In Vitro Cell.Dev.Biol.—Plant (2017) 53:239–248
DOI 10.1007/s11627-017-9811-z

PLANT TISSUE CULTURE

An efficient and reproducible method for development of androgenic haploid plants from *in vitro* anther cultures of *Camellia assamica* ssp. *assamica* (Masters)

Vijay Kumar Mishra[1] · **Ruchira Bajpai**[1] · **Rakhi Chaturvedi**[1]

Received: 27 October 2016 / Accepted: 2 March 2017 / Published online: 3 April 2017 / Editor: Wenhao Dai
© The Society for In Vitro Biology 2017

Abstract This study describes the development of haploid plantlets through androgenesis in *Camellia assamica* ssp. *assamica* (tea). Androgenic haploid embryos were produced through callus formation from microspores during the early-to-late uninucleate stages in anther cultures. A high percentage of callus induction (96%) was obtained on Murashige and Skoog's (MS) medium containing 6% (*w*/*v*) glucose, supplemented with 5 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 5 μM 6-furfurylaminopurine (kinetin), 800 mg L^{-1} L-glutamine, and 200 mg L^{-1} L-serine (callus induction medium). Further proliferation of callus occurred when glucose was replaced with 3% (*w*/*v*) sucrose in the medium. Embryogenesis was achieved in 85% of the androgenic callus cultures on MS medium containing 10 μM 6-benzylaminopurine (BAP), 3 μM gibberellic acid (GA_3), 800 mg L^{-1} L-glutamine, and 200 mg L^{-1} L-serine (embryo induction medium). Maturation of embryos occurred when the concentration of the growth regulators and adjuvants contained in the embryo induction medium were reduced by tenfold. Embryos germinated into complete plantlets in 65% of the cultures when the MS medium was supplemented with 10 μM BAP, 1 μM indole-3-butyric acid (IBA), 0.5 μM GA_3, 80 mg L^{-1} L-glutamine, and 20 mg L^{-1} L-serine. These plantlets continued to grow when the major salt concentration in the embryo germination medium was reduced by half (1/2 MS). The chromosomal constitution of these *in vitro* plantlets was confirmed as $2n = X = 15$ by cytological squash preparation of the root tips. Flow cytometric analysis of leaves from these *in vitro* plantlets confirmed the ploidy status as haploid. This study is an effort to overcome the inherent heterozygosity in tea.

Keywords Anther culture · Androgenesis · *Camellia assamica* · Embryogenesis · Haploid

Introduction

Tea, *Camellia assamica* ssp. *assamica* (Masters) (family *Theaceae*), is an important commercial crop, which is consumed daily by two thirds of the population worldwide (Mondal *et al*. 2004). It is a socio-economic crop and plays an important role in the economic development of tea-growing countries by increasing foreign exchange. Although India contributes only 16.4% of the total tea growing area of the world, it has been recorded among the leading tea-producing countries (Hazarika and Chaturvedi 2013). Tea occurs naturally as a cross-breeding plantation crop throughout Southeast Asia (Mondal *et al*. 2004). The cultivated taxa of tea are comprised of three main hybrids, which are classified on the basis of leaf size. *Camellia sinensis* (L.) O. Kuntze (China type) bears the smallest leaves, *C. assamica* ssp. *assamica* (Masters) (Assam type) bears the largest leaves, and *C. assamica* ssp. *lasiocalyx* (Planch MS) (Cambod type) carries intermediate-sized leaves (Sharma *et al*. 2010). The extensive cross-pollination within species of tea produces highly heterozygous plants with long gestation periods, which poses a hurdle in the production of elite clones or pure breeding/homozygous lines (Mondal *et al*. 2004). Lack of pure breeding lines in woody plants like tea restricts its genetic improvement (Germana 2003). In plants where conventional breeding methods seem impractical as a means to obtain homozygous lines, production of haploid plants using *in vitro* anther/microspore and ovary/ovule culture methods are

✉ Rakhi Chaturvedi
rakhi_chaturvedi@iitg.ernet.in; rakhi_chaturvedi@yahoo.co.uk

[1] Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati 781039, Assam, India

potentially beneficial to establish pure breeding lines (Chaturvedi *et al*. 2003). This could be accomplished in a single step by diploidization of haploids using anti-mitotic agents (Mishra and Chaturvedi 2014). Furthermore, haploid plant production in tea besides being an important tool in tea for genetic research, breeding, and improvement would also help establish genetic stability within the selected clones in a shorter period of time (Evans *et al*. 2003) and assist in overall improvement of this recalcitrant tree. The present study provides a reproducible protocol for developing complete haploid plantlets through androgenesis in *C. assamica* ssp. *assamica* by *in vitro* anther cultures.

Materials and Methods

Plant material and sterilization protocol Healthy, unopened flower buds of Tocklai vegetative (TV21) cultivars of *C. assamica* ssp. *assamica* (Masters) were handpicked between 6.00 and 7.00 AM, during the flowering season, in the months from October to December from field-grown donor plants at the germplasm collection center at the Tocklai experimental station (Tea Research Association) Jorhat, Assam, India. The flower buds, measuring 4 mm in size, with anthers bearing microspores at the early-to-late uninucleate stage, were selected for raising the cultures (Fig. 1*A*–*C*). The collected flower buds were surface sterilized using 50 mL of 0.8% (*v*/*v*) sodium hypochlorite (NaOCl, Merck, Mumbai, India) solution per 15 flower buds, for 7 min, followed by thorough rinsing with sterile distilled water 3–4 times. All of the steps above were performed in a laminar-air-flow cabinet (Saveer Biotech Limited, New Delhi, India). Stages of the microspores were analyzed using 2% (*w*/*v*) aceto-carmine dye (Sigma-Aldrich®, St. Louis, MO) (Srivastava and Chaturvedi 2011). Isolation of the anthers from the flower buds was completed with a stereo-microscope (Nikon, Tokyo, Japan) using 60 × 15 mm pre-sterilized Petri plates (Tarsons Products Pvt. Ltd., Kolkata, India), forceps (12.7-cm length), and fine needles (12.7-cm length) from HiMedia®, Mumbai, India. Any damaged anthers were discarded and the filaments were gently removed. Twenty anthers from a bud (Fig. 1*D*) were cultured in 60 × 15-mm pre-sterilized, disposable Petri plates (Tarsons Products Pvt. Ltd.) with 10 mL of Murashige and Skoog's (MS) medium (described below) either with or without growth regulators. The plates were sealed with Parafilm™ (Tarsons Products Pvt. Ltd.) and incubated as described in the subsequent sections.

Establishing anther cultures and callus induction Anther cultures were raised on MS medium (Murashige and Skoog 1962), supplemented with auxins, including 2,4-dichlorophenoxyacetic acid (2,4-D), α-naphthaleneacetic acid (NAA), and cytokinins, including 6-benzylaminopurine (BAP), 6-furfurylaminopurine (kinetin), and thidiazuron (TDZ), at different concentrations along with additional nitrogen sources of L-glutamine (gln) and L-serine (ser). All growth regulators and additives were purchased from Sigma-Aldrich® Co. Two carbon sources, glucose and sucrose, at different concentrations ranging from 3 to 12% (*w*/*v*) were used for media optimization. All media were solidified with 0.8% (*w*/*v*) bacteriological agar (HiMedia®, Mumbai, India). The pH of the medium was adjusted to 5.8 with 0.1 N NaOH or 0.1 N hydrochloric acid (HCl) before sterilizing in an autoclave at 103.4 kPa pressure and 121°C for 15 min.

Entire flower buds or the anthers in culture were exposed to temperature pre-treatments of cold shock (5°C) or heat shock (33°C), under complete dark incubation for 0 and 5 d. Cultures kept at 25°C, under light 100–200 μmol m^{-2} s^{-1} or complete dark incubation, served as controls. After pre-treatments for defined durations, the cultures were maintained continuously at 25 ± 2°C temperature with 50–60% relative humidity and 16-h photoperiod of irradiance 100–200 μmol m^{-2} s^{-1} provided by cool day light fluorescent tubes (Philips TL 40W, Philips, New Delhi, India). In each set of experiments, 100 anthers were raised and the experiments were repeated at least three times. On regular weekly intervals, morphological changes in the growing cultures were observed. The results were recorded as the percentage of responding cultures.

Callus multiplication and embryogenesis Only those calluses that were induced inside the anther locules (microspores) on the induction medium were considered for further experiments. After three transfers, each of 6-wk duration on the induction medium, the calluses (approximate size of 5 × 5 mm) were transferred to multiplication medium consisting of different combinations of auxins and cytokinins, either alone or in combination with amino acids, gln and ser, for further multiplication and maintenance of callus. Sucrose at a concentration of 3% (*w*/*v*) was used unless mentioned otherwise. To document the callus growth on various multiplication media, the fresh weight of the calluses was recorded at the end of an 8-wk growth cycle. To calculate the percentage cell biomass increase, the following formula was used:

$$\text{Percentage biomass increase} = \frac{(CW_f - CW_i) \times 100}{CW_i}$$

where CW_f and CW_i are the final weight (after 8 wk) and initial weight (at the time of inoculation) of the callus, respectively.

Once the calluses were well established showing potentially unlimited growth and increase in cell biomass on the multiplication medium, the growth cycle was reduced to 3 wk,

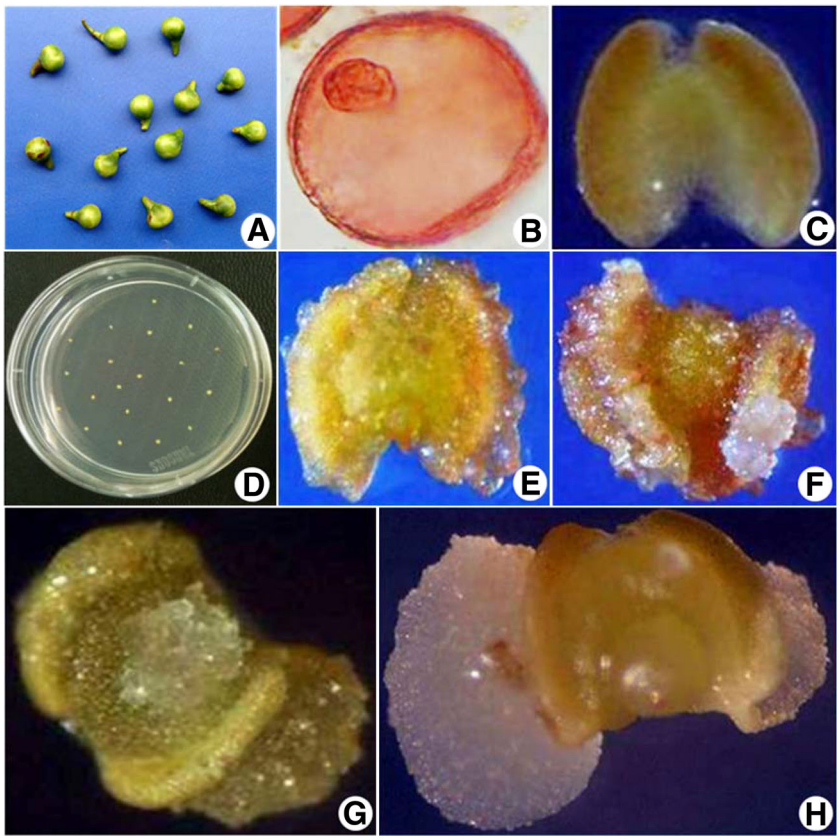

Figure. 1. Tea anther cultures. (*A*) Flower buds of 4-mm size with correct stage of microspores (×1.5). (*B*) A uninucleate microspore (×1200). (*C*) An isolated anther at culture (×75). (*D*) Anthers in Petri dish at the time of culture on MS (6% glucose) + 2,4-D (5 μM) + kinetin (5 μM) + gln (800 mg L^{-1}) + ser (200 mg L^{-1}). A total of 20 anthers were inoculated in each plate (×1.0). (*E*) Five-week-old anther culture incubated in the dark at 5°C for 5 d before being transferred to 25°C dark incubation on MS + (6% glucose) + 2,4-D (5 μM) + kinetin (5 μM) + gln (800 mg L^{-1}) + ser (200 mg L^{-1}). The anthers have been enlarged to triple the original size (×66). (*F*) Same as *E*, after 6 wk, showing broad opening of anther sacs and the release of shiny white transparent callus from inside the locules (×67). (*G*) Same as *F*, after 10 wk of culture initiation. The calluses multiplied further (×40). (*H*) Same as *E*, showing opening of anther sacs and callus proliferation from the surface of the anthers (×56).

and going forward, the calluses were routinely subcultured, every 3 wk onto fresh medium of same composition.

For regeneration through embryogenesis, the calluses were grown on medium containing BAP at varying concentrations, in combination with gibberellic acid (GA_3) and amino acids, including gln and ser. The embryos were transferred to embryo maturation medium every 3 wk. Maturation of embryos occurred when the concentration of the growth regulators and adjuvants in embryo induction medium was reduced by tenfold. Embryos germinated into complete plantlets on MS medium supplemented with BAP, indole-3-butyric acid (IBA), GA_3, gln, and ser. These plantlets continued to grow when the major salt concentration in the embryo germination medium was reduced by half (1/2 MS).

Histological analysis of embryos For histological studies, the material fixation was performed using a mixture of formalin. acetic acid:70% (*v*/*v*) ethanol (FAA) in (5:5:90 *v*/*v*/*v*) for 48 h, followed by preservation of the material in 70% (*v*/*v*) ethanol until used (Chaturvedi *et al.* 2003). Dehydration, wax infiltration, sectioning, and staining of embryogenic materials were done according to Srivastava and Chaturvedi (2011). The stained sections were observed under the microscope (Nikon, Tokyo, Japan).

Scanning electron microscopy Embryogenic anther-derived calluses with different stages of embryos were fixed using 2.5% (*v*/*v*) glutaraldehyde solution, followed by dehydration by dipping in ascending ethanol series (30, 50, 70, 90%, and pure alcohol). Thereafter, the cultures were kept in glass vacuum desiccators (Tarson Products Pvt. Ltd.) for drying at low vacuum of 13 kPa for 30 min. The dried callus material was sputter-coated with gold according to manufacturer manual (Carl Zeiss, Munchen, Germany) and observed under a scanning electron microscope (Leo 1430vp, Carl Zeiss) and the images were recorded (Singh and Chaturvedi 2012).

Statistical analysis Data on percentage culture response and calluses growth were collected at each step of culture initiation, callus induction, and multiplication. The statistical mean value was calculated, the data were analyzed by variance (ANOVA), and the *p* value was determined using SPSS 16.0

software. The results were considered significant at a p value less than 0.05. A *post hoc* Duncan's multiple range test was calculated using SPSS 16.0 software.

Ploidy analysis. Cytological analysis For cytological analysis, root tips from *in vitro*-grown haploid plants and shoot tips from field-grown donor plants (control) were collected at 10 AM to determine the chromosome count. The material was washed with distilled water and pre-treated with 0.02% (*w*/*v*) 8-hydroxyquinoline (Merck, Mumbai, India) at 4°C for 4 h. Thereafter, the material was fixed in modified Carnoy's fixative (7:3:1:1 *v*/*v*/*v*/*v* absolute ethanol:-chloroform:methanol:glacial acetic acid; Merck, Mumbai, India) for 48 h under refrigeration (Chaturvedi *et al.* 2003). The fixed material was positioned in a watch glass; a mixture consisting of 1% (*w*/*v*) aceto-orcein dye (HiMedia Laboratories Pvt. Ltd.) and 1 N HCl (Merck) in 9:1 ratio was added to the fixed material and heated gently over the flame (on and off) for 1 min. The material was allowed to cool and then placed in 1 μL of fresh 1% (*w*/*v*) aceto-orcein stain on a glass slide, and a coverslip was placed over it. The slide was warmed gently over the flame (on and off) for 30 s and the material was squashed. The number of chromosomes in the shoot-tip and root-tip squash preparations were counted under the ×100 objective lens of a Nikon 80i microscope (Nikon, Tokyo, Japan).

Flow cytometry analysis Leaves from donor plants (control) and from *in vitro* regenerated plants were used for the ploidy determination using a BD Accurie C6 flow cytometer (Becton-Dickinson, Franklin Lakes, NJ) with an argon laser (15 mV) at 488 nm encompassing an emission range of greater than 590 nm. The freshly plucked, 50 mg leaf samples were chopped into fine pieces in nuclear isolating-modified woody plant buffer consisting of 0.2 M Tris HCl, 4 mM $MgCl_2.6H_2O$, 2.5 mM EDTA $Na_2.2H_2O$, 86 mM NaCl, 10 mM sodium metabisulfite, 1.5% (*v*/*v*) Triton™ X-100 (all from Merck), and 2% (*w*/*v*) polyvinylpyrrolidone (PVP-10) (Sigma-Aldrich®) on wet ice. The pH of the buffer was adjusted to 7.5 using 1 N HCL or 1 N NaOH. The fine nuclear suspension, obtained after the leaves were chopped, was filtered using a 30.0-μm nylon membrane (Millipore, Temecula, CA). The filtrate was treated with a 50 μg mL^{-1} solution of RNase (Sigma-Aldrich®) and simultaneously stained with 50 μg mL^{-1} solution of propidium iodide (Sigma-Aldrich®) (Hazarika and Chaturvedi 2013). Using instrument gain (photomultiplier voltage and amplitude gain), the position of the G_1 peak nuclei of the reference sample control was established on channel 711 on a 1024 scale. Following this process, the instrument settings were kept constant and the unknown samples were analyzed using the same parameters.

Results

Callus induction Of the various growth regulator combinations and carbon sources tested, only four media (Table 1) showed induction of callus from anthers, and MS2 was the best among the four media. Thereafter, to improve the callus induction response, different concentrations of sucrose and glucose were tested with the best medium (Fig. 2). The MS medium (MS2) with 6% (*w*/*v*) glucose, supplemented with 2,4-D (5 μM), kinetin (5 μM), gln (800 mg L^{-1}), and ser (200 mg L^{-1}), showed the highest percentage of callus induction (96 ± 1.9%).

The application of low and high temperature for a defined duration as a stress pre-treatment is known to promote induction of androgenesis (Saha and Bhattacharya 1992). Temperature pre-treatments (cold and heat) provided to entire flower buds in the present study did not yield any results, but significant callus formation responses were obtained when anther cultures were given pre-treatments (Table 2). Among controls, dark incubation of anthers resulted in fourfold higher response on induction of callus from anthers compared to those anthers maintained in light. Despite higher callus formation at 25°C in dark incubation, the origin of the callus was primarily from anther walls (Table 2). However, when anther cultures were subjected to a cold temperature pre-treatment of 5°C for 5 d in dark incubation, callus formation was induced from inside the anther locules in over 60% of the anthers (Table 2). Heat pre-treatment at 33°C for 5 d in dark incubation induced callus formation from inside the locules in only 18% of the cultures.

On the most responsive medium (MS2) at 5°C incubation in dark for 5 d, within 5 wk of culture, the anthers enlarged almost threefold (Fig. 1*E*). The anther sacs burst open releasing shiny, white, translucent callus from within the anther locules (Fig. 1*F*, *G*) along with some brown granulated callus from the surface of the anther walls (Fig. 1*H*). The calluses that developed from inside the locules were selected and used

Table 1. Composition of media effective for callus induction from anthers

Constituents	MS1	MS2	MS3	MS4
Basal medium	MS	MS	MS	MS
Sucrose (g L^{-1})	30	–	60	40
Glucose (g L^{-1})	–	60	–	–
2,4-D (μM)	5	5	1	3
NAA (μM)	–	–	1	–
BAP (μM)	–	–	5	–
Kinetin (μM)	5	5	–	–
TDZ (μM)	–	–	–	18
Glutamine (g L^{-1})	800	800	–	–
Serine (g L^{-1})	200	200	–	–

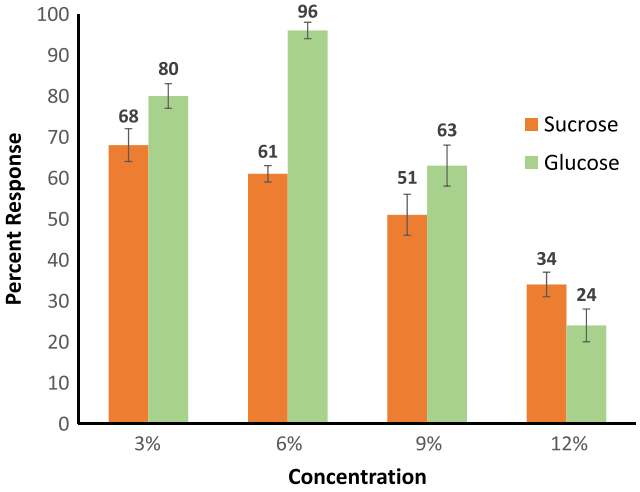

Figure. 2. Effect of various concentrations of carbon source, glucose and sucrose, on callus induction when the anthers were inoculated on MS + 2,4-D (5 μM) + kinetin (5 μM) + L-glutamine (800 mg L^{-1}) + L-serine (200 mg L^{-1}). The cultures were incubated in the dark at 25°C.

in additional experiments, while those that developed from the anther walls were excluded.

Callus multiplication and differentiation of embryos The calluses (approximate size of 5 × 5 mm), which originated from microspores from within the anther locules, were transferred to fresh original medium (MS2) and maintained in diffused light at 25 ± 2°C. The rate of callus proliferation showed no significant increase after three transfers, each of 6-wk duration. Consequently, the calluses were subcultured onto a set of media combinations to increase the rate of callus proliferation (Table 3). Of the media combinations tested, in terms of callus proliferation and increased cell biomass, significant results ($p < 0.05$) were obtained on MS (3% sucrose) + 2,4-D (5 μM) + kinetin (5 μM) + gln (800 mg L^{-1}) + ser (200 mg L^{-1}) (callus maintenance medium). The percentage cell biomass increase was greater than 235% in a single growth cycle of 8 wk on this medium (Table 3). The calluses were green, compact, fresh, and fast growing (Fig. 3*A*). Morphological variations in appearance of the callus were observed in the second subculture, after 8 wk, and the compact green callus was transformed into nodulated green callus (Fig. 3*B*). A view of histological sections of these calluses revealed random meristemoid formation (Fig. 3*C*). Well-developed nodulated callus can be clearly visualized from the scanning electron microscopic (SEM) images (Fig. 3*D*).

The nodulated calluses were transferred onto MS medium supplemented with BAP (10 μM), GA_3 (3 μM), gln (800 mg L^{-1}), and ser (200 mg L^{-1}). Emergence of pro-embryogenic masses was observed on this medium within 6 wk of inoculation and was confirmed by SEM image analysis (Fig. 3*E*). On the second subsequent subculture, these pro-embryogenic masses showed asynchronous embryogenesis within 6 wk in 85% of the cultures (Fig. 3*F*). The rate of embryo development was very high with over 20 opaque white and green embryos developed with smooth shiny surfaces per callus culture. Repetitive secondary embryogenesis was also observed when these primary embryos were inoculated onto the same medium. All the stages of embryos, including globular, heart, torpedo, and dicotyledonous stages, were clearly visible. SEM images shown in Fig. 3*G* display distinct globular and heart-shaped structures. Globular, heart-shaped, and torpedo stage embryos appeared fresh, shiny, off-white, or green (Fig. 4*A–C*). Mature embryos can be seen in Fig. 4*D*. Histological sections revealed the epidermal origin of these embryos with well-differentiated epidermis and compact internal cells (Fig. 4*E–G*). These structures were loosely attached to the explants and displayed pro-vascular strands.

Table 2. Effect of temperature pre-treatments on callus induction from anthers cultured on MS2 medium (MS (6% [*w*/*v*] glucose) + 2,4-D (5 μM) + kinetin (5 μM) + L-glutamine (800 mg L^{-1}) + L-serine (200 mg L^{-1}) and incubated in the dark

Temperature pre-treatments to anthers	Percent of anthers with callus formation response	Percent of anthers with callus formation originating from inside the anther locules
25°C light (control)*	24.0^{d}	0^{d}
25°C dark (control)	96.25^{a}	47 ± 6^{b}
5°C dark for 5 d	76.35^{ab}	60 ± 5^{a}
33°C dark for 5 d	36.25^{c}	18 ± 2^{c}

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

* Diffused light of 16-h photoperiod

Embryo maturation, germination, and plantlet development The embryos differentiated on MS + BAP (10 μM) + GA_3 (3 μM) + gln (800 mg L^{-1}) + ser (200 mg L^{-1}) medium and were transferred for maturation onto the same medium but with a tenfold reduced concentration of growth regulator and adjuvants. On MS + BAP (1 μM) + GA_3 (0.3 μM) + gln (80 mg L^{-1}) + ser (20 mg L^{-1}), complete bipolar embryos were developed. Histological sections of early dicotyledonous stage (Fig. 4*H*) and late-dicotyledonous stage (Fig. 4*I*) embryos revealed the presence of closed vascular strands and well-developed plumular and radicular ends. In addition, aberrant embryos such as embryos without cotyledons or with multiple cotyledons were also observed. These embryos developed either shoots or roots but never both. A histological section of the shoot developed from the germination of a monopolar embryo is represented in Fig. 4*J*. These shoots had very well-developed plumular ends, showed leaf primordia, and had well-developed conducting strands.

The germination of embryos into complete plantlets was achieved within 10 wk when the embryos were transferred to MS medium containing BAP (10 μM), GA_3 (0.5 μM), IBA

Table 3. Cell biomass increase on various callus multiplication media

Media constituents	Initial fresh weight (mg) at the time of culture	Fresh weight (mg) after 8 wk	Percentage biomass increase after 8 wk
MS (3% [*w/v*] sucrose) + 2,4-D (5 μM) + kinetin (5 μM) + L-glutamine (800 mg L^{-1}) + L-serine (200 mg L^{-1})	499.78 ± 11.15	1678.56 ± 17.58	235.95^{a} ± 5.1
MS (3% [*w/v*] glucose) + 2,4-D (5 μM) + kinetin (5 μM) + L-glutamine (800 mg L^{-1}) + L-serine (200 mg L^{-1})	507.90 ± 15.30	1514.00 ± 18.43	198.25^{b} ± 6.29
MS (3% [*w/v*] sucrose) + 2,4-D (3 μM) + TDZ (18 μM)	505.40 ± 15.93	1310.3 ± 18.79	159.53^{c} ± 9.95
MS (3% [*w/v*] sucrose) + 2,4-D (1 μM) + NAA (1 μM) + BAP (5 μM)	497.60 ± 18.59	1096.6 ± 31.69	120.46^{d} ± 3.32

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

(1 μM), gln (80 mg L^{-1}), and ser (20 mg L^{-1}) (Fig. 5*A*, *B*). However, these plantlets continued to grow only when the major salt concentration in the medium was reduced by half (1/2 MS) (Fig. 5*C*, *D*). On 1/2 MS medium supplemented with BAP (10 μM) + GA_3 (0.5 μM) + IBA (1 μM) + gln (80 mg L^{-1}) + ser (20 mg L^{-1}), 4-cm-long, multinodal, single or multiple plantlets developed with well-developed root systems. An interesting phenomenon was seen with these embryos as the embryos grown in clusters developed into complete plantlets by a bipolar germination pattern, while those embryos grown individually often showed a monopolar germination pattern, i.e., either shoot or root.

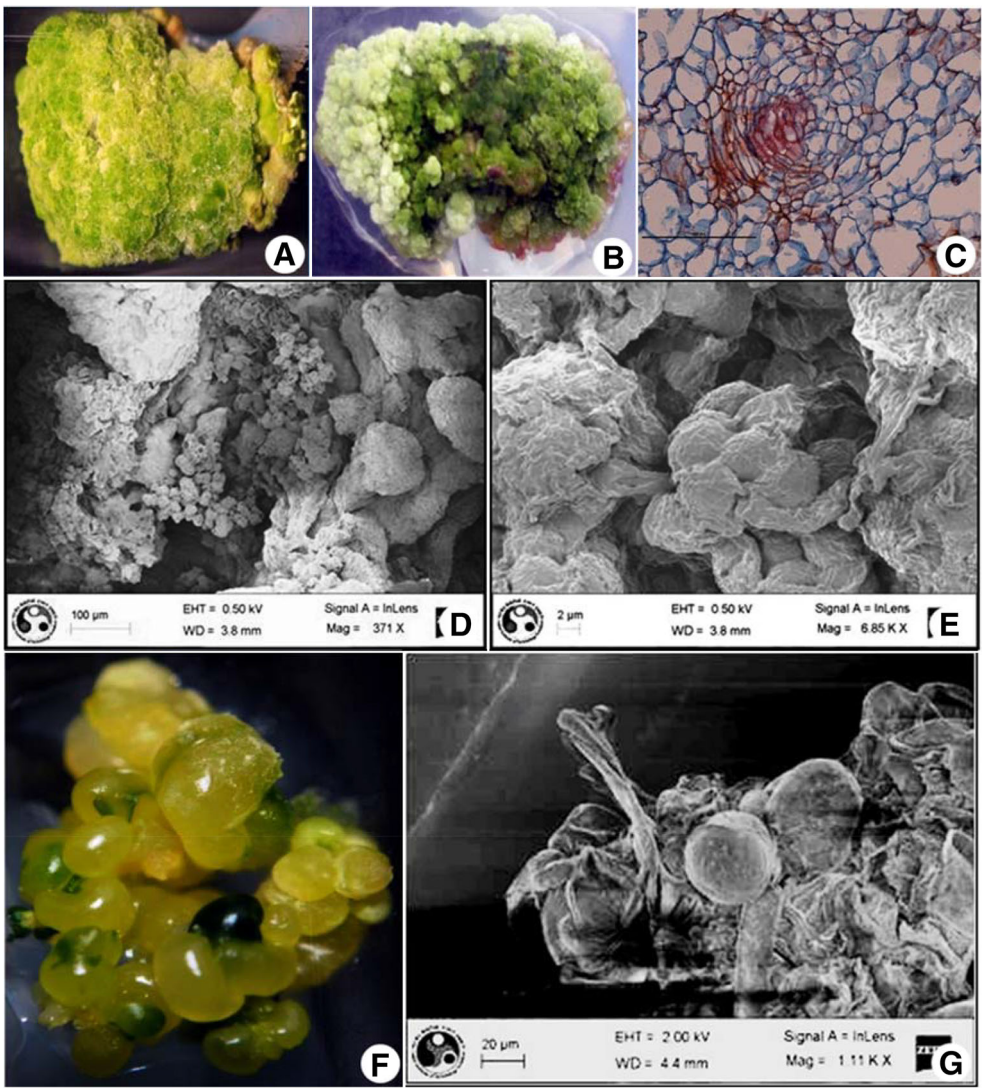

Figure. 3. (*A*) The calluses obtained from the induction medium were subcultured and multiplied on MS (3% sucrose) + 2,4-D (5 μM) + kinetin (5 μM) + gln (800 mg L^{-1}) + ser (200 mg L^{-1}). Note the increase in cell biomass and the development of green, compact callus (×1.9). (*B*) Same as *A*, appearance of nodulated green callus after 8 wk in second subculture (×1.7). (*C*) Same as *B*, showing nests of vascular elements randomly developed in callus tissue (×100). (*D*) Scanning electron micrograph (SEM) image of well-developed nodular structures (×371). (*E*) Nodulated callus from *D*, after 6 wk of culture on MS + BAP (10 μM) + GA_3 (3 μM) + gln (800 mg L^{-1}) + ser (200 mg L^{-1}), showing emergence of pro-embryogenic mass in SEM analysis (×6.85 K). (*F*) Six-week-old culture on MS + BAP (10 μM) + GA_3 (3 μM) + gln (800 mg L^{-1}) + ser (200 mg L^{-1}) showing asynchronous embryogenesis. Note the presence of shiny embryos at various developmental stages including globular, heart, torpedo, and dicot (×5). (*G*) Same as *F*, scanning electron micrograph (SEM) image showing globular and heart-shaped embryos (×1.11 K).

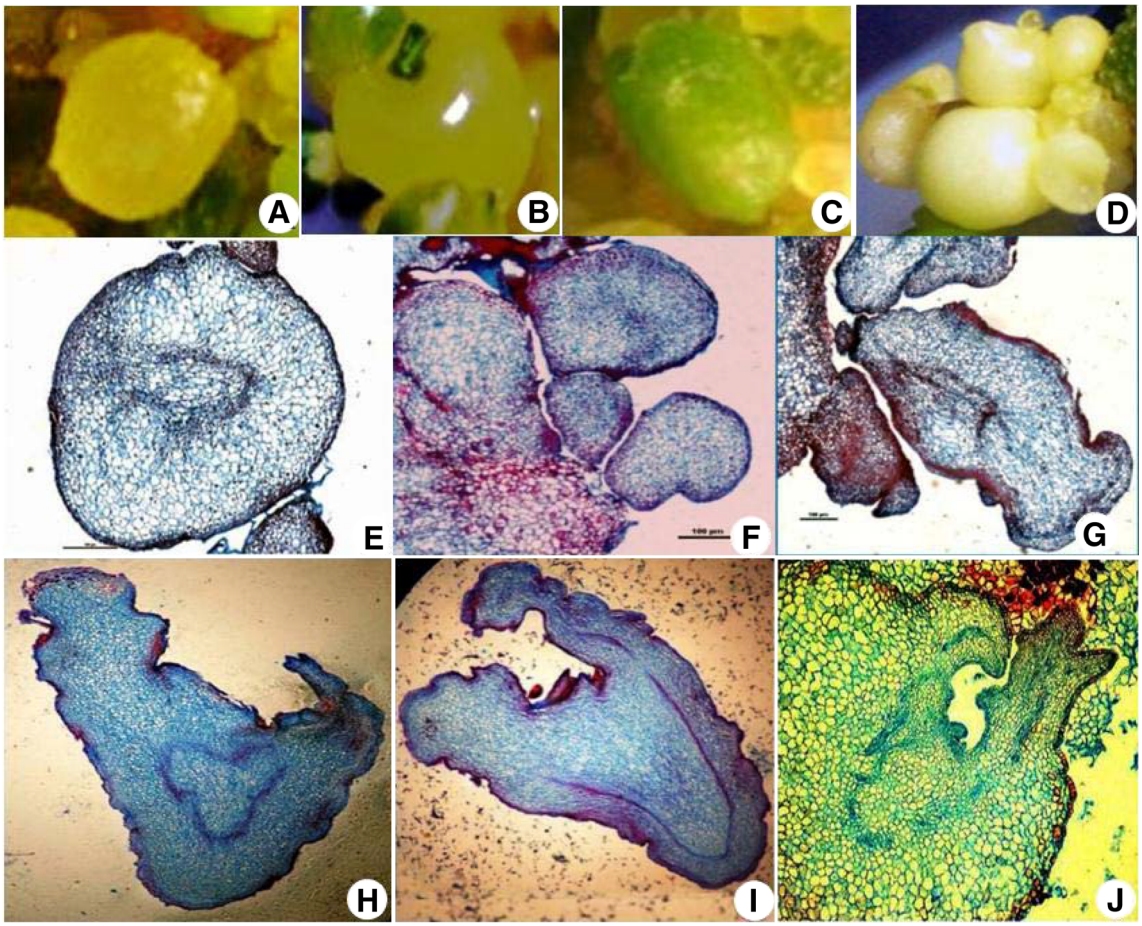

Figure. 4. Embryogenesis in TV21 cultures. (*A*) Globular embryo (×50). (*B*) Heart-shaped embryo (×50). (*C*) Torpedo-shaped embryo (×50). (*D*) Mature embryos (×50). (*E–G*) Histological sections of embryos at various stages of development. Note the presence of well-differentiated epidermis, compactly arranged internal cells, and pro-vascular strands (×125). (*H,I*) Histological sections of early dicotyledonous (H) and late dicotyledonous stage (I) embryos showing well-developed plumular and radicular ends with closed vascular strands (×125). (*J*) A histological section passing through the apex of a shoot showing leaf primordia and conducting strands (×50).

Cytological analysis Cytological analysis of root tip from *in vitro* developed plantlets showed that 90% of the plants regenerated were haploids with chromosome number $2n = X = 15$ (Fig. 6*A*). The remaining 10% were either aneuploids with chromosome number $2n = 2X = 28$ or diploids with chromosome number $2n = 2X = 30$. The shoot tips from the field-grown donor TV21 plants (control) were diploid (Fig. 6*B*).

Flow cytometry analysis The linear fluorescence intensity histograms of relative nuclear DNA content of leaves from the field-grown donor plants (control) and *in vitro* regenerated haploid plantlets were measured. The histogram in Fig. 6*C* represents the ploidy status of leaves from the control donor plant (diploid plant). Two distinct peaks were observed. The first peak at channel position 711 shows the G_0/G_1 peak with coefficients of variation (CVs) less than 5%, while the minor peak at channel position 1425 represents the G_2 phase of the cells. Flow cytometric data obtained from the donor plant was used as a reference comparison with the DNA content from leaves of *in vitro* regenerated plants. The histogram in Fig. 6*D* illustrates the ploidy of regenerated haploid plants with two visible distinct peaks. The dominant peak at channel position 352 signifies the G_0/G_1 phase of the haploid plants, while the small peak at channel position 760 designates the G_2 phase of the cells. The ploidy estimation of *in vitro* grown plants was exactly half of the DNA content obtained in the control plant sample. This result confirmed that the plants obtained through androgenesis of *C. assamica* ssp. *assamica* were haploid in nature.

Discussion

Androgenesis is an indispensable tool that can be used to generate doubled haploids or homozygous diploid plants. Most woody plant species have long reproductive cycles, typically outbreed, and are extremely heterozygous. The inherent nature of woody plant species acts as a barrier in mutant selection, genetic studies, and restricts crop improvement programs. Conventional breeding methods are unpredictable and

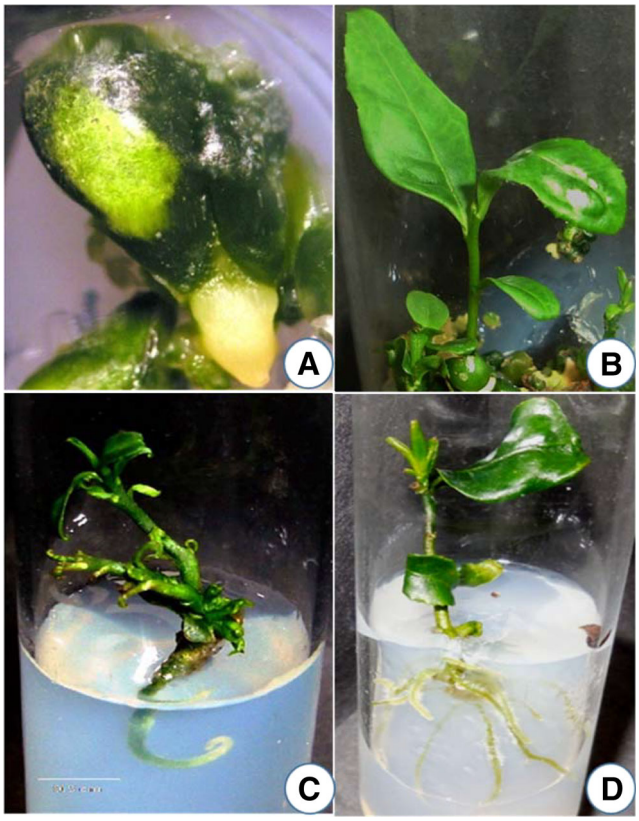

Figure. 5. Embryo germination and plantlet development. (*A*) Eight-week-old embryo grown on MS + BAP (10 μM) + IBA (1 μM) + GA_3 (0.5 μM) + gln (80 mg L^{-1}) + ser (20 mg L^{-1}), showing germination with well-developed radicular and plumular end (×5). (*B*) Same as *A*, after 10 wk, showing well-developed shoot system (×5). (*C*) Twelve-week-old plantlet grown on 1/2 MS + BAP (10 μM) + GA_3 (0.5 μM) + IBA (1 μM) + L-glutamine (80 mg L^{-1}) + L-serine (20 mg L^{-1}), showing a branched, healthy, green, multinodal shoot with a well-defined root at the base of the shoot (×5). (*D*) Sixteen-week-old plantlet showing single, multinodal shoot and well-developed root system (×5).

time consuming and require several generations of selfing the plants to establish pure bred lines with desired traits (Seran 2007; Srivastava and Chaturvedi 2008). In the present study, haploids were produced under *in vitro* conditions by androgenesis, which transform the pollen to develop haploid plants with the morphology of a sporophyte instead of participating in the usual gametophytic mode of development. These haploids are diploidized in a single generation to produce homozygous diploid plants.

The number and extent of studies concerned with generating doubled haploid plants are very limited in tree species including *Camellia* spp. The major bottleneck is the extreme recalcitrance and unpredictable nature of *Camellia* explants in *in vitro* conditions. In the past decades, there have been a few attempts towards plant improvement using *in vitro* anther cultures of *Camellia* spp. The majority of the previous reports demonstrated only haploid callus production from microspores (Pedroso and Pais 1994; Seran *et al*. 1999) or roots (Katsuo 1969; Okano and Fuchinone 1970). Chen and Liao produced complete plantlets by callus production from tea anthers of cultivar Fuyun No. 7 from nine different tea cultivars studied (Chen and Liao 1982). Only a few details are available conveying that three out of the four plants were haploids. In another study, researchers reported development of globular structures in tea, which failed to differentiate further (Saha and Bhattacharya 1992). Embryo production has also been reported in this species but the embryos did not germinate (Shimokado *et al*. 1986).

The present study demonstrates the pioneering successful attempt to obtain well-developed haploid plants in the TV21 cultivar of *C. assamica* ssp. *assamica* (Masters). Various factors were evaluated to obtain androgenic callus induction from anthers cultured at the early-to-late uninucleate stage of microspores. The composition of the culture medium and an optimized concentration of plant growth regulators played an essential role in induction and proliferation of microspore calluses (Sopory and Munshi 1996; Kim *et al*. 2003; Yin *et al*. 2014). The synergistic effect of an auxin and cytokinin (Sarwar 1985; George *et al*. 2008) along with additional nitrogen sources, such as gln and ser, selectively induced callus formation from microspores. Another crucial factor was the type and concentration of the carbon source present in the anther culture medium. In this study, the percentage of anther response varied with the carbon source and concentration, and the maximum percentage of callus formation from inside the anther sacs was observed with a high glucose concentration (6%). This finding is in agreement with reports on androgenesis in *Azadirachta indica* (neem), where the maximum percentage of responding anthers showing callus induction was obtained when higher concentrations of sucrose (9–12%) were used as carbon source in the medium (Chaturvedi *et al*. 2003; Srivastava and Chaturvedi 2011). In addition, temperature pretreatments of cultured anthers can be major triggers for androgenesis (Pescitelli *et al*. 1990; Saha and Bhattacharya 1992).

In the current study, callus induction in 96% of the cultures was obtained on MS medium with 6% glucose and supplemented with 5 μM 2,4-D, 5 μM kinetin, 800 mg L^{-1} gln, and 200 mg L^{-1} ser (callus induction medium). The calluses further multiplied in subsequent subcultures when 6% glucose was replaced by 3% sucrose in the callus maintenance medium. Embryogenesis was achieved from these calluses within 3 months in 85% cultures when grown on MS medium consisting of 10 μM BAP, 3 μM GA_3, 800 mg L^{-1} gln, and 200 mg L^{-1} ser. An average of 20 embryos were induced to develop for each callus culture. Repetitive secondary embryogenesis was obtained from these primary embryos when cultured on the same medium at the end of each growth cycle. The embryos matured on medium containing a tenfold reduction in the concentration of the growth regulators and supplements present in the embryo induction medium. The embryos germinated on MS medium with 10 μM BAP, 1 μM IBA, 0.5 μM GA_3, 80 mg L^{-1} gln, and 20 mg L^{-1} ser, and 65%

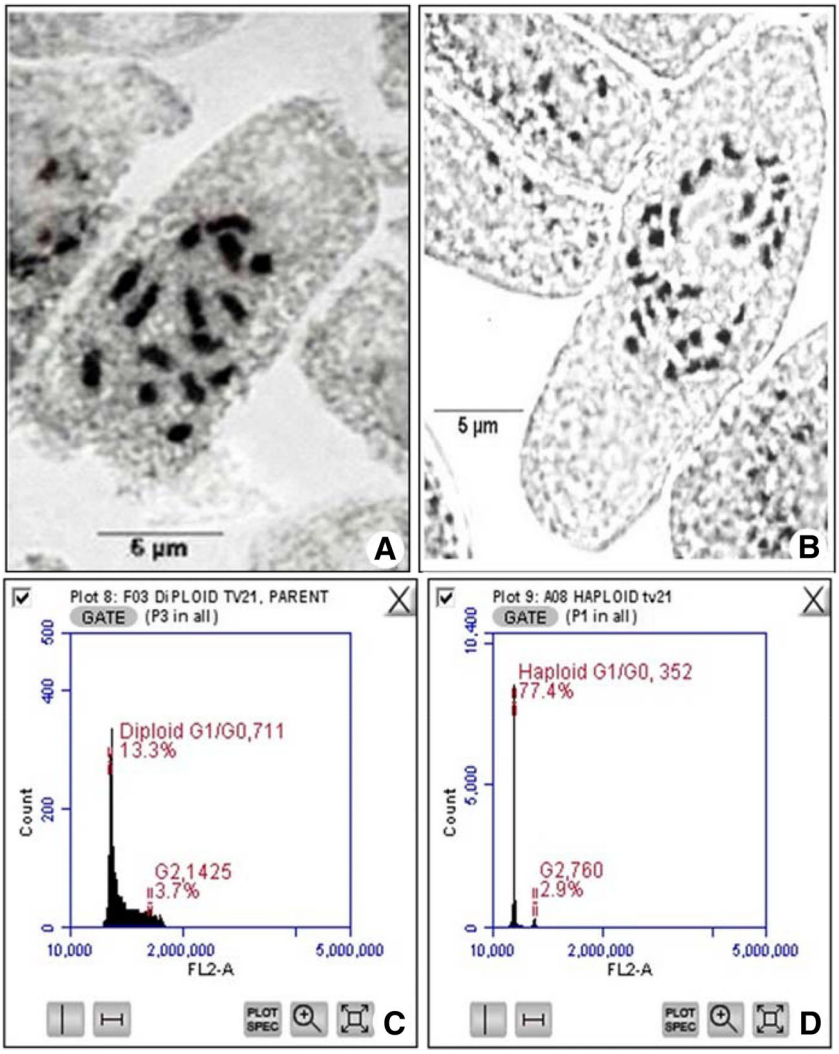

Figure. 6. Ploidy analysis by squash preparation and flow cytometry. (*A*) Squash preparation of root-tip cell from *in vitro* regenerated TV21 plant, showing haploid status with chromosome no. of $2n = X = 15$ (×100). (*B*) Squash preparation of shoot-tip cell from a field-grown donor plant of TV21 cultivar, showing diploid status with chromosome no. of $2n = 2X = 30$ (×100). (*C*) Flow cytometry histogram from leaves of a field-grown TV21 donor plant (control), where the G_1/G_0 peak was obtained at the 711 channel position. (*D*) Flow cytometry histogram from leaves of an *in vitro* regenerated plant of TV21 cultivar, where the G_0/G_1 peak was obtained at the 352-channel position and confirms the plant to be haploid.

of the embryos germinated to complete plantlets. However, further growth and development of the plantlets could be achieved only when the concentration of the major salts in the embryo germination medium was reduced by half (1/2 MS). Glutamine and ser proved to be crucial components in the culture media at all stages of growth and development of the embryos. Glutamine is not only used as a building block in protein and nucleotide synthesis but also regulates the activity of the enzyme nitrate reductase and regulates the expression of genes responsible for transportation of nitrate and ammonium ions. The exact mechanism remains unclear, but gln synthesis pathways have been associated in the activation of certain transcription factors that manage stress tolerance in plants (Dodds and Roberts 1982; Kan *et al.* 2015). Serine on the other hand regulates transfer of methyl groups in folate metabolism, which is one of the important mechanisms that facilitate embryogenesis, development of roots following post-embryonic development, and photorespiration (Srivastava and Chaturvedi 2011; Ros *et al.* 2014).

Naturally occurring tea is diploid ($2n = 30$) having a basic chromosome number of 15 (i.e., $X = 15$) (Bezbaruah 1971; Wang *et al.* 1994). This matches the results with the cytological squash preparation of the haploid regenerants obtained in the present study. The chromosomal constitution of the regenerated haploid plantlets was determined to be $2n = X = 15$ by cytological squash preparation of root tips. The ploidy of these haploid plantlets was further confirmed by flow cytometric analysis using leaves from *in vitro* regenerated plants. This study accomplished a key milestone by establishing a reproducible method for complete haploid plant development, which can be utilized to produce homozygous diploids in this excessively cross-breeding tea plant.

Acknowledgements The authors thank the Department of Biotechnology, Government of India, for the financial assistance and also the Tea Research Association (TRA), Tocklai, Jorhat, for providing the tea plant materials.

References

Bezbaruah HP (1971) Cytological investigation in the family *Theaceae*—I. Chromosome numbers in some *Camellia* species and allied genera. Caryologia 24:421–426

Chaturvedi R, Razdan MK, Bhojwani SS (2003) Production of haploid plants of neem (*Azadirachta indica* A. Juss.) by anther culture. Plant Cell Rep 21:531–537

Chen Z, Liao HH (1982) Obtaining plantlets by anther culture of tea plants. J Chinese Tea 4:6–7

Dodds J, Roberts LW (1982) In: Experiments in plant tissue culture. Cambridge University Press, London, pp 467–470

Evans DC, Coleman JD, Kearns A (2003) In: Plant cell culture. Basics. BIOS Scientific Publication, London, pp 121–128

George EF, Hall MA, Klerk De (2008) In: Plant propagation by tissue culture, 3rd ed. Springer, Netherlands, pp 175–226

Germana MA (2003) Haploids and doubled haploids in *Citrus* ssp. In: Maluszynsky M, Kasha KJ, Foster BP, Szarejko I (eds) Doubled haploid production in crop plants: a manual. Kluwer Academic Publishers, Dordrecht, pp 303–307

Hazarika RR, Chaturvedi R (2013) 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. In Vitro Cell Dev Biol 49:60–69

Kan CC, Chung TY, YAn J, Hsieh MH (2015) Glutamine rapidly induces the expression of key transcription factor genes involved in nitrogen and stress responses in rice roots. BMC Genomics 16:731

Katsuo K (1969) Anther culture in tea plant (a preliminary report). Study of Tea 4:31

Kim KM, Baenzier PS, Rybczynski JJ, Arumugantathan K (2003) Characterization of ploidy levels of wheat microspore-derived plants using laser flow cytometry. In Vitro Cell Dev Biol 39:663–668

Mishra V, Chaturvedi R (2014) An overview on haploid production in trees. In: Ramvat KG, Merillon J, Ahuja MR (eds) Tree biotechnology, CRC press Taylor and Francis group. Science publishers, New York, pp 151–186

Mondal TK, Bhattacharya A, Laxmikumaran M, Ahuja PS (2004) Recent advances of tea (*Camellia sinensis*) biotechnology. Plant Cell Tissue Organ Cult 76:195–254

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco cultures. Physiol Plant 15:473–497

Okano N, Fuchinone Y (1970) Production of haploid plants by anther culture of tea in vitro. JpnJ Breed 20:63–64

Pedroso MC, Pais MS (1994) Induction of microspore embryogenesis in *Camellia japonica* cv. *elegans*. Plant Cell Tissue Organ Cult 37: 129–136

Pescitelli SM, Johnson CD, Petolino JF (1990) Isolated microspore culture of maize: effects of isolation technique, reduced temperature, and sucrose level. Plant Cell Rep 8:628–631

Ros R, Bertomeu JM, Krueger S (2014) Serine in plants: biosynthesis, metabolism, and functions. Trends in Plant Sci 9:564–569

Saha SK, Bhattacharya NM (1992) Stimulating effect of elevated temperatures on callus production of meristemoids from pollen culture of tea (*Camellia sinensis* (L.) O. Kuntze). Indian J Exp Biol 30:83–86

Sarwar M (1985) Callus formation from explanted organs of tea (*Camellia sinensis* L.) J Tea Sci 52:50–54

Seran TH (2007) Anther culture in tea improvement. Med Aromat Plant Sci Biotechnol 1:234–239

Seran TH, Hirimburegama K, Hirimburegama WK, Shanmugarajah V (1999) Callus formation in anther culture of tea clones, *Camellia sinensis* (L.) O. Kuntze. J Natn Sci foundation Sri lanka 27:165–175

Sharma RK, Negi MS, Sharma S (2010) AFLP-based genetic diversity assessment of commercially important tea germplasm in India. Biochem Genet 48:549–564

Shimokado T, Murata T, Miyaji Y (1986) Formation of embryoid by anther culture of tea. Jpn J Breed Suppl 2:282–283

Singh M, Chaturvedi R (2012) Screening and quantification of an antiseptic alkylamide, spilanthol from in vitro cell and tissue cultures of *Spilanthes acmella* Murr. Ind Crop Prod 36:321–328

Sopory S, Munshi M (1996) Anther culture. In: Jain SM, Sopory SK, Veilleux RE (eds) In vitro haploid production in higher plants, vol 1. Kluwer Academic Publishers, Dordrecht, pp 145–176

Srivastava P, Chaturvedi R (2008) In vitro androgenesis in tree species: an update and prospect for further research. Biotechnol Adv 26:482–491

Srivastava P, Chaturvedi R (2011) Increased production of azadirachtin from an improved method of androgenic cultures of a medicinal tree *Azadirachta indica* A. Juss. Plant Signal Behav 6:974–981

Wang YJ, Xuecheng Y, Shu YJ, Xin Jie W (1994) Histo-cytological study on somatic embryogenesis from the leaf of *Camellia sinensis*. J Trop Subtrop Bot 2:47–51

Yin J, Zhang XQ, Pan TH, Wang SQ, Liu QL, Sun LX (2014) Callus induction and haploid plant regeneration from baby primrose (*Primula Forbesii* Franch.) anther culture. Sci Hort 176:273–281