Plant Cell, Tissue and Organ Culture (PCTOC) (2021) 145:127–141
https://doi.org/10.1007/s11240-020-01997-x

ORIGINAL ARTICLE

Recurrent secondary embryogenesis in androgenic embryos and clonal fidelity assessment of haploid plants of Tea, *Camellia assamica* ssp. *assamica* and *Camellia assamica* ssp. *lasiocaylx*

Ruchira Bajpai[1] · **Rakhi Chaturvedi**[1]

Received: 13 July 2020 / Accepted: 19 December 2020 / Published online: 3 February 2021

Abstract

Globular androgenic haploid embryos of TV21 and TV19 cultivars of *Camellia* ssp., obtained on embryo induction medium (EIM), Murashige and Skoog medium with 10 µM 6-benzylaminopurine (BAP), 3 µM gibberellic acid (GA_3), 800 mg l^{-1} L-glutamine and 200 mg l^{-1} L-serine, were pretreated with 18 µM abscisic acid (ABA) and 25 g l^{-1} mannitol for 20 days in liquid medium to attain maturation. The pre-treatment favoured the primary globular embryos to grow and pass through all the developmental stages from globular, heart-shape, torpedo shape to fully matured docotyledonous embryos. Fivefold and threefold increase in secondary embryogenesis (SE) was attained in *Camellia assamica* ssp. *assamica* (TV21; Assam Type) and *Camellia assamica* ssp. *lasiocaylx* (TV19; Cambod Type), respectively, when pre-treated primary embryos were transferred to the liquid embryo development (ED) medium, supplemented with tenfold reduced concentration of growth regulators that were present in EIM. Embryos were germinated well on 10 µM BAP, 0.5 µM GA_3, 1 µM indole-3-butyric acid (IBA), 80 mg l^{-1} L-glutamine and 20 mgl^{-1} L-serine, following liquid-to-semi-solid transition method. Well developed shoots and roots were formed when embryos were initially kept for four weeks in liquid germination medium, followed by their transfer to semi-solid medium on similar medium composition. The method promoted recurrent secondary embryogenesis in lesser time with highest germination rate, 66.6% and 30.3% in TV21 and TV19 cultivars, respectively. Histology and field emission scanning electron microscopy (FESEM) were performed to determine ontological events during embryo development. Clonal fidelity of haploid plantlets was assessed using random amplified polymorphic DNA (RAPD) to ensure no somaclonal variations occurred during the multistep process of secondary embryogenesis, embryo maturation and germination.

Key message

A two-step transition protocol from liquid-semi-solid medium is beneficial for embryos to overcome hyperhydricity. Secondary embryogenesis is the best mode to obtain genetically uniform true-to-type haploid plantlets.

Keywords Recurrent secondary embryogenesis · ABA · Two-step-protocol · FESEM · Histology · RAPD

Abbreviations

ABA	Abscisic acid
BAP	6-Benzylaminopurine
GA_3	Gibberellic acid
IBA	Indol-3-butyric acid
MS	Murashige and Skoog (1962) medium
NAA	1-Naphthaleneacetic acid
PEG	Polyethylene glycol
TV	Tocklai vegetative

Introduction

Camellia sinensis (L.) commonly known as tea, is a member of Theacea family. It is a commercially cultivated beverage crop with potential therapeutic applications (Mondal et al. 2004; Chan et al. 2007). Even though its a tree crop with huge economic significance, varietal improvement in

Communicated by Maria Antonietta Germanà.

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

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

tea is hampered due to its inborn traits, like intense cross-pollination and long juvenile phase (Mukhopadhyay et al. 2016; Mishra et al. 2017). Predominant cross pollination coupled with frequent spontaneous hybridization causing continuous variation in the genus *Camellia* has resulted in highly heterogeneous hybrid plants constituting more than 325 species and 600 different genotypes cultivated globally (Mondal et al. 2004; Orel and Wilson 2012). However, the most admissible explanation classifies cultivable tea taxa into three natural hybrids: *C. assamica* (Masters) or Assam type, with the largest leaf size, followed by *C. assamica* sub ssp. *lasiocalyx* (Planchon ex Watt.), or Cambod tea with intermediate size of leaves and *C. sinensis* (L.) O. Kuntze or China type with the smallest sized leaves (Mondal et al. 2004; Mondal 2014). The natural tea hybrids are known based on their close morphological proximity to either of these three taxa. Conventional propagation of tea by seed propagation and vegetative cuttings have been practiced for years to obtain genetically stable tea plantations. But, these methods are impeded due to the long reproductive cycle, seed-borne variability and poor survivability of the cuttings (Mondal et al. 2004). Further, various diseases and abiotic stress conditions have caused a significant decline in tea production within the last 40 years (Sivapalan and Jain 1999; Akula and Dodd 1998). Thus, it becomes extremely difficult to fulfill the global requirement of elite tea clones using conventional methods of propagation. Furthermore, none of the conventional methods are able to overcome the long existing heterozygosity in the *Camellia* ssp.

In vitro embryogenesis obtained either directly on an explant or indirectly via an intervening callus phase, is considered to be the most efficient micropropagation protocols for large-scale production of genetically uniform plantlets in difficult-to-propagate plants (Jain and Newton 1990; Bano et al. 1991). Embryogenesis offers several advantages over an organogenic system, such as single-cell origin rendering high genetic fidelity, resemblance to zygotic embryos facilitating the study of developmental and regulatory embryogenic pathways and genetic transformation study (Zimmerman 1993). Additionally, the bipolar germination of embryos reduces the cost incurred on rooting of micro shoots by favouring complete plantlet development in a single step (Bhojwani and Razdan 1996). High regeneration ability with independent origin makes embryos an excellent explant for genetic transformation studies (Zimmerman 1993; Guan et al. 2016). Recurrent secondary embryogenesis involves development of multiple fresh embryos on top of the pre-existing primary embryos; and this system is highly beneficial for woody tree species, because it can restore the embryogenic competence of in vitro cultures for years through repeated cycles ensuring high genetic uniformity (Nair and Gupta 2006; Shi et al. 2010; Martinez et al. 2015). Recurrent secondary embryogenesis in liquid medium is advantageous over semi-solid medium providing faster growth, more synchronous development, and easy automation (Burns and Wetzstein 1997; Guan et al. 2016). Although, a highly proficient method for plant regeneration, attaining embryogenesis is crucial and is governed by various factors, such as genotype, age of explant, disease-free explant as well as the medium combination and growth regulators (Pais 2019). Successful plantlet regeneration via somatic embryogenesis has been achieved in tea using various explants (Paratasilpin 1990; Jha et al. 1992; Kato 1996; Ponsamuel et al. 1996; Akula and Akula 1999; Mondal et al. 2002; Sharma et al. 2004 and Seran et al. 2006; Suganthi et al. 2012).

Despite all these reports on in vitro plant regeneration in tea, the major problem on genetic variation remains persistent since the initial explants to raise in vitro cultures were taken from somatic tissues of field grown natural hybrids (parent plant) and, consequently have diluted the quality of the product. Owing to long reproductive cycle and extereme hetrozygosity due to outbreeding nature of tea, the pure breeding lines can only be achieved by developing haploid plants in vitro using gametic tissues and then diploidization of these haploids. Our laboratory was the first to report successful embryogenesis from gametic explants to raise haploids (Mishra et al. 2017). The present investigation is the extension of that work and involves recurrent secondary embryogenesis from the gametic embryos obtained in two cultivars of tea, *Camellia assamica* ssp. *lasiocalyx* (TV19) and *Camellia assamica* ssp. *assamica* (TV21).

Tea has a high potential for developing secondary embryos. But, the phenomenon was not highlighted till Akula et al. (2000b) reported regeneration via recurrent secondary embryogenesis in TRI 2025 clone using temporary immersion system. Recurrent secondary embryogenesis is beneficial as it yields unrestricted induction of secondary embryos. Modified MS medium with reduced concentration of nitrate, sulfate and ammonium salts, and addition of L-glutamine in the medium was recommended for enhanced secondary embryogenesis (Mondal et al. 2002). Effect of ABA in combination with osmoticum, like polyethylene glycol (PEG-400), mannitol and betaine, on inducing direct embryo regeneration and maturation has been reported in *Camellia sinensis* L (Akula et al. 2000a; Ozudogru et al. 2004) and UPASI tea (Suganthi et al. 2012). ABA facilitates the accumulation of food reserves (proteins, lipids, and carbohydrates) in embryos, prevents precocious germination and, thereby promoting normal germination of embryos. Its role in enhanced embryo production and embryo maturation has been reported in many plant species (Guttman et al. 1996; Langhansova et al. 2004). Osmoticum, on the other hand, creates desiccation like condition and stimulates embryogenesis (Ozudogru et al. 2004). Although there are a few reports on somatic embryogenesis in tea, none of the

existing literature reveals recurrent haploid embryogenesis in tea using a combination of ABA and osmoticum in the liquid culture medium.

The present study aims at enhanced secondary embryogenesis and efficient germination of haploid embryos of TV21 and TV19 cultivars of tea. A two-step sequential protocol, involving semi-solid and liquid medium transition under the combined influence of ABA and osmoticum, has been optimized. The objective of this present research was to establish a faster method for large-scale production of haploid plants by recurrent gametic embryogenesis in liquid medium. Enhanced haploid embryogenesis will serve as potential and continuous explant source for diploidization, and, thereby, facilitating the production of genetically stable homozygous lines in tea. Additionally, it could be a source for easy automation and multiplication of elite clones of tea to fulfill the globally rising demand for high quality planting materials of tea.

Materials and methods

Embryo induction, maturation, development and recurrent secondary embryogenesis

The in vitro haploid embryos of the two cultivars *Camellia assamica* ssp. *assamica* (TV21) and *Camellia assamica* ssp. *lasiocaylx* (TV19) of tea were routinely induced from callus, obtained within anther locules bearing uni-nucleate microspores (Mishra et al. 2017). Briefly, the nodulated calli from multiplication medium were cultured for 10 weeks on semi-solid MS (Murashige and Skoog 1962) medium supplemented with BAP, GA_3, L-glutamine and L-serine to induce embryogenesis. Individual globular stage embryos from the embryo induction medium were pre-treated on liquid MS medium consisting of abscisic acid (ABA), alone or in combination with any one of the three osmotica, PEG-400, glycine betaine or mannitol for 20 days to pass the embryos through maturation phase. The embryos in liquid medium were incubated in an incubator shaker and agitated at 120 rpm at 25 ± 2 °C with 16 h photoperiod irradiance 100–200 µmol m^{-2} s^{-1}. After pre-treatment, the embryos were transferred to the embryo development medium, MS fortified with tenfold reduced concentration of BAP, GA_3, L-glutamine and L-serine in both liquid and semi-solid state. MS medium in combination of different growth regulators and other adjuvants was used throughout the study. Medium was supplemented with 3% sucrose unless mentioned differently. The pH of the medium was maintained at 5.8 using 1 N HCl and 1 N NaOH. The medium was sterilized by autoclaving at 121 °C for 20 min at 100 kPa. All the cultures were kept in culture room conditions 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 daylight fluorescent tubes (Philips TL 40 W, Philips, New Delhi, India).

Effect of carbohydrate source on secondary embryogenesis

Two carbohydrate sources, glucose and sucrose in range of 1% to 3% concentrations were added in the liquid embryo development medium, MS consisting of BAP, GA_3, L-glutamine and L-serine, to determine its effect on rate of secondary embryogenesis. The embryos from the embryo development medium were further transferred to the liquid germination medium. All the cultures were incubated in an incubator shaker and agitated at 120 rpm at 25 ± 2 °C and with 16 h photoperiod. All experiments were repeated three independent times, each with 50 replicates and data were analyzed statistically.

Embryo germination and plantlet development

Cluster of fully developed mature haploid embryos from the liquid embryo development medium were transferred to the germination medium, MS supplemented with BAP, GA_3, IBA, L-glutamine and L-serine, in both liquid and semi-solid state for 4 weeks. Cultures were monitored regularly, and the germination % in each physical state was determined. The plantlets were eventually transfered to the potting mixtures and maintained under greenhouse conditions for 8 weeks with controlled temperature and humidity for acclimatization.

Histology and field emission scanning electron microscopic (FESEM) analysis

Histology and FESEM analysis were performed to study anatomical and morphological growth of embryos and to determine if any abnormality was induced with change in physical state of medium. For histological analysis, the embryos were fixed in FAA (5:5:90 v/v/v formaldehyde: acetic acid: 70% ethanol) for 48 h, followed by storage in 70% alcohol until use. Sample preparation involved dehydration of the preserved material by passing it through the series of tertiary-butyl-alcohol (TBA). After dehydration, the sample was infiltrated with paraffin wax (melting point 60 °C, Merck, Germany) and, eventually embedded in pure paraffin wax. Paraffin blocks were prepared and mounted on top of the wooden stubs. The 10 µm-thick sections were made using a manual rotary microtome (Leica, Germany) equipped with a stainless steel knife. Series of sections obtained were mounted on slides, dewaxed with xylene and double-stained with safranin (1%) and astrablue (1%). The sections were photographed (×20) using a Nikon light microscope.

The morphology of the embryos in suspension culture was examined through FESEM analysis. Samples were fixed in 2.5% glutaraldehyde solution for 24 h, followed by dehydration with a graded alcohol series (30%, 50%, 70%, 90%, and pure alcohol). After passing through the dehydration series, samples were dried to remove any trace of water by vacuum drying in desiccators. The dried samples were eventually sputter-coated with gold and observed under an FESEM (Carl Zeiss, Germany).

Ploidy determination

Flow cytometric analysis

Fresh, young leaves from the germinating embryos of TV21 and TV19 cultivars, as well as the leaves from field grown parent plants (control) were utilized for flow cytometric analysis. The collected leaves (~ 50 mg) were finely chopped in 1 ml of woody plant buffer in 90 mm petri dish (Borosil, India) kept on ice. Composition of the buffer used in the experiment is adopted from the established protocol of Loureiro et al. (2007) with little modification. A nuclear suspension was prepared by chopping each of the leaf samples with the help of a scalpel in the buffer and was filtered through 30 µm nylon net filter (Merck Millipore, Ireland). After this, 50 µg/ml propidium iodide (PI; Fluka) and 50 µg/ml RNase A (Sigma, India) was added to the filtered suspension. The suspension was incubated for 15–20 min in the dark and eventually analyzed using BD accurie C6 flow cytometer (Beckton–Dickinson, USA), equipped with an argon laser (15 mV) at 488 nm and encompassing emissions range greater than 590 nm (Mishra et al. 2017).

Chromosome count

Actively growing, 0.5 cm—1 cm long root-tips from in vitro developed haploid as well as from field-grown parent plants (control) of the two cultivars, TV21 and TV19, of tea were excised between 10 AM to 10:30 AM. After washing the excised root-tip samples with distilled water, the materials were pre-treated with 0.02% 8-hydroxyquinoline for 4 h at 4 °C. This was followed by fixation in modified Carnoy's fluid containing absolute alcohol: chloroform: methanol: glacial acetic acid (7:3:3:1 v/v/v/v) for 48 h at 4 °C. After that, the root-tips were stored until use in 70% ethanol (Chaturvedi et al. 2003). For chromosomal count, excised root-tips were placed in 3 N HCl for hydrolysis at 60 ºC for 15 min, followed by washing and drying on filter paper. The dried root-tips were placed in a leucobasic-fuschin solution (Hi-media, India) for 45 min at room temperature (Lamo and Rao 2016). The root-tips were washed and finally squashed in 2% aceto-carmine (Sigma, India). The number of chromosomes per cell in the root-tip squash preparations were counted under the × 100 objective lens of a Zeiss photomicroscope (Carl Zeiss, Germany).

Clonal fidelity assessment

Genomic DNA extraction

The young leaf samples from in vitro haploid shoots as well as the leaves from the parental heterozygous (in vivo) mother plants of both TV21 and TV19 cultivars were utilized. The leaves were collected in zip lock bags and stored overnight at − 80 °C, followed by lyophilization in freeze dryer alfa 1–4 model (Chaist Osterode am Harz, Germany). The lyophilized leaf material (400 mg) was finely grounded in sterilized mortar and pestle with the addition of silica powder (SRL, India) and immediately transferred to the pre-heated extraction buffer incubated at 60 °C in the hot water bath (Julabo, Germany) with gentle shaking at 10 min interval for 1 h. DNA isolation was performed using the CTAB (Cetyl trimethyl ammonium bromide) protocol (Doyle and Doyle 1990). The composition of the buffer used was adopted from (Bhau et al. 2015) with slight modification; (2.5% CTAB, 3% PVP-4000, 2 M NaCl, 20 mM EDTA, 100 mM Tris–HCl, 0.2% β-Mercaptoethanol; pH 8.0). All the components of the buffer were procured from (Himedia, India). Concentration and purity of the DNA were determined using bio-spectrophotometer (Eppendorf, Germany) at (260/280 nm), and also confirmed by 0.8% agarose gel electrophoresis. The gel was observed under Geldoc system (G: BOX, Syngene, and U.K). The final volume of DNA was made to 10 ng.

Polymerase chain reaction (PCR) analysis

PCR amplification was performed using a final reaction volume of 20 µl. The reaction mixture was prepared by addition of 10X PCR buffer consisting of 15 mM $MgCl_2$ (Thermo Fisher Scientific, US), 2.5 mM dNTP Mix (Thermo Scientific, US), (1 unit/µl) Taq DNA polymerase, 1 µl of RAPD primer (Operon Technologies, USA). A total of 20, 10-mer primers were used for this study in TV21 (Table 1) and TV19 (Table 2) cultivars, respectively. Amplification of DNA was carried out on a 96 well Master cycler Pro S (Eppendorf, Germany) using following cycling program, Initial denaturation at 94 °C for 5 min followed by 40 cycles each at 94 °C for 1 min, extension at 72 °C for 2 min and final extension at 72 °C for 7 min. All other conditions were maintained as such during PCR reaction, except for the annealing temperature, which varied between 45–55 °C depending on the Tm (melting temperature) of the primer. The amplified products were electrophoresed on 1.2% (w/v) agarose gel in 1X TBE buffer and stained with ethidium bromide. The gel was visualized under UV light and documented using (G: BOX,

Table 1 List of primers, their sequences and total number of bands and % monomorphism in TV21 cultivar of tea

Primer	Primer sequence (5′-3′)	Total number of bands amplified	Number of monomorphic bands	Monomorphism %
OPA-01	CAGGCCCTTC	8	8	100
OPA-02	TGCCGAGCTG	6	6	100
OPA-03	AGTCAGCCAC	9	9	100
OPA-04	AATCGGGCTG	4	4	100
OPA-05	AGGGGTCTTG	5	5	100
OPA-06	GGTCCCTGAC	4	4	100
OPA-07	GAAACGGGTG	3	3	100
OPA-08	GTGACGTAGG	1	1	100
OPA-09	GGGTAACGCC	6	6	100
OPA-10	GTGATCGCAG	10	10	90
OPA-13	CAGCACCCAC	8	8	100
OPH-02	TCGGACGTGA	10	10	100
OPV-06	ACGCCCAGGT	5	5	100
Total number of bands		79	79	100

Table 2 List of primers, their sequences and total number of bands and % monomorphism in TV 19 cultivar of tea

Primer	Primer sequence (5′-3′)	Total number of bands amplified	Number of monomorphic bands	Monomorphism %
OPA-01	CAGGCCCTTC	4	4	100
OPA-02	TGCCGAGCTG	8	8	100
OPA-03	AGTCAGCCAC	6	6	100
OPA-04	AATCGGGCTG	3	3	100
OPA-05	AGGGGTCTTG	7	7	100
OPA-06	GGTCCCTGAC	5	5	100
OPA-07	GAAACGGGTG	8	8	100
OPA-08	GTGACGTAGG	2	2	100
OPA-09	GGGTAACGCC	6	6	100
OPA-10	GTGATCGCAG	5	5	100
OPA-13	CAGCACCCAC	4	4	100
OPH-02	TCGGACGTGA	1	1	1
OPV-06	ACGCCCAGGT	3	3	100
Total number of bands		62	62	100

Syngene, U.K.) imaging system. The fragmentation pattern of the bands was studied using a 100 bp ladder (Thermo Fisher Scientific, USA).

Data analysis

All the data obtained were analyzed using *Graph Pad Prism 6* software (Graph Pad, California, US). The significant difference between mean values was found via Tukey's multiple comparison test at a 95% confidence level ($P < 0.05$).

Results

Embryo induction, maturation, development and recurrent secondary embryogenesis

The in vitro haploid embryos of the two cultivars, TV21 and TV19, of tea were routinely induced from the callus, obtained from inside anther locules bearing uni-nucleate microspores, on MS medium supplemented with 10 µM BAP + 3 µM GA_3 + 800 mg l^{-1} L-glutamine + 200 mg l^{-1} L-serine, within 4 weeks of culture (Embryo induction medium) (Fig. 1A). Single globular stage embryos from

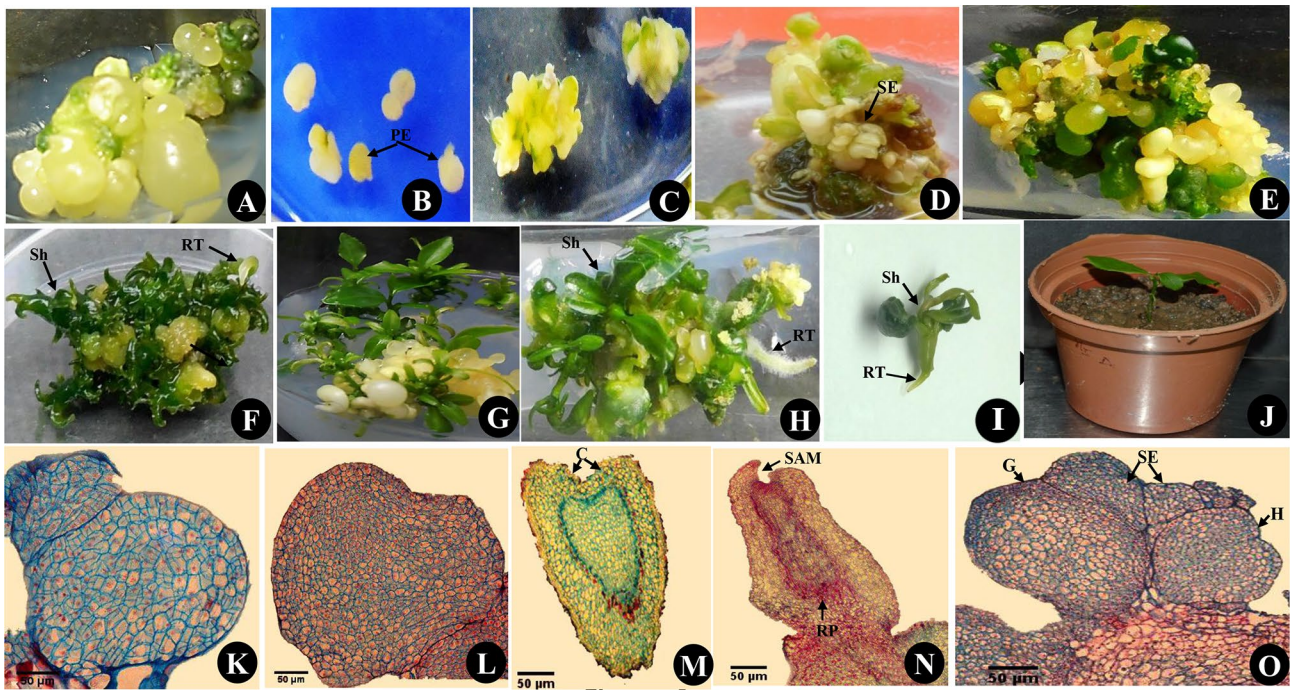


Fig. 1 Induction, maturation and germination in in vitro obtained haploid embryos and confirmation via histological analysis. **A** Induction of embryos on MS + 10 µM BAP + 3 µM GA_3 + 800 mg l^{-1} L-glutamine + 200 mg l^{-1} L-serine (×1.4X). **B** Initial stage single globular embryos with a notch inoculated on embryo maturation medium, MS + 18 µM ABA + 25 g l^{-1} mannitol (×1.7X). **C** Secondary embryos formation from primary embryos on same media as in B, (×1.4). **D** Embryo development on liquid MS + 1 µM BAP + 0.3 µM GA_3 + 80 mg l^{-1} L-glutamine + 20 mg l^{-1} L-serine after pre-treatment with ABA and osmoticum. (x 1.2X). **E** Same as D, Secondary embryogenesis from primary embryos on transfer into semi-solid embryo development medium showing all the developmental stages from globular, heart-shape, topedo shape to fully mature embryos on (×1 X). **F** Germination of embryos into cluster of shoots on prologed exposure to liquid MS + 10 µM BAP + 0.5 µM GA_3 + 1 µM IBA + 80 mg l^{-1} L-glutamine + 20 mg l^{-1} L—serine showing multiple short shoots (Sh) with leaves that did not unfold fully and with small roots (RT) (×0.6X). **G** Germination of embryos on liquid-to-semi-solid transition whereby embryos were inoculated on liquid MS + 10 µM BAP + 0.5 µM GA_3 + 1 µM IBA + 80 mg l^{-1} L-glutamine + 20 mg l^{-1} L—serine medium for first 4 weeks and then shifted to semi-solid medium of same composition. Embryos showing normal pattern of leaf development (×0.8X). **H** Same as **G**, where embryos germinated by giving rise to distinct well devloped cluster of green shoots (Sh) and prominent hairy white roots (RT) (×0.5X). **I** Individual germinated embryo from (**H**) showing complete well developed plantlet. **J** Hardening of plantlet into potting mixture consisting of soil and sand in 1:2 ratio (x 1X). **K** Histological section of initial stage single globular embryo (×50). **L** Histological section of a heart shaped embryo (×50). **M** Cotyledonary stage embryo with arrow marked at cotyledons (C) (×50). **N** Histological section of shoot proliferation from embryo showing arrows indicating shoot apical meristem (SAM) and root pole (RP) shoot with an arrow (×50). **O** Secondary embryos budding on the top of the primary embryos with large cluster of embryos upon transfer into semi-solid maturation medium. The globular embryos marked as (G), Heart (H) and secondary embryos are marked as (SE) (×50)

the embryo induction medium were pre-treated with ABA (18 µM) alone or in combination with one of the three osmotica, namely, 30 g l^{-1} PEG-400, 1 g l^{-1} glycine betaine or 25 g l^{-1}mannitol, for 20 days, to pass the embryos through maturation phase (Fig. 1B). Following the treatment, the primary embryos initiated secondary embryogenesis on embryo maturation medium, 18 µM ABA + 25 g l^{-1} mannitol (Fig. 1C). This is followed by transfer of embryos on to the development medium consisting of ten times reduced concentration of growth regulators present on embryo induction medium. An increase in cluster size with a simultaneous rise in the number of secondary embryos was found on embryo development medium when embryos were pre-treated with MS + 18 µM ABA + 25 g l^{-1} mannitol, for 20 days (Fig. 1D). The pre-treatment also favoured the primary globular embryos to grow and pass through all the developmental stages from globular, heart-shape, torpedo shape to fully matured dicotyledonous embryos on embryo development medium consisting of MS + 1 µM BAP + 0.3 µM GA_3 + 80 mg l^{-1} L-glutamine + 20 mg l^{-1} L-serine (Fig. 1E).

The highest mean number of secondary embryos with fivefold and threefold increase was attained in TV21 (Fig. 2A) and TV19 (Fig. 2B) cultivars, respectively, on maturation medium when primary globular embryos were pre-treated with 18 µM ABA + 25 g l^{-1} mannitol. An average of 59.6 ± 4.1 and 45.3 ± 3.0 embryos were produced in TV21 and TV19 cultivars, respectively. Pre-treatment with glycine

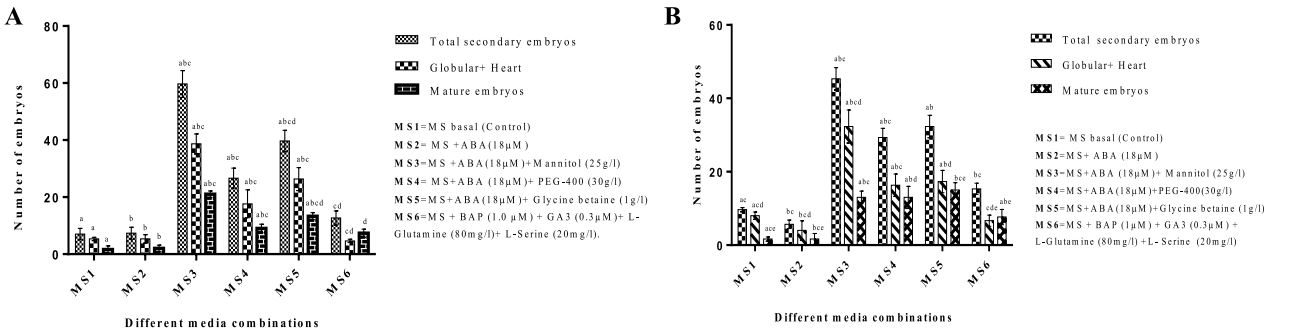


Fig. 2 Effect of different media combinations on secondary embryogenesis in TV21 and TV19 cultivars of tea. (Values are mean of three independent experiments. Mean values sharing the same letter in different media combination do not differ significantly ($p < 0.05$) according to Tukey's multiple range test

betaine or PEG in combination with ABA also enhanced secondary embryogenesis as compared to the development medium without pre-treatment (control) but overall response was inferior to ABA + mannitol treatment. The least number of secondary embryos were attained in medium supplemented with ABA alone.

Effect of carbohydrate source on secondary embryogenesis

The effect of two carbohydrate sources, glucose and sucrose, in the range of 1% to 3% concentrations were tested to promote secondary embryogenesis in liquid embryo development medium on MS + 1 μM BAP + 0.3 μM GA_3 + 80 mg l^{-1} L-glutamine + 20 mg l^{-1} L-serine. The highest multiplication rate with the maximum number of secondary embryos, 36 ± 2.6 in TV21 (Fig. 3A) and 34 ± 3.4 in TV19 (Fig. 3B) were obtained within 4 weeks when liquid medium was supplemented with 3% glucose. However, the number of mature embryos and the embryos showing germination was highest in the medium supplemented with 3% sucrose. This could be due to the slow hydrolysis of sucrose in the medium providing effective source of carbon for longer duration, thereby promoting maturation and germination. The average number of secondary embryos produced on sucrose supplemented media are 13.3 ± 1.5 in TV21 and 13.7 ± 2.0 in TV19.

Embryo germination and plantlet development

The cluster of fully developed mature embryos with small shoots (~ 1 cm) from embryo development medium were transferred to germination medium consisting of MS medium supplemented with 10 μM BAP + 0.5 μM GA_3 + 1 μM IBA + 80 mg l^{-1} L-glutamine + 20 mg l^{-1} L-serine, following liquid-to-semi-solid transition method (Germination medium). The method promoted highest percentage of embryo germination, 66.6% and 30.3% in TV21 and TV19 cultivars, respectively, with well developed shoots and roots when embryos were initially kept for four weeks in liquid germination medium, followed by their transfer to semi-solid medium on similar medium composition (Fig. 4). Liquid medium alone supported

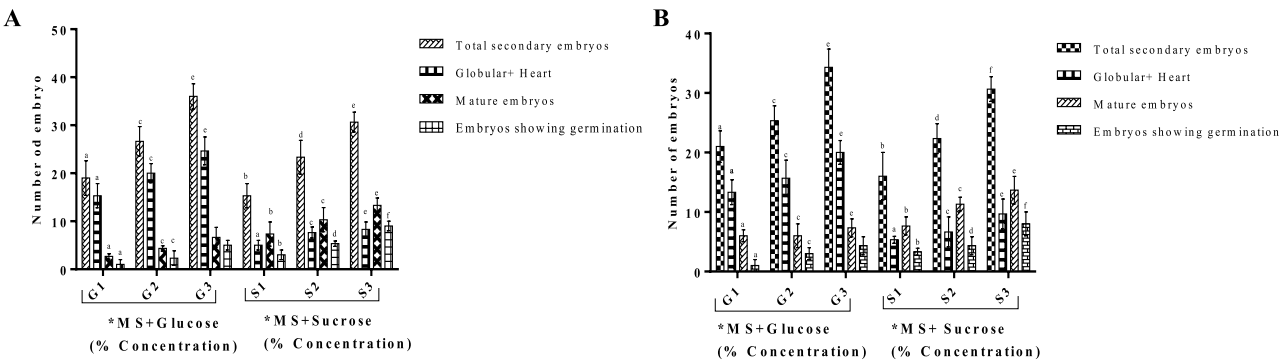


Fig. 3 Effect of carbohydrate source on secondary embryogenesis in TV21 and TV19 cultivars, where 1%, 2% and 3% are concentrations of glucose and sucrose utilized. (Values are mean of three independent experiments. Mean values sharing the same letter in media with different combinations of carbon source do not differ significantly ($p < 0.05$) according to Tukey's multiple range test)

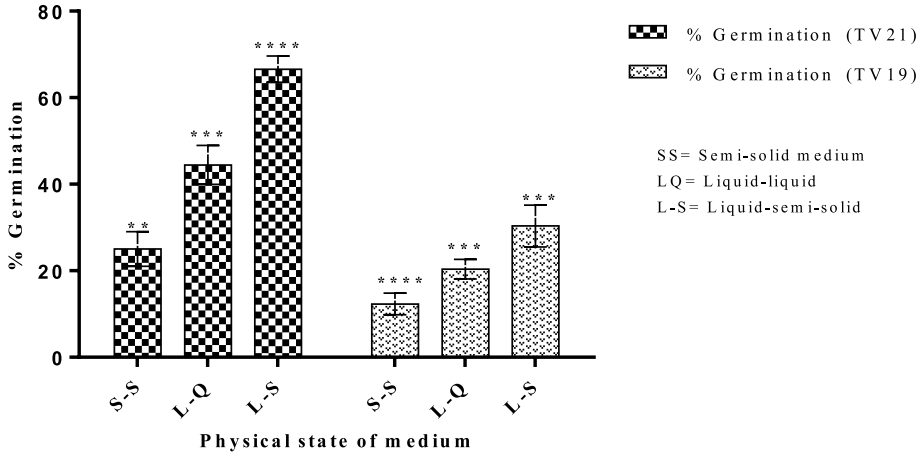


Fig. 4 Effect of physical state of medium on % germination in TV21 and TV19 cultivars of tea. S–S = semi-solid medium, L-Q = liquid medium, L-S = Liquid to semi solid medium transition. (Values are mean of three independent experiments. Mean values sharing the same character do not differ significantly ($p < 0.05$) according to Tukey's multiple range test)

44.4% and 20.4% germination in TV21 and TV19 cultivars, respectively, where majority of embryos exhibiting bipolar germination. However, prolonged exposure in the liquid medium produced short shoots with leaves that did not unfold fully due to hyperhydricity (Fig. 1F). Continuous culture of secondary embryos in semi-solid medium yielded lowest germination percentage, 25% in TV21 and 12% in TV19 cultivars and slow growth. Moreso, it nearly took ten days extra germination time when compared to liquid-to-semi-solid transition method. In liquid-to-semi-solid transition method, higher rate of embryo germination with normal pattern of leaf development was found when the germinating embryos were shifted after four weeks from liquid medium to semi-solid medium with similar medium composition (Fig. 1G). The embryos germinated by giving rise to well developed shoots and roots (Fig. 1H, I). The individual plantlets were hardened successfully on potting mixture consisting of soil and sand in 1:2 ratio and acclimatized (Fig. 1J).

Histological and FESEM analyses

Histological sections at different developmental stages of embryos, globular, heart-shaped, and cotyledonary stage and those showing apical meristem can be seen in (Fig. 1K–N). Figure 1O, on the other hand, represents secondary embryos on the top of the pre-existing primary embryos. Sections of embryos were cut to know, if there are any anatomical abnormalities with the change in the physical state of the medium. The embryos were normal showing apical meristem and closed vascular strand with bipolar axes. In addition to the anatomical studies, the morphology of the somatic embryos was studied through FESEM analysis where each embryo showed secondary embryogenesis on top of the primary embryos (Fig. 5A–D).

Ploidy determination

Flow cytometry was used for rapid determination of ploidy level of a large number of plant samples. The results obtained from flow cytometric analysis of leaves from in vitro haploid plants, and field grown parent (control) plants of TV21 and TV19 cultivars are represented in Fig. 6 and Fig. 7, respectively. The leaves from haploid plants of TV21 cultivar showed G1 peak at channel position 353 and G2 peak at 770 channel (Fig. 6A). In contrast to this, the G1 peak from the leaves of field grown parent plants of TV21 was detected at channel position 732 and G2 peak at 1514 (Fig. 6B), which was approximately double that in haploid plants. A similar data plot was found in TV19 cultivar, where, the leaves of in vitro regenerated haploid plants showed G1 peak at channel position 365 and G2 at 756 (Fig. 7A). The data plot of the leaves from TV19 parent plant (control) showed G1 peak at channel position 716 and G2 at 1389 channel position (Fig. 7B).

Similarly, as an independent verification of ploidy, the chromosome number per cell in the root-tips from the in vitro rooted haploid plantlets and the parent (control) plants were determined. The root-tips of haploid plantlets of TV21 (Fig. 6C) and TV19 (Fig. 7C) revealed a chromosomal count $2n = X = 15$. While the root-tips squash preparations from the parent (control) TV21 (Fig. 6D) and TV19 cultivars (Fig. 7D), exhibited a chromosome count $2n = 2X = 30$.

Clonal fidelity assessment

Clonal fidelity assessment was performed to ensure the genetic stability among 11 in vitro regenerated haploid plant lines of TV21 cultivar and 10 in vitro regenerated haploid plant lines of TV19 cultivar. DNA samples from in *vivo* donor plant (control) of both the varieties were also analyzed to confirm its similarity with regenerants. A total

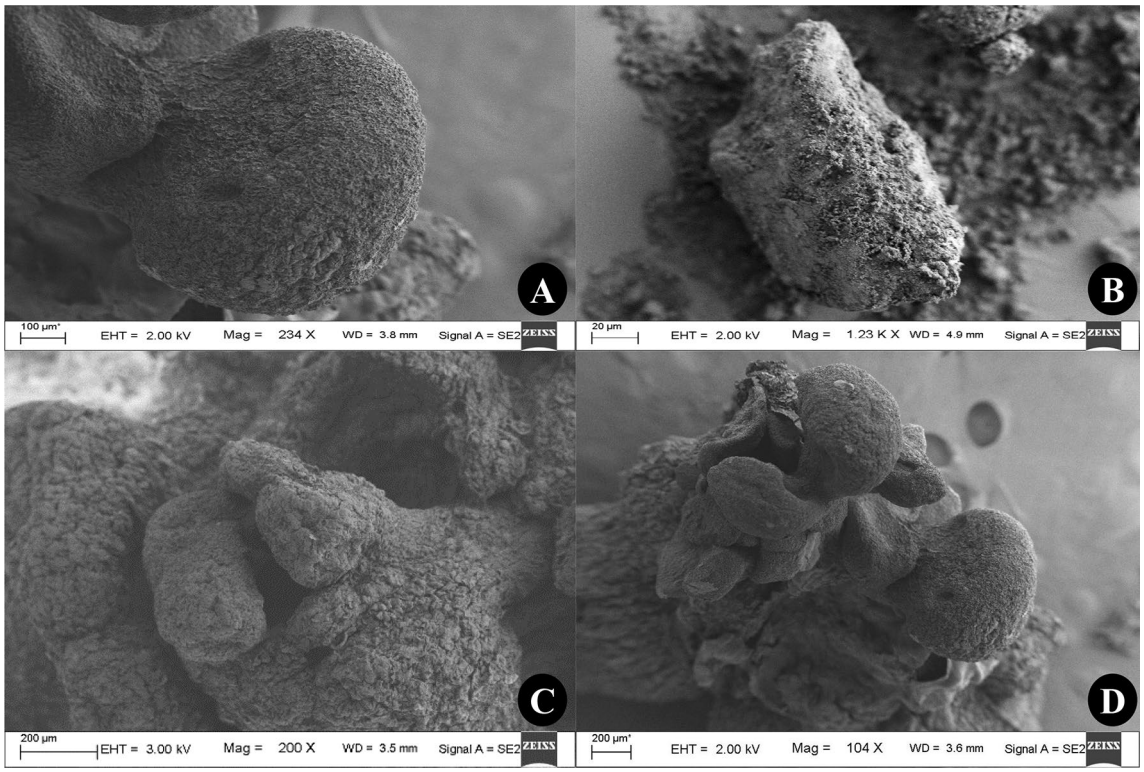


Fig. 5 Field Emission Scanning Electron Microscopic Analysis. **A** Initial stage single globular embryo (×234). **B** A torpedo-shaped embryo (×123). **C** Cluster of embryos with a globular and a heart shaped embryo (×200). **D** Secondary embryogenesis (×104)

of 20 RAPD primers were initially screened, among which only 13 primers gave clear scorable bands. The number of clearly amplified bands varied from one to ten with different primers in TV21 cultivar. Total 79 bands were obtained with an average number of about 6 bands per primer (Table 1). The number of scorable bands in TV19 varied from one to eight depending on the primer. Total 62 bands were obtained in total from 13 primers, with an average number of 4.8 bands per primer (Table 2). Distinct banding pattern in both the cultivars in a range of 100 bp to 2500 bp was obtained. The maximum number of bands (10) was attained in TV21 variety with OPA-10 and OPH-02 primer. While maximum 8 bands were found in TV19 with OPA-07 and OPA-02. No polymorphism was detected with any of the primers, ensuring genetically uniform lines of in vitro haploid plants in both TV21 (Fig. 8A) and TV19 (Fig. 8B). The pattern of bands obtained in control (donor) in vivo plant samples of bothTV21 and TV19 resembled the banding pattern of their respective in vitro regenerated plants confirming lack of variation between the androgenic haploid plantlets and their parental lines. The feature of achieving plantlets via secondary embryogenesis, which is reported to generate true-to-type plants could be the reason for zero polymorphism.

Discussion

Recurrent secondary embryogenesis is an efficient method for regeneration in difficult-to-propagate plant species, specifically trees. The technique is not only useful for the production of a large number of plants in a relatively short time, but also is highly advantageous providing benefits of independent cell origin, maintaining genetic integrity within micropropagated lines and facilitating easy automation (Burns and Wetzstein, 1997; Guan et al. 2016). Development of genetically stable, pure breeding lines in perennial trees, such as tea, through traditional methods is impractical (Mishra et al. 2017). In vitro haploid plant production in such plants, serves as the only feasible option for generation of homozygous elite clones (Germana 2011). Strenuous efforts have been going on for obtaining haploid plants in tea for years, but, almost all of them remained confined to the generation of haploid callus (Seran et al. 1999). Mishra et al. (2017), for the very first time, reported the development of complete haploid plantlets. Initial attempts of establishing embryogenic lines from androgenic cultures in tea was taxing and time-consuming. Thus, we aimed to maintain and preserve

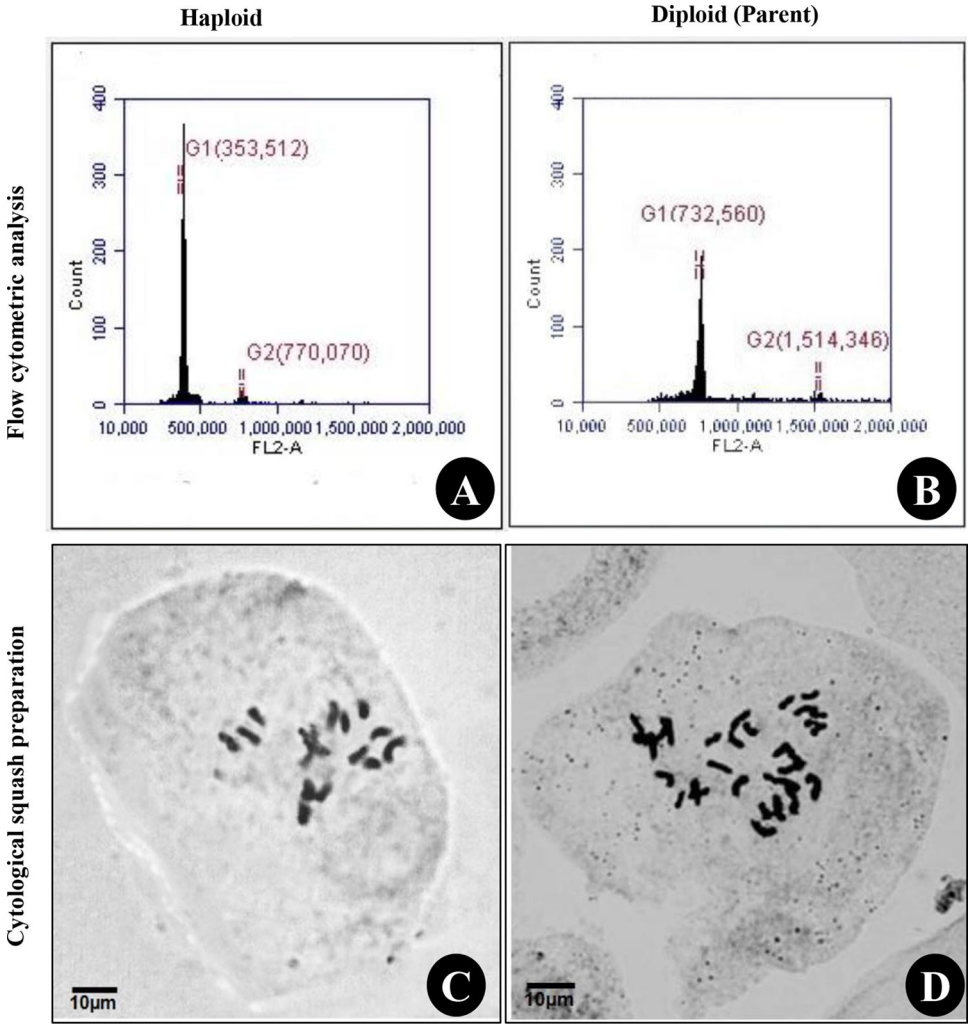


Fig. 6 Ploidy Determination in vitro regenearted haploid and parent (control) plants of TV21 cultivar. **A** Flow cytometric data from in vitro regenerated haploid shoots showing G1 peak at 353 and G2 peak at 770 channel positions. **B** Flow cytometric data from parent plant (control) showing G1 peak at 732 and G2 peak at 1514 channel positions respectively. **C** Cytological squash preparation from root-tip of in vitro regenerated haploid palntlets showing chromosome number 2n = X = 15 (×100). **D** Cytological squash preparation from field grown parent plant showing chromosome number 2n = 2X = 30 (×100)

these precious resources via regular subculture of primary embryos to produce secondary embryogenesis in both TV21 and TV19 cultivars. The haploid embryogenic cultures were multiplied using liquid-to-semi-solid transition method, and they underwent complete bipolar germination, and formed cluster of shoots. Haploid embryos of tea demonstrated a high potential for plant regeneration via secondary embryogenesis. However, the number of secondary embryos produced was very less. A pre-treatment of initial globular stage embryos with ABA and osmoticum for 20 days in liquid medium followed by transfer to embryo development medium, effectively increased the rate of secondary embryo formation by fivefold in TV21 cultivar and a threefold in TV19, respectively.

ABA is one of the five essential growth regulators that promote somatic embryo induction (Balasubramanium et al. 2000). It causes accumulation of food reserves and promotes embryo maturation and germination (Guttman et al. 1996; Langhansova et al. 2004) Osmoticum, on the other hand, creates desiccation like condition that promotes embryogenesis (Ozudogru et al. 2004). It has a tendency of creating water stress, thereby enhancing embryo maturation (Suganthi et al. 2012). ABA alone in majority of reports including tea, has been found to cause no positive effect (Roberts 1991; Akula et al. 2000a, b). Nevertheless, when added in combination with osmoticum like PEG, mannitol, sorbitol, betaine, stimulates both embryogenesis and embryo maturation (Suganthi et al. 2012). The results of our study also fall in line with these reports. In the current study, ABA alone did not cause any rise in number of secondary embryos, however, when added in combination with either of the three osmotica; mannitol, PEG and glycine betaine caused a rapid increase in the number of secondary embryo formation with simultaneous maturation. The best response among the three osmotica, however, was attained on MS media supplemented with ABA in combination with mannitol. Besides acting as

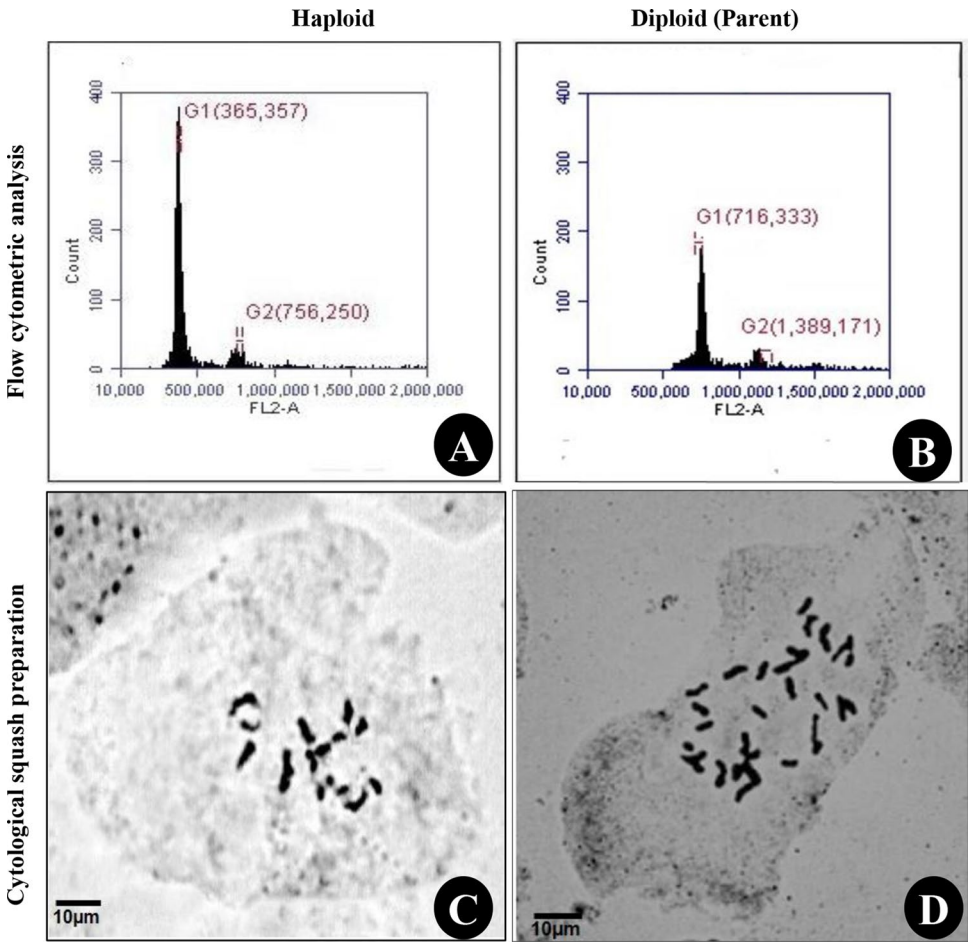


Fig. 7 Ploidy Determination of in vitro regenearted haploid and parent (control) plants of TV19 cultivar.**A** Flow cytometric data from in vitro regenerated haploid shoots showing G1 peak at 365 and G2 peak at 756 channel positions. **B** Flow cytometric data from parent plant (control) showing G1 peak at 716 and G2 peak at 1389 channel position. **C** Cytological squash preparation from root-tip of in vitro regenerated haploid palntlets showing chromosome number 2n = X = 15 (×100). **D** Cytological squash preparation from field grown parent plant showing chromosome number 2n = 2X = 30 (×100)

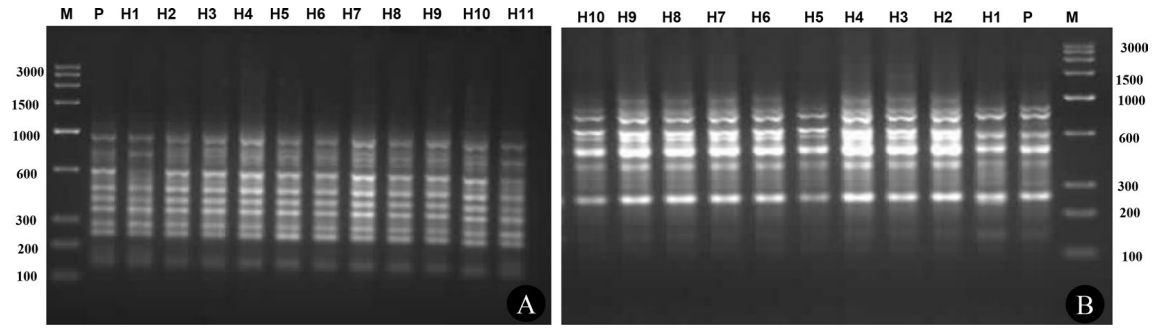


Fig. 8 Clonal fidelity assessment in in vitro regenerated haploid lines and parent plant of TV21 and TV19 cultivars of tea. **A** RAPD amplification profile with primers OPA-13. Lane 1 represents the 100 bp ladder (M) followed by the in vivo parent plant (P) in lane 2. Lane 3–13 labelled H1-H11 are the in vitro regenerated haploid plants lines. **B** RAPD amplification profile with primers OPA-09. Lane 1 represents the 100 bp ladder (M) followed by the in vivo parent plant (P) in lane 2. Lane 3–12 labelled H1-H10 are the in vitro regenerated haploid plants lines

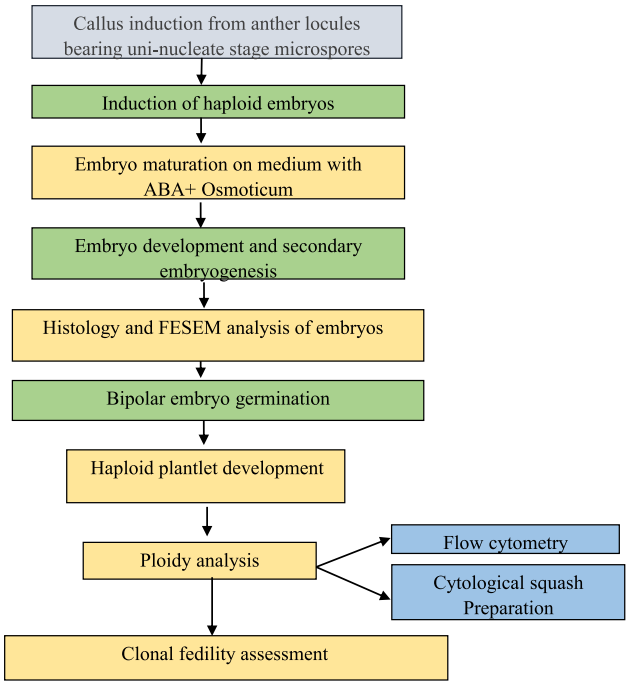
a source osmoticum, mannitol is also a source of carbohydrate. Therefore, when added in medium, it plays a dual role of acting as a carbon source and an osmoticum resulting in increased secondary embryogenesis (Roberts 1991; Ozudogru et al. 2004). The combination of ABA and mannitol has also been reported to increase the rate of embryo formation and maturation to cotyledonary stage embryos in *Picea glauca engelmanni* complex (Roberts 1991) and in *Cucumis sativus* L. cv. 'Shimoshirazu'(Lou and Kako 1995) and has also been reported to increase somatic embryogenesis in tea (Ozudogru et al. 2004; Aoshima 2005). None of the previous reports on Tea received secondary embryogenesis in haploid embryos of *Camellia assamica* ssp. *assamica* (TV21) and *Camellia assamica* ssp. *lasiocalyx* (TV19). Akula et al. (2000a, b), reported embryogenesis on media containing ABA with glycine betaine while inducing direct somatic embryogenesis from mature seeds in *Camellia sinensis* (L) O. Kuntze. Suganthi et al. (2012) on the other hand reported direct somatic embryogenesis using cotyledonary explant from seeds of UPASI-9, UPASI-10, ATK-1, and UPASI-17 cultivars of tea on a medium consisting of ABA in combination with all three osmoticum mannitol, PEG and glycine betaine. The authors, however, found less response in medium with ABA alone as compared to that with osmoticum. The response varied with variety but in general maximum response was reported in medium consisting of PEG followed by medium containing with mannitol. In our findings on tea, ABA alone did not give a promising result, but an increment secondary embryogeneis was obtained on media having ABA with at least one osmoticum. However, maximum number of secondary embryos in our case was obtained in media containing ABA with mannitol. Better maturation with early germination of embryos was found after pre-treatment with ABA and osmoticum in liquid medium followed by transfer into medium containing BAP and GA_3. It could be because BAP is a cytokinin that promotes cell division and is known to overcome any dormancy induced by ABA (Guan et al. 2014). GA_3 also breaks ABA-induced dormancy in cultures and promotes germination (Dunwell 1986).

Sandal et al. (2001) reported abnormality in liquid cultures while performing shoot proliferation in tea. In the current investigation, we studied the effect of the physical state of the medium on embryo germination in both TV21 and TV19 cultivars. Three different states of medium semi-solid, liquid–liquid, and a liquid-semi-solid transition were tried. Maximum germination with 66.6% and 30.3% were found respectively in TV21 and TV19 cultivars, in liquid-to-semi-solid medium transition. Liquid medium was equally good, but continuous immersion in liquid caused abnormal foliature in leaves. The lowest yield was found in semi-solid medium alone. The liquid medium is always chosen over semi-solid medium due to uniform nutrient distribution, better uptake of media components by explants and dilution of the phenolic exudates, which reduce growth (Akula et al. 2000a, b). But, its utility is limited due to the physiological abnormality such as swollen leaves or anatomical defects observed in the plants during continuous immersion (Sandal et al. 2001). A liquid-to-semi-solid medium transition is, therefore, a better alternative of attaining a higher multiplication with no abnormality in plants. We report successful plant regeneration following liquid-semi-solid medium transition with no anatomical and morphological defect in regenerated plants. Techniques such as histology and FESEM were tried to elucidate any morphological or anatomical abnormality.

Clonal fidelity of the plants was accessed to rule out any somaclonal variation induced during repeated cycles of subculture. Twenty different RAPD primers were used to check the genetic stability within in vitro cultures and their similarity with their respective in vivo parental (control) plants. Zero polymorphism was found among the in vitro haploid plants in both cultivars. The banding pattern of the in vitro lines were highly resembled with their respective parental plants representing that the androgenic plants have been originated from the parental plants with no variation. Secondary embryogenesis is reported as one of the best modes for true-to-type plant regeneration (Bano et al. 1991; Akula et al. 2000a, b). Thus the haploid plantlets obtained in the present study were genetically uniform.

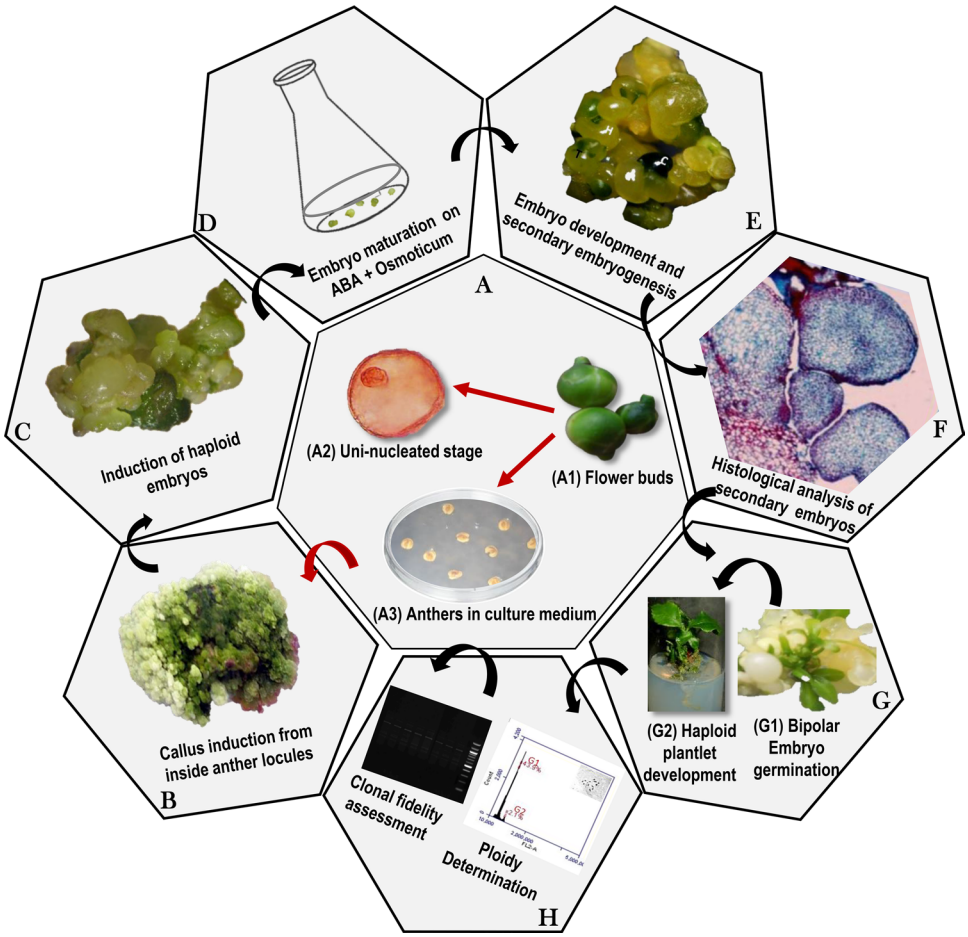
Conclusion

It is concluded that ABA alone did not promote embryogenesis in tea. However, when added with an osmoticum, it promoted recurrent repetitive secondary embryogenesis. Among all the osmotica tried, ABA in combination with mannitol was the best to facilitate maximum secondary embryogenesis. Complete plants with intact anatomical and morphological development was achieved, following liquid-semi-solid medium transition strategy. Clonal fidelity of the plants was assessed to ruled out any somaclonal variation induced during repeated cycles of embryo subculture. The banding pattern of the in vitro lines were highly resembled with their respective parental plants



Flow Chart Recurrent embryogenesis in Tea

suggesting that the androgenic plants originated from the parental plants with no variation. Secondary embryogenesis is reported as one of the best modes for true-to-type plant regeneration. The haploid plantlets obtained in the present study via recurrent secondary emnbryogenesis were genetically uniform. The protocol for the recurrent embryogeneis established in this research (see Flow Chart) could be used for easy scale-up using photobiorecator for large scale production of true-to-type haploid plantlets. Schematic representation of recurrent secondary embryogenesis from androgenic haploid embryos, ploidy determination and clonal fidelity assessment of haploid plants using RAPD marker is presented in Scheme 1. The protocol established would also serve as an excellent explant source for chromosome duplication to generate planting material with novel genetic constitution, attaining which otherwise would not be fessible.



Scheme 1 Schematic representation of recurrent secondary embryogenesis from androgenic haploid embryos and clonal fidelity assessment of haploid plants using RAPD marker. Pictures labelled as A-H in the figure, indicates the step-wise methods involved in development of haploid plants, ploidy determination and clonal fidelity assessment. The arrows in red highlight initiation of haploid callus

Acknowledgements The authors thank Department of Biotechnology, Government of India, for financial assistance. We also wish to thank Prof. B.S Bhau, and Miss Marine Hussain for their help in conducting experiments on marker analysis.

Author contributions The corresponding author, RC conceived and designed the research and co-author, RB conducted the expeiments. Both the authors have written, read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Akula A, Akula C (1999) Somatic embryogenesis in tea (*Camellia sinensis* (L.) O. Kuntze). In: Jain SM, Gupta PK, Newton RJ (eds) Somatic embryogenesis in woody plants. Kluwer Academic Publishers, Netherlands, pp 239–259

Akula A, Dodd WA (1998) Direct somatic embryogenesis in a selected tea clone, TRI-2025' (*Camellia sinensis* (L.) O. Kuntze) from nodal explants. Plant Cell Rep 17:804–809

Akula A, Akula C, Bateson M (2000a) Betaine a novel candidate for rapid induction of somatic embryogenesis in tea (*Camellia sinensis* (L.) O. Kuntze). Plant Growth Regul 30:241–246

Akula A, Becker D, Bateson M (2000b) High-yielding repetitive somatic embryogenesis and plant recovery in a selected tea clone, 'TRI-2025', by temporary immersion. Plant Cell Rep 19:1140–1145

Aoshima Y (2005) Efficient embryogenesis in the callus of tea (*Camellia sinensis*) enhanced by the osmotic stress or antibiotics treatment. Plant Biotechnol J 22:277–280

Balasubramanium S, Marimuthu S, Rajkumar R, Haridas V (2000) Somatic embryogenesis and multiple shoot induction in *Camellia sinensis* (L.) O Kuntze. J Plant Crops 28:44–49

Bano Z, Rajarathnam S, Mohanty BD (1991) Somatic embryogenesis in cotyledon cultures of tea (*Thea sinensis*, L.). J Hort Sci 66:465–470

Bhau BS, Gogoi G, Baruah D, Ahmed R, Hazarika G, Borah B, Wann SB (2015) Development of an effective and efficient DNA isolation method for *Cinnamomum* species. Food Chem 188:264–270

Bhojwani SS, Razdan MK (1996) Plant tissue culture: theory and practice (revised edn). Elsevier, Amsterdam, Lausanne, New York

Burns JA, Wetzstein HY (1997) Development and characterization of embryogenic suspension cultures of pecan. Plant Cell Tiss Org Cult 48:93–102

Chan EWC, Lim YY, Chew YL (2007) Antioxidant activity of *Camellia sinensis* leaves and tea from a lowland plantation in Malaysia. Food Chem 102:1214–1222

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

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12:39–40

Dunwell JM (1986) Pollen, ovule and embryo culture as tools in plant breeding. In: Alderson PG, Withers LA (eds) Plant tissue culture and its agricultural applications. Butterworth, London, pp 375–404

Germana MA (2011) Anther culture for haploid and doubled haploid production. Plant Cell Tiss Org Cult 104:283–300

Guan C, Wang X, Feng J, Hong S, Liang Y, Ren B, Zuo J (2014) Cytokinin antagonizes abscisic acid-mediated inhibition of cotyledon greening by promoting the degradation of abscisic acid insensitive5 protein in Arabidopsis. Plant Physiol 164:1515–1526

GuanY LSG, Fan XF, Su ZH (2016) Application of somatic embryogenesis in woody plants. Front Plant Sci 7:938–943

Gutmann M, Von Aderkas P, Label P, Lelu MA (1996) Effects of abscisic acid on somatic embryo maturation of hybrid larch. J Exp Bot 47:1905–1917

Jain SM, Newton RJ (1990) Prospects of biotechnology for tea improvement. Proc Natl Acad Sci India Sect B Biol Sci 56:441–448

Jha T, Sen SK (1992) Micropropagation of an elite Darjeeling tea clone. Plant Cell Rep 11:101–104

Kato M (1996) Somatic embryogenesis from immature leaves of *in vitro* grown tea shoots. Plant Cell Rep 15:920–923

Langhansova L, Konradova H, Vaněk T (2004) Polyethylene glycol and abscisic acid improve maturation and regeneration of Panax ginseng somatic embryos. Plant Cell Rep 22:725–730

Lou H, Kako S (1995) Role of high sugar concentrations in inducing somatic embryogenesis from cucumber cotyledons. Sci Hortic 64:11–20

Martínez MT, Vieitez AM, Corredoira E (2015) Improved secondary embryo production in *Quercus alba* and *Q. rubra* by activated charcoal, silver thiosulphate and sucrose: influence of embryogenic explant used for subculture. Plant Cell Tiss Org Cult 121:531–546

Mishra VK, Bajpai R, Chaturvedi R (2017) An efficient and reproducible method for development of androgenic haploid plants from *in vitro* anther cultures of *Camellia assamica* ssp. *assamica* (Masters). Vitro Cell Dev-Plant 53:239–248

Mondal TK (2014) Breeding and biotechnology of tea and its wild species. Springer Science & Business Media, New York

Mondal TK, Bhattacharya A, Sood A, Ahuja PS (2002) Factors affecting germination and conversion frequency of somatic embryos of tea [*Camellia sinensis* (L.) O. Kuntze]. J Plant Physiol 159:1317–1321

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

Mukhopadhyay M, Mondal TK, Chand PK (2016) Biotechnological advances in tea (*Camellia sinensis* [L.] O. Kuntze): a review. Plant Cell Rep 35:255–287

Nair RR, Gupta SD (2006) High-frequency plant regeneration through cyclic secondary somatic embryogenesis in black pepper (*Piper nigrum* L.). Plant Cell Rep 24:699–707

Orel G, Wilson PG (2012) *Camellia cherryana* (Theaceae) a new species from China. Ann Bot 49:248–254

Ozudogru EA, Ozden-Tokatli Y, Akcin A (2004) Effect of mannitol and ABA on induction of somatic embryogenesis in tea (*Camellia sinensis* L.). In: International symposium on *in vitro* culture and horticultural breeding. Vol 725, pp 411-416

Pais MS (2019) Somatic embryogenesis induction in woody species. The future after omics' data assessment. Front Plant Sci 10:240

Paratasilpin T (1990) Comparative studies on somatic embryogenesis in *Camellia sinensis* var. *sinensis* and *C. sinensis* var. *assamica* (Mast.) Pierre. J Sci Soc 16:23–41

Ponsamuel J, Samson NP, Ganeshan PS, Sathyaprakash V, Abraham GC (1996) Somatic embryogenesis and plant regeneration from the immature cotyledonary tissues of cultivated tea (*Camellia sinensis* (L). O. Kuntze). Plant Cell Rep 16:210–214

Roberts DR (1991) abscisic acid and mannitol promote early development, maturation and storage protein accumulation in somatic embryos of interior spruce. Physiol Plant 83:247–254

Sandal I, Bhattacharya A, Ahuja PS (2001) An efficient liquid culture system for tea shoot proliferation. Plant Cell Tiss Org Cult 65:75–80

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

Seran TH, Hirimburegama K, Gunasekare MTK (2006) Direct somatic embryogenesis from explants obtained from *in vitro* germinated embryonic axes of *Camellia sinensis* (L.) O Kuntze. J Hortic Sci Biotech 81:883–890

Sharma P, Pandey S, Bhattacharya A, Nagar PK, Ahuja PS (2004) ABA associated biochemical changes during somatic embryo development in *Camellia sinensis* (L.) O Kuntze. J Plant Physiol 161:1269–1276

Shi X, Dai X, Liu G, Zhang J, Ning G, Bao M (2010) Cyclic secondary somatic embryogenesis and efficient plant regeneration in camphor tree (*Cinnamomum camphora* L.). In Vitro Cell Dev Biol Plant 46:117–125

Sivapalan P, Jain NK (1999) Pest management in tea. In: Jain NK (ed) Global advances in tea science. Aravalli Books, New Delhi, pp 625–646

Suganthi M, Arvinth S, Kumar RR (2012) Impact of osmotica and abscisic acid on direct somatic embryogenesis in tea. Int J Plant Res 2:22–27

Zimmerman JL (1993) Somatic embryogenesis: a model for early development in higher plants. Plant Cell 5: 1411