Journal of Biotechnology 361 (2023) 89–98

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

In vitro production of doubled haploid plants in *Camellia* spp. and assessment of homozygosity using microsatellite markers

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ARTICLE INFO

Keywords:
Haploid
Colchicine
Doubled haploid
Chromosome
Flow cytometry
SSR marker

ABSTRACT

In this report, *in vitro* doubled haploid (DH) plants were established in two tea (*Camellia* spp) cultivars, TV21 (Assam Type) and TV19 (Cambod Type). Androgenic globular stage haploid embryos, obtained via callusing from microspores at an early-to-late uninucleate stage in anther cultures, were diploidized by colchicine treatments at varying concentrations and durations under dark incubation at 25 ± 2 °C temperature. Thereafter, treated embryos were transferred to development medium, Murashige and Skoog (MS) medium supplemented with 6-benzylaminopurine (BAP; 1 μM) + gibberellic acid (GA_3; 0.3 μM) + L-glutamine (80 mg l^{-1}) + L-serine (20 mg l^{-1}) and incubated in diffused light. Ploidy of germinating embryos was evaluated by flow-cytometry and cytological squash preparation. High chromosome doubling, 76.89% and 67.34%, was obtained in embryos of TV21 and TV19, respectively, at 0.2% colchicine treatment for 24 h. The DH plants were further multiplied via axillary-bud proliferation on multiplication medium, MS + glucose (30 g l^{-1}) + BAP (5 μM) + GA_3 (0.5 μM) + IBA (0.5 μM) + L- glutamine (80 mg l^{-1}) + L-serine (20 mg l^{-1}). Rooting of shoots was achieved on $^1/_3$ MS basal medium within 50 days of inoculation when shoots were pre-treated with IBA (175 μM) for ten days. The rooted plants were acclimatized in field. Homozygosity in diploidized plants was validated by SSR marker.

1. Introduction

Camellia assamica ssp. commonly known as "Tea" is an agro-industrially important crop, and a globally recognized non-alcoholic beverage crop that is highly valued for its stimulating effect, aroma and health benefitting properties (Mondal et al., 2004; Hazarika et al., 2013). It is an evergreen, perennial tree with immense medicinal value and vast commercial significance. It provides means of livelihood for thousands of tea industry workers and generates monetary benefits to countries producing it (Majumder et al., 2010; Hazarika et al., 2013). Although the global demand for tea is rising with each year, the absence of genetically stable elite clones poses challenge to suffice the requirements. Obtaining genetic improvement in this tree has always been a challenge, owing to its inherent, intense cross-pollinating nature and long gestation period (Mondal et al., 2004; Mukhopadhyay et al., 2016; Mishra et al., 2017).

Tea cultivation via conventional methods, involving vegetative cuttings and seeds, has been practiced since decades to meet the growing industrial demand (Mukhopadhyay et al., 2016). But, obtaining a sufficient amount of elite planting material remains operose due to slow multiplication rate, poor survivability of cuttings, and high variability obtained in seed-borne plants (Mondal et al., 2004). Attaining genetic improvement in woody perennials following traditional breeding strategy has always been inept and unpredictable, as it requires 7–8 recurrent cycles of inbreeding (Srivastava and Chaturvedi, 2008). The tea is excessively out-breeding tree and, hence, conventional methods cannot be implemented. However, *in vitro* induction of haploid plants followed by artificial chromosomes doubling has been suggested to be the most reliable, speedy, single-step method for attaining homozygous breeding lines (Srivastava and Chaturvedi, 2008; Mishra et al., 2017). The technique has been successfully implemented in several plants, such as *Pyrus communis* L. (Bouvier et al., 2002), *Prunus avium* L. (Höfer and Grafe, 2003), *Quercus suber* L. (Pintos et al., 2007), *Plantanus acerifolia* (Liu et al., 2007), *Eucalyptus grandis* W. Hill ex Maiden (Silva et al., 2019).

Decades of strenuous efforts were made to obtain haploid plants in tea. However, all of it remained confined to the generation of haploid callus, until a pioneering report on regeneration of complete haploid plantlet in tea by Mishra et al. (2017). This breakthrough achievement of haploid plant generation in tea became an inspiration to conduct the current investigation for the successful attainment of homozygous

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https://doi.org/10.1016/j.jbiotec.2022.11.019
Received 30 March 2022; Received in revised form 25 November 2022; Accepted 30 November 2022
Available online 5 December 2022

breeding lines in tea via artificial chromosome duplication method.

Chromosome duplication finds immense application in plant breeding programs facilitating the speedy accomplishment of fertile, homozygous breeding lines from *in vitro* or *in vivo* developed sterile haploid plants. Haploids are diploidized spontaneously though a rare phenomenon in nature (Pintos et al., 2007). Diploidization could also be induced artificially by the application of antimitotic agents, like colchicine, oryzaline, amiprophos-methyl (APM), trifluralin, and pronamide at a particular concentration for the specific time duration (Pintos et al., 2007). Antimitotic agents are chemical compounds, which prevent microtubule polymerization and hinder the formation of spindle fibers. This, in turn, restricts the movement of chromosomes towards the poles, which consequently get arrested in the metaphase stage (Shao et al., 2003). Although, various doubling agents with comparatively less toxicity such as oryzaline, amiprophos-methyl (APM), and trifluralin are available but colchicine still is a most commonly used antimitotic agent. It has been efficiently used in different plant species, including many trees resulting in high doubling efficiency (Shao et al., 2003).

Treatment with the antimitotic agent is mostly performed by soaking the whole plantlet, nodal segments or microspore derived embryos into it, or, by culturing them directly into the medium supplemented with the antimitotic agent (Fletcher et al., 1998). Immersing whole plants into the antimitotic solution is cumbersome and requires a higher amount of chemicals raising the cost of the experiment. Using smaller explants like haploid embryos, on the other hand, is safe, as they can be easily immersed in Petri-plates with easy handling, requiring less amount of antimitotic agent, and hence, reducing the overall cost involved. Additionally, the chances of getting chimera are least while using haploid embryos (Fletcher et al., 1998; Mohammadi et al., 2012). Another significant advantage of the diploidized embryos is that they serve as directly available material for transformation and mutation studies (Mohammadi et al., 2012).

One big challenge faced post diploidization is to distinguish haploids from doubled haploids. Various indirect methods involving analysis of morphological and anatomical traits, such as leaf size, stomata size and density, have been used to identify the ploidy. However, these methods may not be able to identify mixoploids. Flow cytometry is a simple, high throughput technique for screening a large population in less time. It is advantageous as it not only segregates the haploids and doubled haploids but also detects any aneuploids, if generated. Chromosome counting via cytological squash preparation is classical, yet a highly reliable approach of determining ploidy in plants (Salma et al., 2017).

Microsatellite markers are used to confirm homozygosity among diploidized individuals. These are highly polymorphic, co-dominant markers; therefore, can easily differentiate between the homozygous or heterozygous alleles at the same locus (Murovec and Bohanec, 2012). The present study aims to achieve diploidization of *in vitro* developed haploids by applying colchicine at various concentrations and time durations to standardize the optimal combination for maximum diploidization. Various techniques, such as the study of morphological and anatomical traits, flow cytometry, chromosomal counting, and microsatellite marker analysis, have been deployed to confirm the development of homozygous diploids in tea.

2. Materials and methods

2.1. Plant material and culture conditions

The *in vitro* haploid embryos were obtained via callusing of microspores during the early-to-late uninucleate stages in anther cultures, according to the protocols mentioned earlier, in two cultivars of tea, *Camellia assamica* ssp. *assamica* (TV21; Assam Type) (Mishra et al., 2017) and *Camellia assamica* ssp. *lasiocalyx* (TV19; Cambod Type) (Mishra et al., 2022). Briefly, callus formation occurred from microspores from inside the anther locules on MS (Murashige and Skoog, 1962) medium containing glucose (60 g l^{-1}), 2,4-dichlorophenoxyacetic acid (2,4-D; 5 μM), 6-furfurylaminopurine (kinetin; 5 μM), L-glutamine (800 mg l^{-1}) and L-serine (200 mg l^{-1}). The calluses were further proliferated only when glucose was replaced with sucrose (30 g l^{-1}) in the MS medium. Embryogenesis in calluses occurred on embryo induction medium consisting of MS medium supplemented with 6-benzylaminopurine (BAP; 10 μM), Gibberellic acid (GA_3; 3 μM), L-glutamine (800 mg l^{-1}) and L-serine (200 mg l^{-1}). The globular stage haploid embryos served as explants for colchicine treatment. Histological analysis of the similar embryo, was performed to determine the intact globular embryo as described in (Bajpai and Chaturvedi, 2021). The colchicine treated embryos were regularly multiplied in numbers by the process of secondary embryogenesis on embryo development medium. The embryo development medium constitutes 10 times reduced concentration of growth regulators present in the embryo induction medium. The fully developed mature embryos germinated by differentiating into a cluster of small haploid shoots (~1 cm) on MS medium consisting of BAP (10 μM), GA_3 (0.5 μM), Indole-3-butyric acid (IBA; 1 μM), L-glutamine (80 mg l^{-1}) and L-serine (20 mg l^{-1}). These differentiating shoots were then transferred for elongation on ½ MS (only major salts reduced to half) medium comprising BAP (10 μM), GA_3 (0.5 μM), IBA (1 μM), L-glutamine (80 mg l^{-1}) and L-serine (20 mg l^{-1}). Multiplication of shoots was performed via forced axillary bud proliferation, using single nodal explants, on MS medium supplemented with glucose (30 g l^{-1}) in combination with BAP (5 μM), GA_3 (0.5 μM), IBA (0.5 μM), L- glutamine (80 mg l^{-1}) and L-serine (20 mg l^{-1}). Repeated multiplication was performed to preserve the haploid lines. For rooting, the elongated shoots (~3 cm in length) were pre-treated with IBA (175 μM) for ten days, followed by transfer to $^1/_3$ MS (only major salts reduced to one third strength) basal medium. All the growth regulators used in the experiments were procured from (Sigma, India).

2.2. Colchicine treatment

The solution of colchicine (Sigma, India) was prepared by dissolving the required amount of colchicine (0.05%−0.3%) in 1% (v/v) dimethyl sulphoxide (DMSO) (Merck, India). The DMSO was used to achieve higher permeability. The solution was filtered using 0.2 μm PVDF sterile filter (Axiva, India) and 10 ml of filtered colchicine solution (0.05%, 0.1%, 0.2%, and 0.3%) was dispensed into each 60 × 15 mm presterilized disposable Petri plates (Tarson Products Pvt. Ltd, India). Single globular haploid embryos from the cluster of embryos developed on the embryo induction medium were immersed in filtered colchicine solution in Petri plates and the plates were sealed with Parafilm (Pechiney, USA). For each treatment, 50 embryos were used and the experiment was repeated at least thrice. The embryos were exposed to different colchicine concentrations for varying time intervals (4, 8, 12, 24, 48 h). Filter sterilization and immersion of embryos was performed inside the laminar-air-flow cabinet (Saveer Biotech Limited, India), using forceps and scalpel (HiMedia, India). Haploid embryos immersed in sterile distilled water served as control during the experiment. The cultures undergoing treatment were incubated in completely dark conditions at 25 ± 2 ℃ on the rotatory incubator shaker (Scigenics, India) for the required duration. After each colchicine treatment, embryos were washed thrice with sterile distilled water and surface dried on Whatman filter grade-1 (GE Health care, U.K). These treated embryos were then inoculated on embryo development medium for 4 weeks. The fully developed mature embryos were transferred onto the embryo germination medium. The survival rate and germination rate (percentage) of embryos was calculated using the following formula (Tang et al., 2010):

$$\{Survival\ \ rate(\%) = Number\ \ of\ \ surviving\ \ embryos / total\ \ number\ \ of\ \ treated\ \ \ embryos \times 100\}$$

$$\{Germination\ \ rate(\%) = Number\ \ of\ \ embryos\ \ proliferating\ \ \ shoots / total\ \ number\ \ of\ \ embryos \times 100\}$$

Percent population of plants at each ploidy level, haploid, intermediate cytotype, doubled haploid and tripoid was determined using the equation mentioned below (Tang et al., 2010):

$$\{Ploidy(\%) = Number\ \ of\ \ plants\ \ \ at\ \ a\ \ particular\ \ \ ploidy / total\ \ number\ \ of\ \ treated\ \ \ plants\ \ \times 100\}$$

$$\{Total\ \ \ number\ \ \ of\ \ \ treated\ \ \ plants \times 100\}$$

2.3. Shoot elongation and multiplication

The cluster of small shoots (~ 1 cm) of TV21 and TV19 cultivars, obtained from haploid and doubled haploid embryos germinating on MS medium consisting of BAP, GA_3, IBA, L-glutamine and L-serine, were transferred for elongation. The shoots were passaged for elongation treatment for three transfer cycles, each of 4-week duration. In the second passage, the individual shoots were separated from the cluster of shoots and inoculated on fresh elongation medium on ½ MS (major salts reduced to half strength) medium supplemented with BAP, GA_3, IBA, L-glutamine, and L-serine. After attaining a height of ~ 3 cm, the shoots were multiplied via forced axillary bud proliferation using single node segments as explants on the multiplication medium, MS comprising of glucose (30 g l^{-1}) along with BAP, GA_3, IBA, L-glutamine and L-serine.

2.4. Rooting and hardening of the plants

Rooting of both haploid and doubled haploid shoots of TV21 and TV19 cultivars, measuring 3 cm in length, were obtained on $^1/_3$ MS (only major salts reduced to one third) basal medium. Initially, the shoots were pre-treated with a high concentration of IBA (175 μM) on $^1/_3$ MS medium for ten days, followed by transfer to $^1/_3$ MS basal medium for next 50 days for root emergence. Since the *in vitro* rooting took prolonged time, an alternate method of *ex-vitro* rooting of shoots was also tested along with the hardening process. The cut end of each microshoot, measuring 3 cm in length, was gently dipped into Rooton-3 for 1–2 min, followed by transfer of shoots into different potting mixtures. Shoots in the potting mix were directly placed in the greenhouse at 75–80% relative humidity.

2.5. Ploidy determination

2.5.1. Flow cytometric analysis

Fresh, young leaves from the colchicine treated and untreated germinating embryos of TV21 and TV19 cultivars, as well as the leaves from field grown parent plants (reference plant) 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 plate (Borosil, India) kept on ice. Composition of the buffer used in the experiment is adopted from the established protocol of Loureiro et al. (2007) after 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^{-1}ml propidium iodide (PI; Fluka) and 50 μg ml^{-1} 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).

2.5.2. Chromosome count

Actively growing, 0.5–1 cm long root-tips from *in vitro* developed haploid and doubled germinating embryos, 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 et al., 2016). The root-tips were washed and finally squashed in 2% aceto-carmine (Sigma, India). The number of chromosomes in the root-tips squash preparations from each sample were counted under the × 100 objective lens of a Zeiss, photomicroscope (Carl Zeiss, Germany).

2.6. Screening of morphological and anatomical characteristics

The morphological trait analysis was performed to screen haploid and doubled haploid plants. The observations with regard to leaf size and the number, size and density of stomata per mm of leaf area were recorded between haploid and doubled haploid plants. Leaves were excised from haploid and doubled haploid plants. The average leaf size and width were measured for ten leaf samples. The measurements related to the length and width of the leaf was recorded on a graph paper considering 1square = 1 cm. The stomatal analysis was performed by applying clear nail enamel to the abaxial surface of the leaf. The surface was allowed to dry slightly and gradually peeled off using an adhesive tape. The peeled epidermal layer was placed gently on the microscopic slide and observed under 20X lens of Nikon 80i microscope. Ten randomly selected microscopic fields of 50 μm each from three different leaf samples in each haploid, doubled haploid, and heterozygous diploid plant case were analyzed, and data were recorded. The stomatal count, length, and width of each was determined using Image J software (LOCI, USA).

2.7. Data analysis

The data obtained was analysed 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 95% confidence level ($P < 0.05$).

2.8. SSR marker analysis

2.8.1. DNA extraction

The fresh leaf samples were collected from *in vitro* haploid and doubled haploids plants and from field-grown heterozygous diploid (control) plants of both TV21 and TV19 cultivars of tea. The leaves were collected in zip lock bags and stored overnight at − 80 ºC, followed by lyophilization in the 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 (Hi-Media, India). Concentration and purity of the DNA were determined using bio-spectrophotometer (Eppendorf, Germany) at (260/280 nm) and 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 15 ng.

2.8.2. 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 $MgCl_2$ (ThermoScientific, US), 2.5 mM dNTP Mix (Thermo Scientific, US), (1 unit/ µl) Taq DNA polymerase, 1.4 µl (10 pM) of SSR primer including both forward and reverse primer (Bio serve, India). The total of the 15 primers (Table 1), used in the present study, were synthesized from the sequences adopted from the already published report on microsatellite marker analysis in *Camellia* spp. (Bali et al., 2013). Amplification of DNA was carried out on a 96 well Master cycler Pro S (Eppendorf, Germany) using following cycling program, Denaturation at 94 °C for 4 min followed by 35 cycles each at 94 °C for 1 min, extension at 72 °C for 2 min, followed by a 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 for different primer sets depending on the Tm (melting temperature) of the primer. The amplified products were electrophoresed on 2.2% (w/v) agarose gel in 1X TBE buffer using 100 bp ladder and stained with ethidium bromide. Gel images were obtained using (G: BOX, Syngene, U. K.) imaging system. The gel was visualized under UV light and documented using (G: BOX, Syngene, U.K.) imaging system. The pattern of the bands was studied using a 100 bp ladder (Thermo Fisher Scientific, USA).

3. Results

3.1. Embryo induction, development and secondary embryogenesis in haploid embryos

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 embryo induction medium, within 4 weeks of culture. This was followed by transfer of embryos on to the development medium consisting of ten times reduced concentration of growth regulators present in embryo induction medium. An increase in number of embryos and secondary embryogenesis was observed on embryo development medium in TV21 (Fig. 1 a) and TV 19 (Fig. 2a) cultivars.

3.2. Colchicine treatment

The individual globular haploid embryos of TV21 (Fig. 1g) and TV19 (Fig. 2g) cultivars from embryo induction medium were utilized for colchicine treatments at varying concentrations and time intervals (Table 2 and Table 3). Following treatments, the embryos were inoculated onto the development medium for 4 weeks where they showed further development and secondary embryogenesis in the two cultivars, TV21 and TV19.

A low survival frequency (%) of embryos was observed at increased concentration 0.3% and high exposure time (48 h) of colchicine in both the cultivars, TV21 and TV19 (Supplementary Figs. S1 and S2) Consequently, there was a decrease in % cultures of treated embryos showing shoot differentiation in TV21 and TV19 (Figs. S3 and S4) data added in supplementary sheet) cultivars during the first two transfer cycles. On the other hand, the 100% survival was observed among untreated embryos (control) where they all differentiated into shoots. Slow germination among survived and treated embryos initially may be due to the residual effect of colchicine. However, the growth resumed to normal during third transfer of these treated embryos into the same medium. Highest % survival, 97.86% and 91.34%, was found in TV21 and TV19 cultivars, respectively, with 0.05% colchicine treatment for 4 h but it did not result into doubled haploids. A decrease in percentage survivability of embryos was observed with an increase in the concentration and exposure time of colchicine. Maximum response in terms of % doubled haploid production, 76.89% in TV21 (Tables 2) and 67.34% in TV19 (Table 3) cultivars, was found when haploid embryos were treated with 0.2% colchicine for 24 h. With 0.1% colchicine treatments, the % survival and shoot differentiation were more but it resulted in lower number of doubled haploids embryos and higher number of intermediate cytotype or haplo-diploid population. Colchicine concentration at 0.3% was highly unfavorable and the maximum necrosis (95%) was found with 48 h colchicine exposure. The results obtained in this study revealed that the concentration and exposure time of antimitotic agent treatments are the two critical factors that determine the efficacy of attaining diploidization in any plant species.

3.3. Embryo germination, shoot elongation and multiplication

The germination of water immersed haploid embryos (control) of TV21 (Fig. 1b) and TV19 (Fig. 2b), as well as the colchicine treated, doubled haploid embryos of TV21 (Fig. 1h,i) and TV19 (Fig. 2h,i) were obtained on MS + BAP (10 µM) + GA_3 (0.5 µM) + IBA (1 µM) + L-glutamine (80 mg l^{-1}) + L- Serine (20 mg l^{-1}) after two cycles of 4 weeks each. A few germinating embryos showed bipolar germination with shoot and root development simultaneously while the majority of the embryos showed monopolar germination by giving rise to only multiple small shoots towards plumular region. Small bunch of 4–5 shoots (~1 cm size) were transferred for elongation on the same composition as that of the germination medium where full MS is replaced with ½ MS (only major salts reduced to half concentration). The shoot elongation was achieved after 3 cycles, each of 4 weeks duration. After the first passage of growth period of 4 weeks, the individual shoots, which grew sufficiently in the bunch, were transferred to the fresh shoot elongation medium. The individual haploid shoots of TV21 (Fig. 1c) and TV19 (Fig. 2c) cultivars as well as doubled haploid shoots of TV21 (Fig. 1j,k) and TV19 (Fig. 2 j,k) cultivars grew upto an average height of

Table 1
List of SSR primer sequences used for the study (Bali et al., 2013).

Marker	Forward primer	Reverse primer	Annealing temperature °C
Csin01	CAATCCCTCCTCCATCCTTT	GAGCCACTACCGCCTCCAG	63
Csin02	GACGAGGATGAGGATGAGGA	CACCAAACAAAACCACAGGA	55
Csin04	ATTTTGAAGTCCTCTCAGAACCAT	CATCGTGAACCGCATCTGTAG	63
Csin05	GGACTACCTGCTCCTGTTCTACTACC	CCCTTGAAGTCCTCTCAGAACCAT	57
Csin06	CGGGCAC TCAATGGAAAGCAC	TGGCATCTGTTGGCGTGGTG	60
Csin07	CAACCCAACTCAGGCAGA	GCTACAACCACCTTCAACACCT	60
Csin24	CCAAGTAGAAGGACGCACTC	GGAGCATAGCATAGCATAGC	62
Csin25	GCTATGCTATGCTATGCTCC	CATCCACAATTTACACCGTG	62
Csin31	GAGGGTTGTAATCTGCTGCCGC	GGTGCTAAACCCATAGGGGC	65
Csin33	CCAGTCGGGAAACCTGTCG	TAGTCCTGTCGGGTTTCGC	60
Csin41	CCCTCAACTCCATCAGCAAT	CCCAAAACGAAAACCGACTA	64
Csin49	CTCCAGCAGCAACATTATTACG	GACCTCAGAAAACTCCCCTTG	62
Csin50	CCGAAGAGGCTGAGTATGAT	CTCCAAGGCACTGAACTGG	55
Csin57	GAGGAGGAGGTCAGTGAAGGAACTG	AGGACTCTGAATGATCTACCAATTCGTC	56
Csin65	TCAGGTGCTCCTATCTCTACC	GTATCTGATACTCACATTGTAGCTG	54

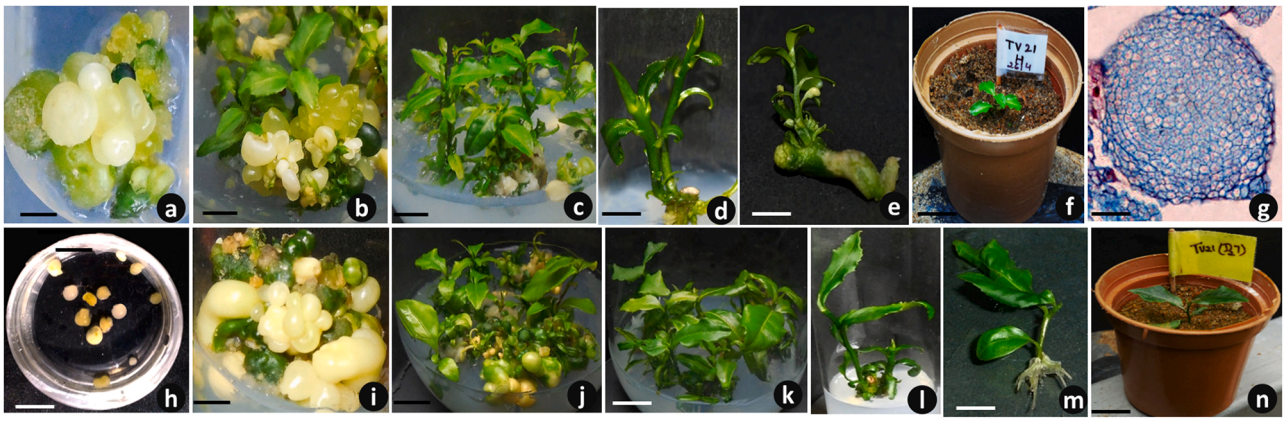

Fig. 1. : Plantlet development in TV21 cultivar of Tea from Haploid and doubled haploid embryos; a-g, Haploid plantlet development from haploid embryos and -h-n, Doubled haploid plantlet development from colchicine treated haploid embryos: **a)** Cluster of embryos undergoing development and secondary embryogenesis on MS + BAP (1 μM) + GA_3 (0.3 μM) + L-glutamine (80 mg l^{-1}) + L-serine (20 mg l^{-1}) (x 1.4X) **b)** Germination of embryos developing multiple shoots at plumular end on MS + BAP (10 μM) + GA_3 (0.5 μM) + IBA (1 μM) + L-glutamine (80 mg l^{-1}) + L-serine (20 mg l^{-1}) (x 1.1 X) **c)** Shoot elongation on ½ MS + BAP (10 μM) + GA_3 (0.5 μM) + IBA (1 μM) + L-glutamine (80 mg l^{-1}) + L-serine (20 mg l^{-1}) (x 1.0X) **d)** Shoot multiplication by axillary bud proliferation from nodal explant on MS + Glucose (30 g l^{-1}) + BAP (5 μM) + GA_3 (0.5 μM) + IBA (0.5 μM) + L-glutamine (80 mg l^{-1}) + L-serine (20 mg l^{-1}) (x 1.5 X) **e)** A rooted haploid shoot obtained on $^1/_3$ MS basal medium (x 0.5X) **f)** Hardening of haploid shoot ready to be transferred to field (x 1.0 X) **g)** Histological section of the globular embryo (x 50X) **h)** Colchicine treatment of globular embryos (x 1 X) **i)** Cluster of doubled haploid embryos undergoing development and secondary embryogenesis on MS + BAP (1 μM) + GA_3 (0.3 μM) + L-glutamine (80 mg l^{-1}) + L-serine (20 mg l^{-1}) (x 1.4 X) **j)** Germination of embryos showing multiple shoots at plumular end on MS + BAP (10 μM) + GA_3 (0.5 μM) + IBA (1 μM) + L-glutamine (80 mg l^{-1}) + L-serine (20 mg l^{-1}) (x 1.2 X) **k)** Shoot elongation on ½ MS + BAP (10 μM) + GA_3 (0.5 μM) + IBA (1 μM) + L-glutamine (80 mg l^{-1}) + L-serine (20 mg l^{-1}) of doubled haploid shoots on same medium as in **c** (x 1.2 X) **l)** Multiplication of shoots via forced axillary bud proliferation from nodal explant on MS + Glucose (30 g l^{-1}) + BAP (5 μM) + GA_3 (0.5 μM) + IBA (0.5 μM) + L-glutamine (80 mg l^{-1}) + L-serine (20 mg l^{-1})1.3X) **m)** Similar as (e), rooted doubled haploid shoot of TV21 cultivar obtained on $^1/_3$ MS basal medium (x 0.4 X) **n)** Hardening of shoots on potting mixture consisting of soil and sand (1:2) (x 0.5 X).

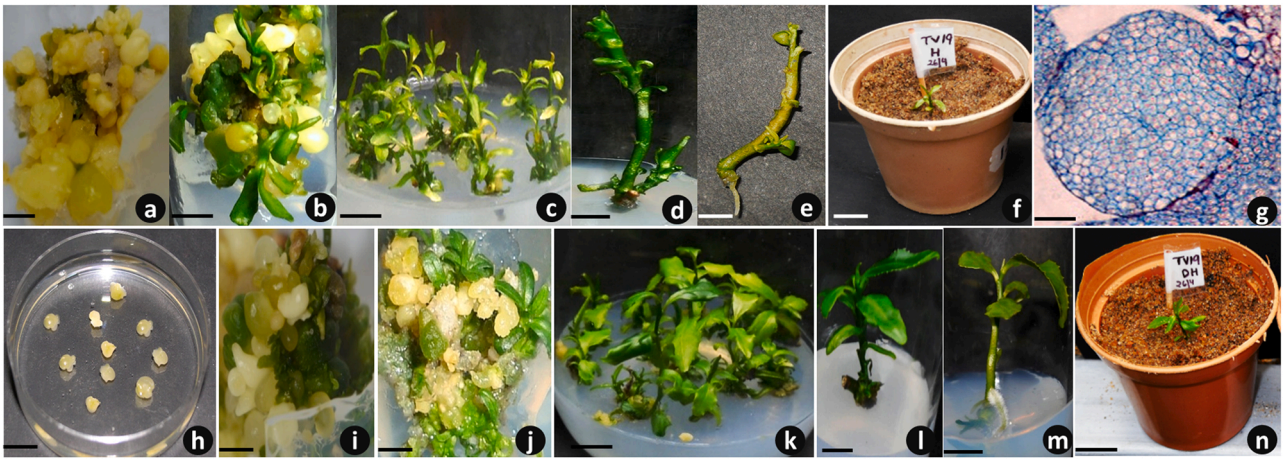

Fig. 2. : Plantlet development in TV19 cultivar of Tea from Haploid and doubled haploid embryos; a-g, Haploid plantlet development from haploid embryos and h-n, Doubled haploid plantlet development from colchicine treated haploid embryos: **a)** Cluster of embryos undergoing development and secondary embryogenesis on MS + BAP (1 μM) + GA_3 (0.3 μM) + L-glutamine (80 mg l^{-1}) + L-serine (20 mg l^{-1}) (x 1.4X) **b)** Germination of embryos developing multiple shoots at plumular end on MS + BAP (10 μM) + GA_3 (0.5 μM) + IBA (1 μM) + L-glutamine (80 mg l^{-1}) + L-serine (20 mg l^{-1}) (x 1 X) **c)** Shoot elongation on ½ MS + BAP (10 μM) + GA_3 (0.5 μM) + IBA (1 μM) + L-glutamine (80 mg l^{-1}) + L-serine (20 mg l^{-1}) (x 1.2X) **d)** Shoot multiplication by axillary bud proliferation from nodal explant on MS + Glucose (30 g l^{-1}) + BAP (5 μM) + GA_3 (0.5 μM) + IBA (0.5 μM) + L-glutamine (80 mg l^{-1}) + L-serine (20 mg l^{-1}) (x 1.6 X) **e)** A rooted haploid shoot obtained on $^1/_3$ MS basal medium (x 0.4X) **f)** Hardening of haploid shoot ready to be transferred to field (x 0.9 X) **g)** Histological section of the globular embryo (x 50X) **h)** Colchicine treatment of globular embryos (x 1.2 X) **i)** Cluster of double haploid embryos undergoing development and secondary embryogenesis on MS + BAP (1 μM) + GA_3 (0.3 μM) + L-glutamine (80 mg l^{-1}) + L-serine (20 mg l^{-1}) (x 1.2 X) **j)** Germination of embryos showing multiple shoots at plumular end on MS + BAP (10 μM) + GA_3 (0.5 μM) + IBA (1 μM) + L-glutamine (80 mg l^{-1}) + L-serine (20 mg l^{-1}) (x 2 X) **k)** Shoot elongation on ½ MS + BAP (10 μM) + GA_3 (0.5 μM) + IBA (1 μM) + L-glutamine (80 mg l^{-1}) + L-serine (20 mg l^{-1}) of doubled haploid shoots on same medium as in **c** (x 1.3 X) **l)** Multiplication of shoots via forced axillary bud proliferation from nodal explant on MS + Glucose (30 g l^{-1}) + BAP (5 μM) + GA_3 (0.5 μM) + IBA (0.5 μM) + L-glutamine (80 mg l^{-1}) + L-serine (20 mg l^{-1}) (x 1.5X) **m)** Similar as (e), rooted doubled haploid shoot of TV21 cultivar obtained on $^1/_3$ MS basal medium (x 1.3 X) **n)** Hardening of shoots on potting mixture consisting of soil and sand (1:2) (x 0.7 X).

3 cm, at the end 3rd cycle, each of 4 weeks. The haploid and doubled haploid shoots of TV21 (Fig. 1 d, 1l) and TV19 (Fig. 2 d, 2 l) cultivars were further multiplied via forced axillary bud proliferation using single node segment as explant on multiplication medium.

3.4. Rooting, hardening and acclimatization shoots

For rooting, the elongated shoots (~3 cm in length) of haploid and doubled haploid plants of TV21 and TV19 were pre-treated with IBA

Table 2
Effect of varying concentrations and durations of colchicine treatments on frequency (%) of different ploidy generation in TV21 cultivar of tea.

Concentration (%)	Time (h)	% Haploids	% Intermediate Cytotype	% Doubled haploid	% Polyploid
0.05	4	98.87 ± 1.30^{a}	1.1 ± 0.09^{a}	0	0
	8	91.1 ± 1.91^{ab}	8.8 ± 1.92^{ab}	0	0
	12	84.4 ± 5.00^{b}	15.5 ± 5.01^{bc}	0	0
	24	73.44 ± 3.51^{bc}	26.37 ± 3.32^{bc}	0	0
	48	69.09 ± 1.70^{c}	29.80 ± 3.10 ^{cd}	1.10 ± 0.92 ^{ns}	0
0.10	4	87.77 ± 6.32^{a}	12.22 ± 3.92^{a}	0	0
	8	82.53 ± 5.33^{a}	17.47 ± 5.11^{ab}	0	0
	12	69.99 ± 3.33^{bc}	28.93 ± 1.73^{bc}	1.11 ± 0.69^{a}	0
	24	59.00 ± 1.70^{c}	33.33 ± 5.71 ^{cd}	5.55 ± 0.59^{a}	2.11 ± 0.80^{a}
	48	39.66 ± 3.51^{d}	46.67 ± 3.50^{d}	6.72 ± 0.06^{a}	6.74 ± 1.42^{a}
0.20	4	80.17 ± 3.69^{a}	19.80 ± 3.33^{a}	0	0
	8	72.12 ± 5.01^{a}	27.87 ± 5.02^{a}	0	0
	12	43.32 ± 6.52^{b}	21.30 ± 1.94^{a}	28.60 ± 4.93^{a}	6.78 ± 1.33^{a}
	24	**5.33 ± 1.74^{c}**	**8.87 ± 5.03^{b}**	**76.89 ± 7.01^{b}**	**8.9 ± 3.81^{a}**
	48	3.20 ± 0.12^{c}	14.26 ± 3.71^{ab}	53.55 ± 6.10^{c}	29.00 ± 8.50^{b}
0.30	4	74.44 ± 5.00^{a}	21.00 ± 1.95^{a}	4.46 ± 0.82^{a}	0
	8	71.12 ± 5.03^{a}	18.83 ± 5.04^{a}	8.93 ± 1.92^{a}	1.11 ± 0.31^{a}
	12	46.60 ± 6.60^{b}	34.48 ± 5.06^{b}	13.33 ± 3.34^{a}	5.56 ± 1.91^{a}
	24	3.34 ± 0.30^{c}	28.88 ± 5.21^{ab}	50.0 ± 1.00^{b}	17.77 ± 5.02^{b}
	48	2.21 ± 0.90^{c}	8.90 ± 2.90^{c}	38.88 ± 5.01^{c}	50.03 ± 3.33^{c}

The data has been compared according to different concentrations within columns. Values are mean of three independent experiments. Mean values sharing the same letter do not differ significantly ($p < 0.05$) according to Tukey's multiple range test.

Table 3
Effect of different concentration and duration of colchicine on % survival, differentiation and each ploidy % in TV19 cultivar of tea.

Concentration (%)	Time (h)	% Haploids	% Intermediate Cytotype	% Doubled haploid	% Polyploid
0.05	4	98.79 ± 1.92^{a}	1.20 ± 0.20^{a}	0	0
	8	95.57 ± 1.80^{ab}	4.39 ± 1.92^{a}	0	0
	12	86.66 ± 6.63^{b}	13.33 ± 6.63^{b}	0	0
	24	79.89 ± 3.15^{b}	20.00 ± 3.31^{b}	0	0
	48	65.56 ± 5.01^{c}	33.33 ± 3.33^{c}	1.11 ± 0.2 ^{ns}	0
0.10	4	96.63 ± 3.34^{a}	3.33 ± 3.30^{a}	0	0
	8	85.55 ± 5.01^{ab}	14.45 ± 5.14^{ab}	0	0
	12	81.10 ± 5.06^{b}	18.87 ± 5.03^{b}	0	0
	24	69.83 ± 5.50^{c}	30.16 ± 5.15^{c}	0	0
	48	61.66 ± 6.61^{c}	35.33 ± 7.26^{c}	3.00 ± 0.26 ^{ns}	0
0.20	4	94.66 ± 5.03^{a}	5.33 ± 4.90^{a}	0	0
	8	76.40 ± 2.10^{b}	18.22 ± 2.01^{b}	5.37 ± 0.22^{a}	0
	12	36.68 ± 5.70^{c}	50.00 ± 6.07^{c}	11.0 ± 0.30^{a}	2.3 ± 0.11^{a}
	24	**8.89 ± 1.61^{d}**	**20.33 ± 5.09^{b}**	**67.34 ± 7.52^{b}**	**3.34 ± 1.30^{a}**
	48	5.56 ± 3.81^{d}	9.86 ± 1.30^{a}	59.11 ± 5.30^{c}	25.20 ± 5.03^{b}
0.30	4	88.88 ± 5.07^{a}	11.11 ± 3.01^{a}	0	0
	8	80.00 ± 6.83^{a}	20.00 ± 7.00^{a}	0	0
	12	41.22 ± 4.90^{b}	39.00 ± 6.63^{b}	14.44 ± 1.92^{a}	5.33 ± 6.93^{a}
	24	5.78 ± 1.65^{c}	13.40 ± 3.57^{a}	60.79 ± 5.27^{b}	20.0 ± 3.70^{b}
	48	3.11 ± 3.00^{c}	8.99 ± 3.73^{a}	51.12 ± 5.07^{b}	36.70 ± 3.34^{c}

The data has been compared according to different concentrations within columns. Values are mean of three independent experiments. Mean values sharing the same letter do not differ significantly ($p < 0.05$) according to Tukey's multiple range test

(175 μM) for ten days, followed by transfer to $^{1}/_{3}$ MS (only major salts reduced to one third strength) basal medium. Rooting of haploid and doubled shoots was observed within 50 days of transfer to the basal medium in TV21 (Figs. 1e, 1dm) and TV19 (Fig. 2 e, 2 m). The rooted plantlets were transferred to the greenhouse for hardening into a potting mixture consisting of soil and sand in 1:2 ratio and then eventually moved to the field conditions for acclimatization. The Fig. 1**f, 1 n** show the hardened haploid and doubled haploid plantlet in TV21 cultivar while Fig. 2f, 2n represent the hardened haploid and doubled haploid plants of TV19 plantlets. Since the *in vitro* rooting took prolonged time, an alternate method of *ex-vitro* rooting of shoots was also tested along with the hardening process by following the process as mentioned in Materials and Methods section.

3.5. Ploidy determination

3.5.1. Flow cytometric analysis

Flow cytometry being accurate and fast method was used as the primary method for screening the ploidy of in vitro developed plantlets. The results obtained from flow cytometric analysis of leaves from in vitro haploid, doubled haploid and field-grown parent (reference plants) of TV21 and TV19 cultivars are represented in Fig. 3 and Fig. 4, respectively. The leaves from haploid plants of TV21 cultivar showed G1 peak at channel position 377 and G2 peak at 720 channel (Fig. 3a). In contrast to this, the G1 peak from the leaves of doubled haploid shoots was detected at channel position 710 and G2 peak at 1381 (Fig. 3b), which is double to that achieved in haploid. A similar observation was made in case of TV19, where, the data plot obtained from the leaves of *in vitro* regenerated haploid plants showed G1 peak at channel position 368 and G2 at 714 (Fig. 4a). The histogram plot of the doubled haploid shoots of TV19 showed G1 peak at channel position 726 and G2 at 1425 (Fig. 4b). Leaves from field grown parent plants in both the cultivars TV21 and TV19 were used as reference for both the haploid and doubled haploid lines. The G1 peak for TV21 parent plant (reference) was found at channel position 756 and G2 at 1538 (Fig. 3c). While the histogram plot of the TV19 parent plant (control) showed G1 peak at channel position 707 and G2 at 1404 channel position (Fig. 4c). The results obtained in the doubled haploid plants of TV21 and TV19 exhibited a ploidy almost double to their haploid counterparts and resembled with the data collected in heterozygous diploid (control) plants, confirming diploidization of haploids in both TV21 and TV19 cultivars respectively.

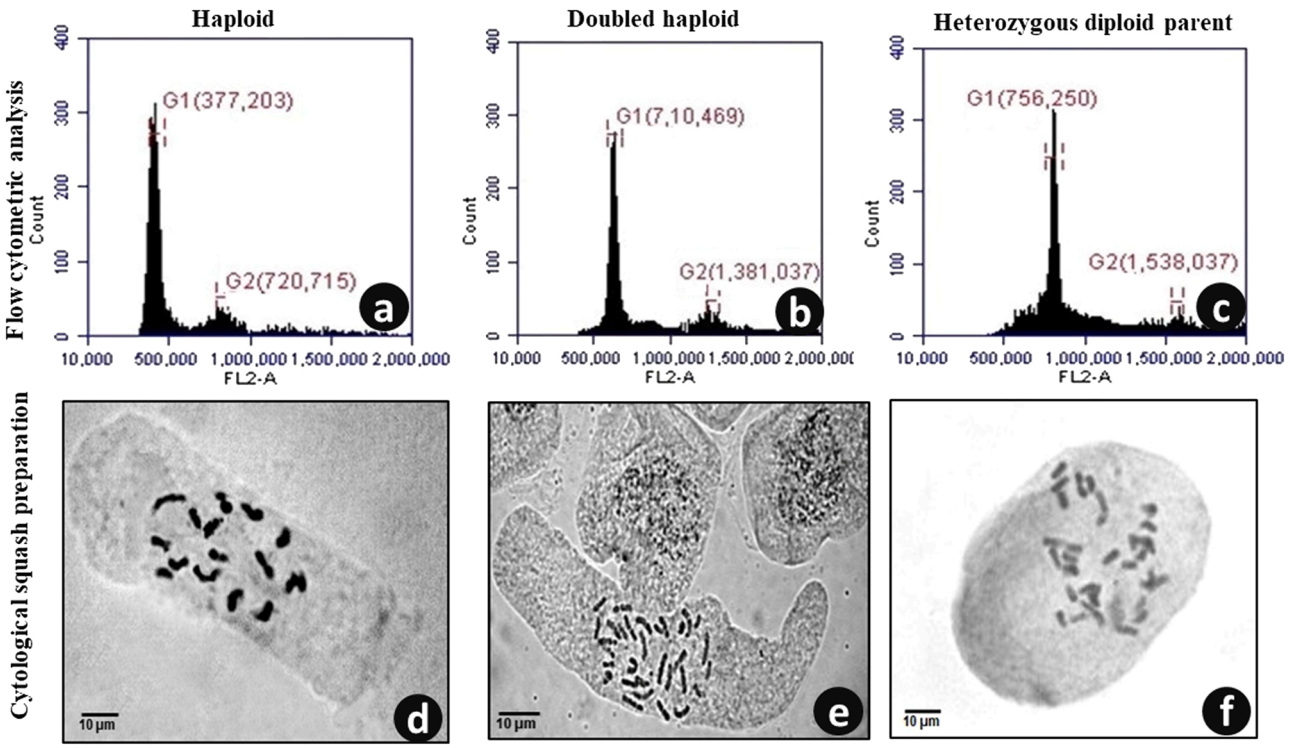

Fig. 3. : Flow cytometry and cytological squash preparation in TV21 cultivar of tea**; a-c** Flow cytometric analysis of leaves from *in vitro* regenerated haploid, doubled haploid and field grown parent plant; **d-f** Cytological squash preparation from haploid, doubled haploid and parent plant **a)** Flow cytometric data from *in vitro* regenerated haploid shoots showing G1 peak at 377 and G2 peak at 720 channel positions **b)** Flow cytometric analysis from leaves of doubled haploid shoots showing G1 peak at 710, and G2 peak at 1381channel positions **c)** Flow cytometric analysis of TV21 parent plant (reference) showing G1 peak at 756 and G2 peak at 1538 channel positions **d)** Cytological squash preparation from root-tip of *in vitro* regenerated haploid shoots showing chromosome number n = X = 15 (x 100) **e)** Cytological squash preparation from root-tip of *in vitro* regenerated doubled haploid shoots showing chromosome number 2 n = 2X= 30 (x 100) **f)** Cytological squash preparation from root-tip of field grown parent (reference plant) showing chromosome number 2 n = 2X= 30 (x 100).

3.5.2. *Cytological squash preparation*

Cytological squash preparation from root-tips of the plantlets is considered to be the most efficient and rational method for ploidy analysis. The root-tip of haploid plantlets of TV21 (Fig. 3d) and TV19 (Fig. 4 d) cultivars of tea was found to be n = X = 15. While the root-tip squash preparations from doubled haploid plantlets showed a chromosomal count of 2 n = 2X= 30 in TV21 (Fig. 3e) and in TV19 (Fig. 4e). The results obtained from the haploid and their respective doubled haploids confirmed that the diploidization of haploid plants was successfully achieved. However, a confirmation for the same was done by comparing the chromosomal count from the root-tips of the parent (reference) TV21 (Fig. 3f) and TV19 cultivars (Fig. 4f), where the chromosome count was same as that of the diploidized plants 2 n = 2X = 30. Thus, it was confirmed that the application of colchicine was fruitful in generating doubled haploid plants.

3.6. *Screening of morphological and anatomical characteristics*

The variation in leaf size and width from the haploid and doubled haploid leaves of TV21 and TV19 was analyzed respectively from ten random leaf samples Table 4. A significant difference in the leaf size as well as the size of stomata was found between haploid and doubled haploid plants in both cultivars. However, the number of stomata did not vary much with variation in ploidy. Similar observations have been reported earlier in tea while studying polyploidy (Wachira et al., 1994).

The noticeable variation in color and size of leaves was also observed between in vitro haploid and doubled haploid plants of both the cultivars. The doubled haploid plants in both TV21 and TV19 had large and greener leaves when compared to their respective haploids (Fig. 5**a, d)**. A variation in size and number of stomata was also found. Fig. 5b and Fig. 5c represent the difference in number and size of stomatal between the haploid and doubled haploid TV21 leaves. The variation in the stomatal count and size between haploid and double haploid leaves of TV19 cultivar is shown in Fig. 5 e and Fig. 5f, respectively.

3.7. *SSR marker analysis*

The diploidized plants confirmed via flow cytometry and cytological studies were further analyzed to validate homozygosity using microsatellite markers. Fifteen primer pair sets as listed in Table 1 were utilized in the present study. Among the listed primers, only three gave amplification wherein the majority of the primers gave bands with high polymorphism, generating no allelic variation. However, clear bands distinguishing the heterozygous parent plant from the *in vitro* haploids and doubled haploids was observed with primer Csin 06 at 350- bp position in TV21 cultivar (Fig. 6a) and 180–200 bp position in TV19 cultivar (Fig. 6b). The heterozygous parent in both the cultivars showed two alleles at the locus marked with an arrow, a single band of lower width at same locus was obtained with microspore derived haploid plants, while the homozygous doubled haploid plants showed a single broad band at the same locus matching either of the two allele in heterozygous diploid (control). Thereby, confirming attainment of homozygosity in diploidized plants.

4. Discussion

Artificial doubling of chromosomes is an attractive alternative strategy for attaining homozygous breeding lines in woody perennials with inherent heterozygosity, extreme high inbreeding depression and long reproductive cycle (Srivastava and Chaturvedi, 2008; Mishra et al., 2017). The technique has a dual advantage as it reduces the labor cost involved, and indubitably produces homozygous lines in a single step,

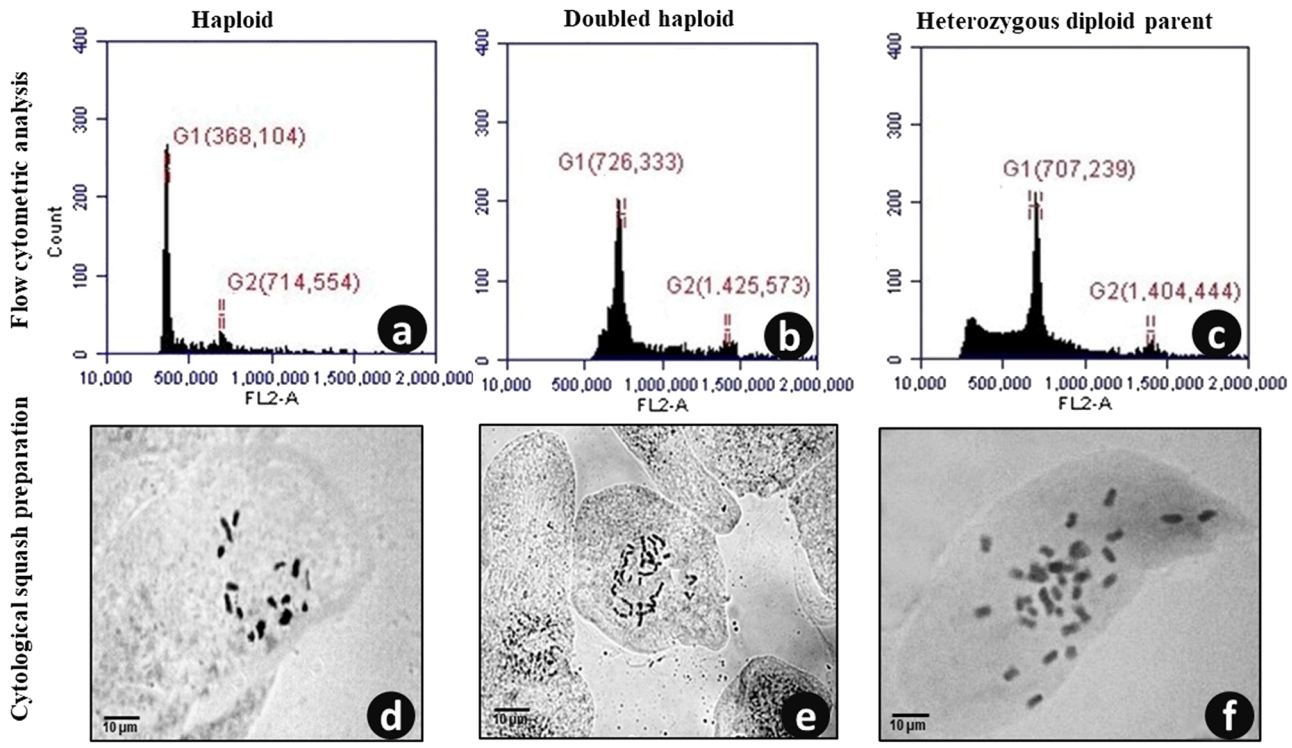

Fig. 4. : Flow cytometry and cytological squash preparation in TV19 cultivar of tea; **a-c** Flow cytometric analysis of leaves from *in vitro* regenerated haploid, doubled haploid and field grown parent plant; **d-f** Cytological squash preparation from the haploid, doubled haploid and parent plant. **a)** Flow cytometric data from *in vitro* regenerated haploid shoots showing G1 peak at 368 and G2 peak at 714 channel positions. **b)** Flow cytometric analysis from leaves of doubled haploid shoots showing G1 peak at 726, and G2 peak at 1425 channel positions. **c)** Flow cytometric analysis of TV19 parent plant (reference plant) showing G1 peak at 707 and G2 peak at 1404 channel positions. **d)** Cytological squash preparation from root-tip of *in vitro* regenerated haploid shoots showing chromosome number n = X = 15 (x 100). **e)** Cytological squash preparation from root-tip of *in vitro* regenerated doubled haploid shoots showing chromosome number 2 n = 2X= 30 (x 100). **f)** Cytological squash preparation from root-tip of field grown (reference plant) showing chromosome number 2 n = 2X= 30 (x 100).

Table 4
Comparative assessment of morphological characteristics between haploid and doubled haploid population.

Cultivar	Ploidy	Average Leaf length (cm)	Average Leaf width (cm)	Stomatal Length (cm)	Stomatal width (cm)	Number of stomata/field
TV21	Haploid	1.43 ± 0.23^{a}	0.77 ± 0.25^{a}	2.18 ± 0.36^{a}	1.94 ± 0.34^{a}	38.30 ± 5.20^{a}
	Doubled haploid	2.10 ± 0.35^{b}	1.28 ± 0.44^{b}	2.74 ± 0.23^{b}	2.27 ± 0.35^{b}	40.70 ± 6.01^{b}
TV19	Haploid	1.02 ± 0.19^{a}	0.33 ± 0.14^{a}	1.75 ± 0.35^{a}	1.51 ± 0.28^{a}	32.30 ± 6.13^{a}
	Doubled haploid	1.81 ± 0.23^{b}	0.98 ± 0.40^{b}	2.44 ± 0.37^{b}	2.21 ± 0.39^{b}	36.60 ± 4.16^{b}

Values are mean of three independent experiments. Mean values sharing the same letter do not differ significantly ($p < 0.05$) according to Tukey's multiple range test.

thereby overcoming the shortfalls of the classical breeding methods (Pintos et al., 2007; Mohammadi et al., 2012). In spite of being an unsurpassable method, chromosome duplication in the tree such as *Camellia* species is limited, owing to its recalcitrant nature and slow growth under *in vitro* conditions. Strenuous efforts have been made for more than a decade to obtain haploid plants in tea; however, most of them remained confined to the generation of haploid callus formation from microspore explant (Seran et al., 1999; Mondal et al., 2004) until the first report on the generation of complete haploid plantlet in tea (Mishra et al., 2017). The present study is an extension of our previous work on haploid production in tea (Mishra et al., 2017), and reveals that *in vitro* regenerated haploid embryos can serve as an efficient source of attaining doubled haploid plants in recalcitrant species such as tea. Although no report for doubled haploid production in tea is available so far, the sources available for the polypoid induction in tea suggest that higher concentration of colchicine 0.2% or even more up to 0.5% could be used for effective polyploidy (Katsuo, 1966). However, the results varied according to the type of explant and a higher percentage 30% of triploid plants were obtained when (Osone, 1958), treated pollen from the diploid plants with 0.05% of colchicine.

In our study, we conclude that the optimized concentration and duration of the antimitotic agent are key players, and perform a critical role in inducing diploidization. Our finding is in agreement with published reports on doubled haploid production in other species such as in *Pyrus communis* L. (Bouvier et al., 2002) and *Brassica napus* L. (Mohammadi et al., 2012). Choosing an appropriate explant is another crucial requirement for inducing diploidization. Chromosome duplication has been achieved in many plants using various explants such as nodal explants, complete plantlet (Fletcher et al., 1998). However, utilization of embryos as an explant followed by direct regeneration into plantlets minimizes chances of chimera formation, which is one of the most desirable aspects in the field of plant breeding (Fletcher et al., 1998; Mohammadi et al., 2012). Therefore, we used haploid embryos for attaining doubled haploids in tea. The treated embryos formed secondary embryos with simultaneous differentiation into shoots. The growth of shoots, differentiating from doubled haploid embryos, was slow during the first two cycles when compared to water immersed, control (haploid embryos). This delayed response may be due to the residual effect of colchicine. Similar observations have been made in *Morus alba* L. (Chakraborti et al., 1998), *Platanus acerifolia* (Liu et al., 2007). However, the growth regressed to normal with the subsequent transfer into fresh medium. The leaves from shoots that differentiated

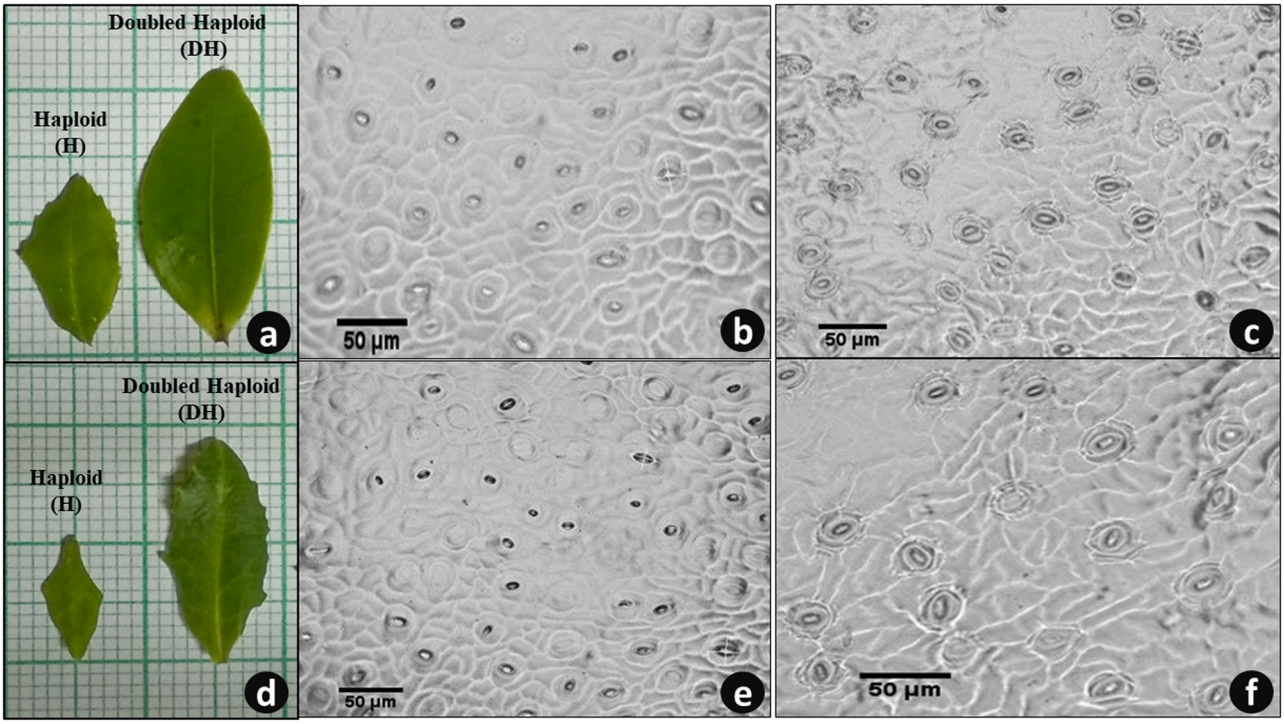

Fig. 5. : Morphological and anatomical analysis of leaves from haploid and doubled haploid plants of TV21 and TV19 cultivars of tea**; a-c** TV21 cultivar**, d-f** TV19 cultivar. **a)** Visual difference in leaf size of haploid (H) and doubled haploid (DH) leaves of TV21 cultivar. **b)** Stomatal analysis from haploid leaves of TV21(Bar =50 µm). **c)** Stomatal analysis from leaves of doubled haploid plants of TV21 (Bar =50 µm). **d)** Visual difference in leaf size of haploid (H) and doubled haploid (DH) leaves of TV19 cultivar. **e)** Stomatal analysis from haploid leaves of TV19 cultivar. **f)** Stomatal analysis from doubled haploid leaves of TV19 cultivar.

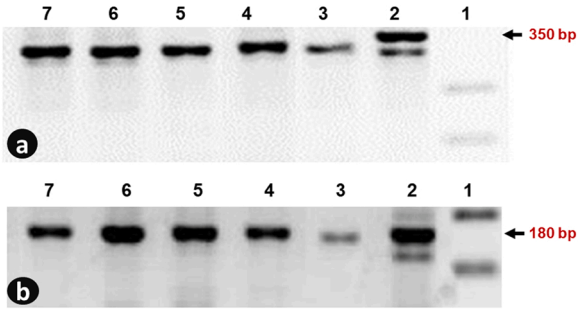

Fig. 6. : SSR marker analysis to determine homozygosity in *in vitro* regenerated doubled haploid plants of TV21 and TV19 cultivars of tea. **a)** SSR marker analysis to determine homozygosity in *in vitro* regenerated doubled haploid plants of TV21 cultivar **Lane 1 is** 100 bp ladder, **Lane 2** contains the Heterozygous parental plant showing two alleles at the marked locus, followed by low width single band from *in vitro* regenerated haploid TV21 plant in **lane 3. Lanes 4–7** consist of the DNA samples from the doubled haploid plants showing single band with more width than haploid sample. **b)** SSR marker analysis to determine homozygosity in *in vitro* regenerated doubled haploid plants of TV19 cultivar. **Lane 1 is 100 bp ladder, Lane 2** contains the Heterozygous parental plant showing two alleles at the marked locus, followed by low width single bands from *in vitro* regenerated haploid TV19 plant in **lane 3**. **Lanes 4–7** consist of the DNA samples from the doubled haploid plants showing single band with more width than haploid sample.

from the doubled haploid embryos were bigger with large sized stomata when compared to their haploid counterparts exhibiting direct correlation with the increase in ploidy. The length and width of the stomata in doubled haploid plants were approximately one fold more than the haploid plants. These observations are in agreement with reports on doubled haploid production in other species (Chakraborti et al., 1998) Flow-cytometry is a high throughput and highly reliable technique for determination of ploidy and DNA content in plants (Doležel and Bartoš, 2005). We determined the ploidy of haploid embryos undergoing differentiation into shoots before antimitotic treatment to ensure haploidy and was rechecked post-treatment to estimate the change in ploidy. Flow cytometry was preferred as primary method to monitor variation in ploidy and identify mixoploids. Chromosome counting is one of the impeccable methods for ploidy determination and was performed in the present study to confirm the change in the number of chromosomes in diploidized plants. The root-tips of *in vitro* grown haploids, doubled haploids as well as *in vivo* grown heterozygous field-grown parent (reference plant) in both TV21 and TV19 cultivars of tea were utilized to study the change in the number of chromosomes.

The doubled haploid plants confirmed via flow cytometry and cytological studies were further analyzed to validate homozygosity via microsatellite markers. SSRs are co-dominant markers and are found in abundance in the genome of the eukaryotic systems. They are multi-allelic, short sequences that can extensively cover the genome, enabling easier detection (Sharma et al., 2010). Similar studies using SSR markers for confirming homozygosity have been performed in other plant species like *Pyrus communis* (Bouvier et al., 2002), *Cocos nucifera* L. (Perera et al., 2008). The authors in the above reports obtained similar results, revealing the difference between homozygous and heterozygous plants with a single primer pair. They concluded that even if a single primer is capable of segregating alleles in a parental population, it is sufficient to confirm homozygosity; as all these plants originated from the same donor. Thus, we report the successful generation of the homozygous doubled haploid lines in TV21 and TV19 cultivars of tea. This study would mark a forward leap for attaining long-awaited genetic improvement in tea.

5. Conclusion

In our study, we conclude that the optimized concentration and duration of the antimitotic agent are key players, and play a critical role in inducing diploidization. Choosing an appropriate explant is another

crucial requirement for inducing diploidization. We report the successful generation of the homozygous doubled haploid lines in TV21 and TV19 cultivars of tea. This study would mark a forward leap for attaining long-awaited genetic improvement in tea.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Rakhi Chaturvedi reports equipment, drugs, or supplies was provided by Indian Institute of Technology Guwahati. Rakhi Chaturvedi reports a relationship with Indian Institute of Technology Guwahati that includes: employment. Rakhi Chaturvedi has patent pending to IIT Guwahati.

Data Availability

Data will be made available on request.

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 experiments on SSR marker analysis.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jbiotec.2022.11.019.

References

Bajpai, R., Chaturvedi, R., 2021. Recurrent secondary embryogenesis in androgenic embryos and clonal fidelity assessment of haploid plants of Tea, *Camellia assamica* ssp. *assamica* and *Camellia assamica* ssp. *lasiocaylx*. Plant Cell Tiss. Org. Cult. 145, 127–141.

Bali, S., Raina, S.N., Bhat, V., Aggarwal, R.K., Goel, S., 2013. Development of a set of genomic microsatellite markers in tea (*Camellia* L.)(*Camelliaceae*). Mol. Breed. 32, 735–741.

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

Bouvier, L., Guerif, P.H., Djulbic, M., Durel, C.E., Chevreau, E., Lespinasse, Y., 2002. Chromosome doubling of pear haploid plants and homozygosity assessment using isozyme and microsatellite markers. Euphytica 123, 255–262.

Chakraborti, S.P., Vijayan, K., Roy, B.N., Qadri, S.M.H., 1998. In vitro induction of tetraploidy in mulberry (*Morus alba* L.). Plant Cell Rep. 17, 799–803.

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

Doležel, J., Bartoš, J.A.N., 2005. Plant DNA flow cytometry and estimation of nuclear genome size. Ann. Bot. 95, 99–110.

Doyle, J.J., Doyle, J.L., 1990. Isolation of plant DNA from fresh tissue. Focus 12, 39–40.

Fletcher, R., Coventry, J., Kott, L.S., 1998. Doubled haploid technology for spring and winter *Brassica napus*. Technical Bulletin. OAC Publ. 1–42.

Hazarika, R.R., Mishra, V.K., Chaturvedi, R., 2013. In vitro haploid production—a fast and reliable approach for crop improvement. In: Tuteja, N., Gill, S. (Eds.), Crop Improvement Under Adverse Conditions. Springer, Heidelberg, pp. 171–212.

Höfer, M., Grafe, C., 2003. Induction of doubled haploids in sweet cherry (*Prunus avium* L.). Euphytica 130, 191–197.

Katsuo, K., 1966. Methods of inducing the polyploid tea plant by colchicine treatment of the axillary bud. Study Tea 33, 1–4.

Lamo, J.M., Wahlang, D.R., Rao, S.R., 2016. Comparative analysis of heterochromatin distribution pattern in wild and cultivated species of *Curcuma* L. Med. Aromat. Plants 3, 6–10.

Liu, G., Li, Z., Bao, M., 2007. Colchicine-induced chromosome doubling in *Platanus acerifolia* and its effect on plant morphology. Euphytica 157, 145–154.

Loureiro, J., Rodriguez, E., Doležel, J., Santos, C., 2007. Two new nuclear isolation buffers for plant DNA flow cytometry: a test with 37 species. Ann. Bot. 100, 875–888.

Majumder, B.A., Bera, B., Rajan, A., 2010. Tea Statistics: Global Scenario. Inc. J. Tea. Sci. 8, 121–124.

Mishra, V.K., 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). Vitr. Cell Dev. -Plant 53, 239–248.

Mishra, V.K., Bajpai, R., Chaturvedi, R., 2022. Androgenic haploid plant development via embryogenesis with simultaneous determination of bioactive metabolites in Cambod tea (*Camellia assamica* ssp. lasiocalyx). Plant Cell Tiss. Org. Cult. 148, 515–531.

Mohammadi, P.P., Moieni, A., Ebrahimi, A., Javidfar, F., 2012. Doubled haploid plants following colchicine treatment of microspore-derived embryos of oilseed rape (*Brassica napus* L.). Plant Cell Tiss. Org. Cult. 108, 251–256.

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

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

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

Murovec, J., Bohanec, B., 2012. Haploids and doubled haploids in plant breeding. In: Abdurakhmonov, D.I. (Ed.), Plant Breeding. In Tech Rijeka, Croacia, pp. 87–106.

Osone, K.I., 1958. Studies on the breeding of triploid plants by diploidizing gamete cells. Jpn. J. Breed. 8, 171–177.

Perera, P.I., Perera, L., Hocher, V., Verdeil, J.L., Yakandawala, D.M.D., Weerakoon, L.K., 2008. Use of SSR markers to determine the anther-derived homozygous lines in coconut. Plant Cell Rep. 27, 1697–1703.

Pintos, B., Manzanera, J.A., Bueno, M.A., 2007. Antimitotic agents increase the production of doubled-haploid embryos from cork oak anther culture. J. Plant Physiol. 164, 1595–1604.

Salma, U., Kundu, S., Mandal, N., 2017. Artificial polyploidy in medicinal plants: advancement in the last two decades and impending prospects. J. Crop Sci. Biotechnol. 20, 9–19.

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

Shao, J., Chen, C., Deng, X., 2003. In vitro induction of tetraploid in pomegranate (*Punica granatum*). Plant Cell Tiss. Org. Cult. 75, 241–246.

Sharma, R.K., Negi, M.S., Sharma, S., Bhardwaj, P., Kumar, R., Bhattachrya, E., Bera, B., 2010. AFLP-based genetic diversity assessment of commercially important tea germplasm in India. Biochem Genet 48, 549–564.

Silva, A.J., Carvalho, C.R., Clarindo, W.R., 2019. Chromosome set doubling and ploidy stability in synthetic auto-and allotetraploid of Eucalyptus: from in vitro condition to the field. Plant Cell Tiss. Org. Cult. 138, 387–394.

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

Tang, Z.Q., Chen, D.L., Song, Z.J., He, Y.C., Cai, D.T., 2010. In vitro induction and identification of tetraploid plants of *Paulownia tomentosa*. Plant Cell Tiss. Org. Cult. 102, 213–220.

Wachira, F.N., Waugh, R., Powell, W., Hackett, C.A., 1994. Detection of genetic diversity in tea (*Camellia sinensis*) using RAPD markers. Genome Biol. 38, 201–210.