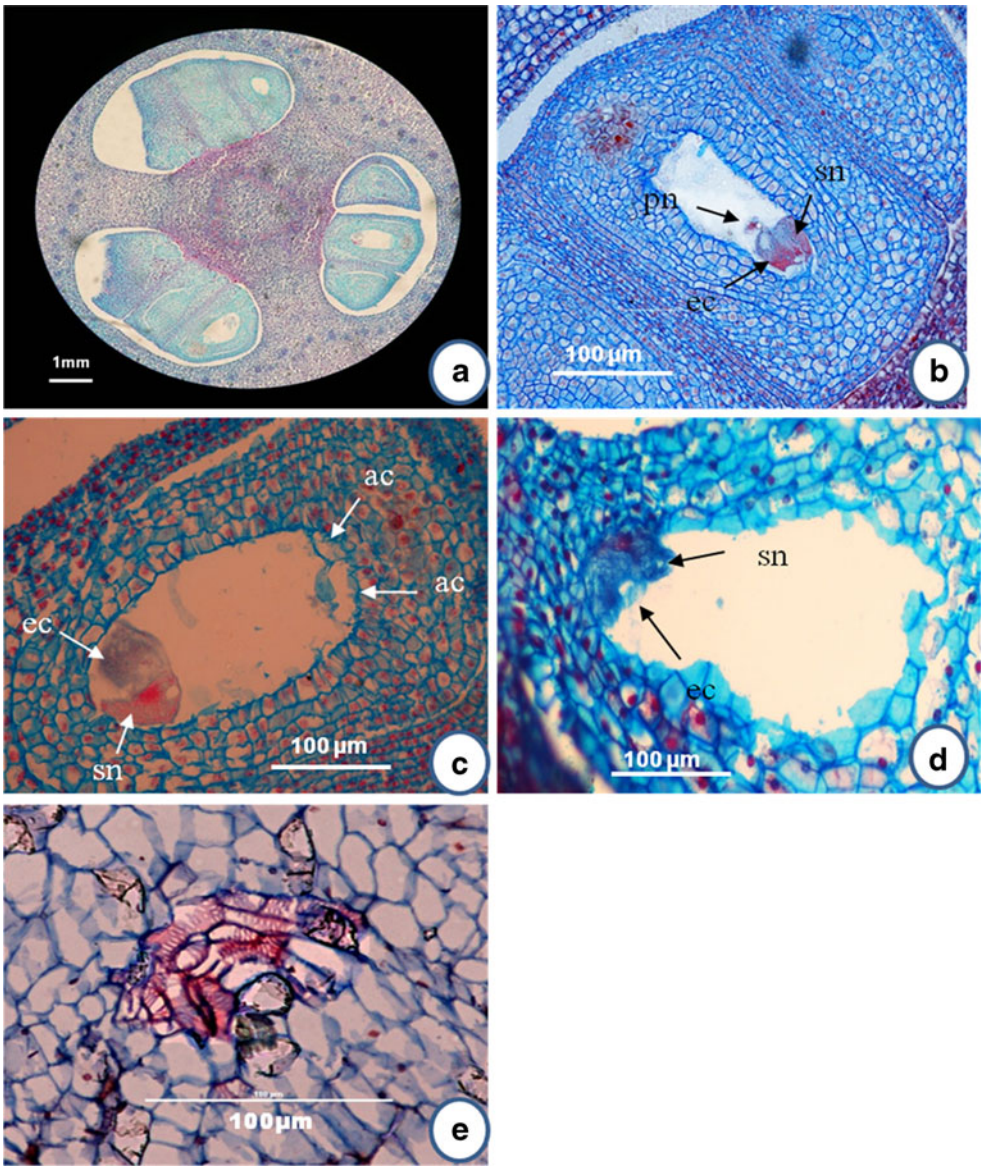

Figure 2. Histology of cultured ovules and callus tissue derived from ovules of tea cultivar TV18 stained with astra blue and safranin. (*a*) Transverse section of an ovary fixed at the time of culture showing ovules with mature embryo sacs; note that the ovules are at different stages of development. (*b*, *c*) Longitudinal section of an ovule with a mature embryo sac; note the *arrowheads* showing an egg cell (*ec*), polar nucleus (*pn*), synergid cell (*sn*), and antipodal cells (*ac*). (*d*) Embryo sac after 2 wk of culture; note that the synergid cell has shrunk while the egg cell has enlarged and become multicelled. (*e*) 24-wk-old callus tissue subcultured on MS+1.8 µM TDZ+5.0 µM TIBA for 8 wk, showing formation of tracheids. *Bars*= 1 mm (*a*); 100 µm (*b–e*).

Table 3. Effect of temperature and treatment duration on ovule response in *Camellia sinensis* (L.) O. Kuntze cv. TV18 under dark incubation

Temperature	Treatment duration	Medium A (%)	Medium B (%)	Medium C (%)
25°C dark (control)	Continuous	41.67[a]	41.67[a]	62.5[bc]
4°C dark (cold shock)	5 d	12.50[ab]	37.50[a]	33.33[d]
	10 d	16.67[ab]	16.67[ab]	58.33[bc]
	15 d	0.00[b]	25.0[ab]	33.33[d]
33°C dark (heat shock)	5 d	20.83[ab]	0.00[b]	66.67[ab]
	10 d	20.83[ab]	0.00[b]	91.67[a]
	15 d	0.00[b]	25.0[ab]	50.00[cd]

Means followed by the same *letter* are not significantly different at the 5% level according to Duncan's multiple range test (DMRT)

After successful induction, the callus tissue was maintained on MS+22.2 μMBA+9.8 μM IBA, which served as the maintenance medium. The maintenance medium promoted sustained and fast proliferation of green, nodulated callus (Fig. 3*i*). In tea culture, the addition of BA and IBA to the culture medium is suitable for both initiation and multiplication of shoots (Mondal *et al.* 2004) and germination of *Camellia japonica* somatic embryos (Vieitez and Barciela 1990; Pedroso and Pais 1993).

On medium containing 1.8 μM TDZ and 5.0 μM antiauxin 2,3,5-triiodobenzoic acid TIBA), the callus formed compact nodules; histological investigation revealed the presence of well-formed tracheids in the callus tissue (Fig. 2*e*). The cytokinin TDZ stimulates adventitious shoot-bud formation in a number of species and especially, woody plants (Niedz *et al.* 1989). Huetteman and Preece (1993) reported that the concentrations at which TDZ is most effective are 10–1,000 times lower than for other plant growth regulators in woody plants. TDZ appeared to be a potent cytokinin for micropropagation of *C. sinensis*, which is a woody, perennial plant with high proliferation rates (Mondal *et al.* 1998). The use of cytokinins along with TIBA has yielded positive results in several woody plant species. In mulberry, the counteracting of endogenous auxins by the application of exogenous cytokinins (BA and TDZ) and blockage of endogenous auxin influx from the neighboring tissues by TIBA promoted direct shoot regeneration from the basal tissues of leaves (Sugimura *et al.* 1998; Singh and Syamal 2000). All these reports clearly support our finding that TDZ and TIBA proved to be a suitable combination for inducing initiation of vascularization in the tea ovary callus.

Effect of temperature pretreatments coupled with dark incubation. Temperature shocks improve gynogenesis by diverting normal gametophytic development into a sporophytic pathway leading to the formation of haploid embryos (Shalaby 2007). The exact mechanisms that act on plantlet formation following temperature pretreatments are still unknown. The shock provided by low or high temperature probably establishes cellular conditions that are important for switching from the gametophytic to sporophytic developmental pathway. For different explants, the optimum types, levels, and durations of pretreatment are different and the regeneration efficiencies also vary (Chen *et al.* 2011). Dark incubation favors gynogenesis and minimizes somatic callusing (Bhagyalakshmi 1999; Gemes-Juhasz *et al.* 2002).

In the present study, the effects of heat shock (33°C) and cold shock (4°C) treatments under dark incubation for 5, 10, and 15 d were evaluated for cultivar TV18. Two distinct types of gynogenic response were observed upon application of temperature shock treatments. Application of cold shock treatment at 4°C promoted both callus induction from the ovules (Fig. 3*d*) and formation of some globular structures in a few ovary slice sections on medium B that failed to differentiate further (Fig. 3*j*). Heat shock (33°C) promoted the bursting of ovules and emergence of callus tissue from within (Fig. 3*e, f*). No callusing from the carpellary wall was observed.

On medium A, the maximum gynogenic response was 41.67% under control (25°C) dark conditions, and neither cold shock nor heat shock improved the overall response. Similar was the case with medium B, where 41.67% of the cultures responded. Only on medium C did the application of heat shock improved gynogenic response in the cultured ovary slice sections. Application of heat shock (33°C) for 10 d to the cultured ovary sections induced 91.67% callus initiation in comparison to 62.5% callus induction under control (25°C) dark conditions. On the same medium, cold shock treatment for 10 d induced 58.33% callus induction from the cultures. The duration of treatment also affected *in vitro* gynogenic response. For all three media compositions, 10 d of temperature pretreatment (whether heat or cold shock) was optimum for inducing ovule response in the cultured ovary sections. Temperature pretreatment for 15 d was least favorable for inducing ovule response, with exception of medium B. The application of heat treatment promoted bursting of ovules and emergence of callus tissue from within (Fig. 3*e*, *f*). Cold shock treatment promoted callusing from ovules, but the frequency was less than under heat shock treatment.

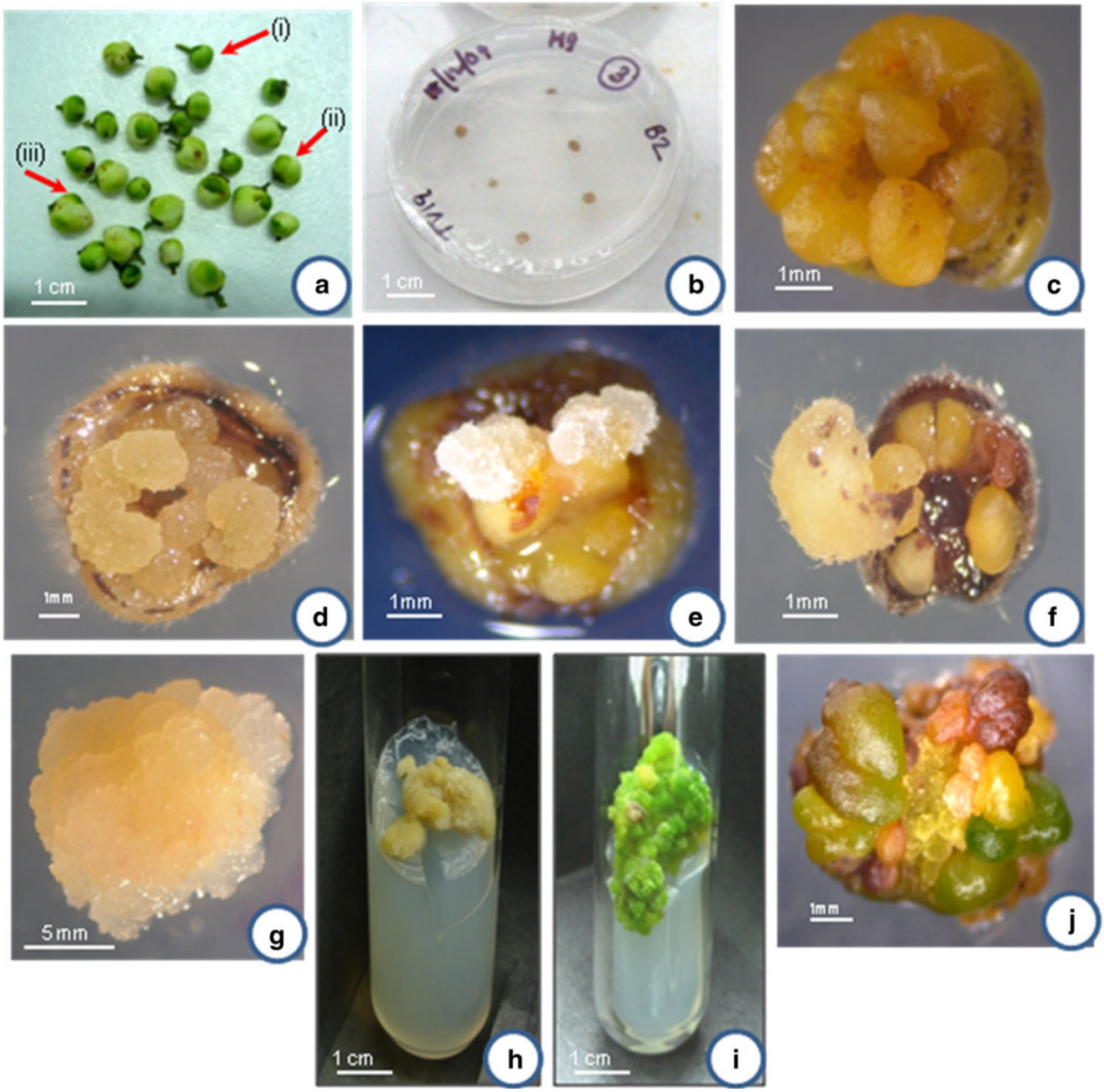

Figure 3. (*a*) Unfertilized flower buds of *Camellia sinensis* (L.) O. Kuntze "TV18" at the time of culture. Buds labeled (*i*) are 4 mm, (*ii*) are 6 mm, and (*iii*) are 8 mm. (*b*) Petri dish containing transverse sections of ovary cultured on MS medium (with 3% sucrose) supplemented with 8.5 µM BA+4.5 µM 2,4-D. (*c*) Same, 1 wk after culture, showing swollen ovules. (*d*) An 8 wk-old ovary slice section from (*b*), showing callused ovules subjected to cold shock treatment (4°C dark for 5 d); note that callusing has occurred only from the ovules and not from the outer carpellary wall of the ovary. (*e*, *f*) 8-wk-old ovary slice section from (*b*), subjected to heat shock treatment (33°C dark for 10 d) showing bursting of ovules and emergence of callus tissue from within. (*g, h*) Callus tissue obtained from within ovules; note that the callus is white, light brown to light green, and friable. (*i*) Green, nodulated callus tissue subcultured on MS medium supplemented with 1.8 µM TDZ+5.0 µM TIBA. (*j*) 12-wk-old ovary slice section subjected to cold shock treatment (4°C light for 10 d) on MS+5.0 µM 2,4-D+ 5.0 µM kinetin+0.8 g/l glutamine+0.2 g/l serine, showing formation of some globular structures that failed to differentiate further; note the formation of some green, loosely attached structures. *Bars*=1 cm (*a*, *b*, *h, i*); 5 mm (*g*); 1 mm (*c–f*, *j*).

Effect of sucrose concentration. MS medium supplemented with sucrose at five concentrations (3%, 6%, 9%, 12%, and 15%) was used along with 8.5 µM BA and 4.5 µM 2,4-D to test the response of cultivar TV18 to sucrose concentration. Sucrose concentration significantly affected gynogenic response in cultured ovary slice sections (Fig. 4): 9% sucrose

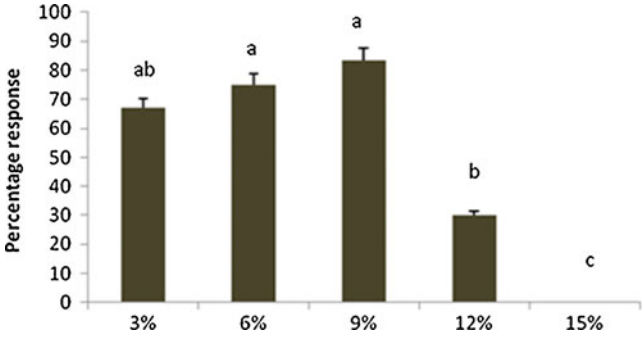

Figure 4. Effect of sucrose concentration on ovule response under dark incubation. Ovules were cultured on MS medium supplemented with 8.5 μM BA and 4.5 μM 2,4-D and sucrose concentrations from 3% to 15%. Within 1 wk of culture the ovules were swollen, and emergence of callus tissue from within the ovules was observed after 4 wk. Means followed by the same *letter* are not significantly different at the 5% level according to Duncan's multiple range test (*DMRT*). Error bars indicate SD.

promoted the highest gynogenic response (83.33%) among the sucrose concentrations tested. Increasing the sucrose concentration from 3 to 9% promoted a gradual increase in the percentage ovule response. However, at 12% sucrose concentration, there was a marked decrease in ovule response, and at 15% sucrose, the response was nearly zero (Fig. 4). For inducing ovule response, sucrose is generally supplied at 2–3% concentration. It has been observed in several species that an increase in sucrose concentration can lead to beneficial morphogenic potential (Sopory and Munshi 1996; Agarwal *et al.* 2006) by suppressing the proliferation of somatic tissues (Ouyang *et al.* 1973). High sucrose concentration (8–10%) in the culture medium is helpful in sweet potato (Kobayashi *et al.* 1993) and onion (Campion *et al.* 1992). The sucrose concentration in the induction medium affects osmosis, which in turn influences the development of embryos (Wakizuka and Nakajima 1975).

Ploidy analysis. Tea contains high levels of secondary metabolites; therefore, a suitable woody plant buffer containing 2% PVP was devised according to the method of Loureiro *et al.* (2007), to perform flowcytometric analysis. The analysis revealed that the control (leaf) samples were diploid (Fig. 5*a*), whereas most of the tested callus cells from *in vitro* gynogenic cell lines of *C. sinensis* were haploid (1*n*=1*x*=15) (Fig. 5*b*), indicating that the callus had originated from haploid tissues. This is further supported by the histological evidence that the egg cell was intact at the time of culture (Fig. 2*a–c*), and after 2 wk of culture, the synergid cell had shrunk while the egg cell had enlarged and become multicelled (Fig. 2*d*).

Total phenolic content and antioxidant activity of the callus cell lines. The total phenol content was estimated for two haploid TV18 cell lines: RM 1, maintained on MS+22.2 μM BA+9.8 μM IBA, and RM 2, maintained on MS+1.8 μM TDZ+5 μM TIBA. Fresh *C. sinensis* leaves collected from field-grown TV18 shrubs were used as the control. Growth regulators are very crucial in secondary product formation (Duangporn and Siripong 2009). The type and concentration of auxin and cytokinin or the auxin/cytokinin ratio may dramatically alter both growth and product formation in cultured plant cells (Mantell and Smith 1984). TV18 leaf samples (control) yielded 55.28% extract, compared with 1.87% and 4.15% extract yield from callus cell lines RM 1 and RM 2, respectively (Table 4). These extracts were dissolved in methanol and analyzed for total phenolic content. The total phenolic content of tea leaf (control) was estimated to be 7.50±0.18 GAE mg/g dry

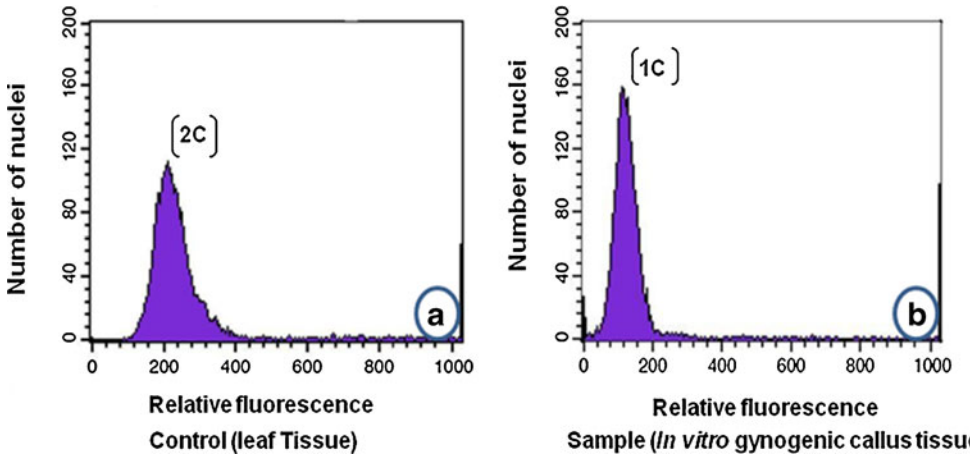

Figure 5. Flow cytometric study of callus derived from tea ovary; note that the fluorescence pulse was measured on a 1,024-channel analyzer. Results represent measurements of 10,000 individual nuclei. (*a*) Mature leaves of field-grown cultivar TV18 were used as a diploid control after staining with propidium iodide. Reference peak 1 was obtained at channel 200. (*b*) Peak 2 corresponds to the histogram of fluorescence intensity of nuclei showing haploid DNA content after staining with propidium iodide. Note the peak at channel 100.

Table 4. Total phenolic content and free-radical scavenging activity of TV18 callus cell lines

Sample	Extraction yield (%)	Total phenolic content as GAE mg/g dry weight[z]	IC_{50} (μg/ml)
Gallic acid	–	–	4.78
Ascorbic acid	–	–	9.18
Vanillic acid	–	–	4,120
Leaf (control)	55.28	7.50±0.18	5.95
RM 1	1.87	3.47±0.21	2,530
RM 2	4.15	2.39±0.12	3,170

GAE gallic acid equivalent

[z] Mean±SD

weight, compared with 3.47±0.21 and 2.39±0.12 GAE mg/g dry weight for RM 1 and RM 2, respectively (Table 4). The determination of antioxidant activity of the leaf sample (control), RM1, and RM2 cell lines was carried out by the DPPH method, which is based on the reduction of alcoholic DPPH solution in the presence of a hydrogen-donating antioxidant; this reaction causes the formation of the non-radical form DPPH-H (Gulcin 2006). Gallic acid, ascorbic acid, and vanillic acid were used as internal standards. Since IC_{50} is a measure of inhibitory concentration, a lower IC_{50} value reflects greater antioxidant activity of the sample. The IC_{50} value of leaf sample (control) was 5.95 μg/ml, suggesting that its antioxidant activity was higher than that of ascorbic acid or vanillic acid (Table 4). RM 1 and RM 2 showed very weak antioxidant activity (IC_{50} values of 2,530 and 3,170 μg/ml, respectively) in comparison to that of the leaf sample (control). These results showed that there was a positive linear correlation between antioxidant activity and total phenolic content, which suggested that the phenolic compounds contributed significantly to the antioxidant capacity of the *in vitro* cell lines. These results were consistent with the findings of many research groups who reported such positive correlation between total phenolic content and antioxidant activity in various plant species (Zheng and Wang 2001; Cai *et al.* 2004). However, both the *in vitro* ovary cell lines showed antioxidant activity comparatively higher than that of vanillic acid. Matkowski (2008) discussed that a close association existed between the expression of secondary metabolites and morphological and cytological differentiation in cultures. This is clearly supported by the present findings that secondary products are normally found in highly differentiated parts of plants and constitute an important aspect of cellular differentiation (Yeoman 1987). Both RM 1 and RM 2 were dedifferentiated callus cell lines, so it may be possible that the intricate metabolic changes that accompany the process of cellular differentiation in plants also initiate biosynthetic pathways resulting in the accumulation of phenolics.

Conclusions

The present study indicated that ovary slice sections of *C. sinensis*, consisting of unpollinated ovules with mature embryo sacs, can be efficiently used for gynogenic haploid callus induction. The dedifferentiated haploid callus tissue can be used as a potential source of phenolics with measurable antioxidant activity.

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