Androgenic Haploid Plant Development Via Embryogenesis With Simultaneous Determination of Bioactive Metabolites in Cambod Tea (*Camellia Assamica* ssp. *Lasiocalyx*)

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Research Article

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Abstract

This pioneering work reports successful androgenic plant development via embryogenesis from microspore calluses in anther cultures and estimation of bioactive metabolites in in vitro regenerants and parent plant (control) of Cambod tea, *Camellia assamica* ssp. *lasiocalyx* (Planch MS) cultivar TV19. Anthers bearing microspores at early-to-late uni-nucleate stage were selected to initiate androgenesis. A pre-treatment of 5^0 C for five days in the dark was most effective to initiate profusely growing white callusing from microspores within 10 weeks of culture on MS medium (6% sucrose) supplemented with high cytokinins/ auxin ratio maintained by benzyl adenine (BAP) and 2,4-dichlorophenxyacetic acid (2, 4-D). Nodular structures on the callus surface differentiated into embryos. Further developement of the embryos occurred on embryogenesis medium but, with ten times reduced concentration of growth regulators and additives. Germination of embryos into complete plantlets was achieved when major salts in medium were reduced to half MS (½ MS) and augmented with BAP, GA_3 and IBA along with glutamine and serine. Cytological examination of the root-tip cells revealed that regenerated plantlets were haploids (2n=x=15), which was further confirmed through flow cytometry. The hot-water extracts from in vitro haploid calluses, embryos and field-grown donor plant were utilized for quantification of (+)-catechin, (-)-epicatechin, (-)-epigallocatechin gallate, caffeine and theophylline. Our findings revealed that the metabolite profile of in vitro regenerated haploid cultures is comparable to that of the mother plant, thereby presenting them as potential source for genome duplication and development of genetically stable homozygous pure breeding lines.

Key Message

This is first report on haploids in out-breeding tree, Cambod tea. Its a significant achievement towards generating homozygous lines, which is impossible using conventional methods. Haploids showed consistent metabolite production.

Introduction

Camellia assamica ssp. *lasiocalyx* (Planch MS) or Cambod tea belongs to the Theaceae family (Parmar et al. 2012; Meegahakumbura et al. 2018). It is an evergreen perennial tree, and exhibits high cross pollinating behavior (Olaniyi et al. 2014; Xia et al. 2020). Several studies have associated consumption of tea with prevention of serious health ailments, such as cardiovascular disorders, cancer and diabetes (Khan and Mukhtar 2013; Singhal et al. 2017). More than 300 different kinds of tea are produced presently from the leaves of the tea plant, depending upon the mode of processing, which clearly demonstrates demand for high quality tea with rich aroma and revitalizing flavor (Sang et al. 2011; Mondal 2014). Thus, the primary concern for both; the producers and the consumers remains the quality of tea, which in turn is highly dependent on the amount of specific secondary metabolites and the concentration of each metabolite (Zeng et al. 2019). The therapeutic potential and aromatic characteristics of this commercially valuable crop is mainly attributed to the presence of polyphenol catechins including (+)- catechin (C), (-)-epicatechin (EC), (-)-gallocatechin (GC), (-)-epigallocatechin (EGC), (-)-catechin gallate (CG), (-)-gallocatechin gallate (GCG), (-)-epicatechin gallate (ECG) and (-)-epigallocatechin gallate (EGCG) and purine alkaloids, such as caffeine, theobromine, theophylline and theacrine (Wei et al. 2011; Khan and Mukhtar 2019). The unique amalgamation of bioactive metabolites in tea contributes diverse biological activities limiting occurrence of various fatal diseases as well as antimicrobial potential. Polyphenols (TP) are a class of compounds with free radical scavenging property that monitor the action of various oxidases found in the body (Yan et al. 2020). This characteristic of polyphenols is due to the phenolic hydroxyl structure, where the electrons exhibit conjugation effect that weakens the binding ability of the hydrogen making it more susceptible to dissociation. The hydrogen ions, thus produced, effectively deactivate the free radicals and other reactive oxygen species (Zuo et al. 2018). The phytochemical composition of any plant in vivo may vary depending on several abiotic factors, like elevation, rainfall, drought, shade as well as the biotic components, such as microbes, insects and pests (Scott et al. 2020). The above listed biotic and abiotic elements in combination with the highly variable global environment lead to inconsistent metabolite production reducing the flavor quality of this valuable cash crop (Ahmed et al. 2019).

Conventional methods of tea farming, including propagation via vegetative cuttings and seed propagation have their own limitations and give rise to plants with variable genetic constitution (Mondal et al. 2004; Mondal 2014). Nonexistence of genetically stable clones in tea not only hinders efficient breeding programs but also metabolite extraction and impede thorough utilization of bioactive compounds. Several reports related to in vitro micropropagation in tea by somatic embryogenesis either via callusing or directly from different explants, such as cotyledon, leaves, nodes, have been published (Mondal et al. 2004, Borchetia et al. 2009). But, none of these reports were able to overcome the long persisting problem of self-incompatibility and inbreeding depression in existing varieties of tea. Production of elite tea clones has, therefore, been severely challenged in the absence of genetically pure lines (Kumarihami and Song 2018). The inherent heterozygosity and long reproductive cycle of *Camellia* spp. restrict development of pure breeding lines, thereby impairing varietal improvement of existing tea clones (Mishra et al. 2017). Production of pure lines in tree species via traditional breeding methods is taxing and unpredictable owing to multiple cycles of selfing (Chaturvedi et al. 2003; Srivastava and Chaturvedi 2008). In such a situation, in vitro regeneration of haploid plants from pollen grains or egg cells using either anther or ovary/ovule explants is the only viable option for the production of homozygous plants via chromosome duplication.

Androgenic haploid plants have been achieved successfully in *Azadirachta indica* A. Juss, a tree species (Chaturvedi et al. 2003; Srivastava and Chaturvedi 2011) via application of external stress, like heat shock, cold pre-treatment, starvation and dark incubation. First initiative for androgenesis in tea was made by Katsuo (1969) and Okano and Fuchinone (1970). They attempted regeneration but, obtained roots from the anther derived calluses. Later, Saha and Bhattacharya (1992) observed occurrence of globular structures from anther cultures of *C. sinensis* (L.) O. Kuntze, and Pedroso and Pais (1994), also reported generation of embryogenic calluses from isolated microspore cultures of *C. japonica* L. But, neither of these structures grew further or developed into complete haploid plants. Mondal et al (2004), also attempted haploid regeneration in tea but it remained limited up to the development of micro-calluses. Kumar et al. (2014; 2019) attempted regeneration of haploids in Chinery and UPASI tea cultivars of tea, but remained confined to micro-calluses and embryoid formation, respectively. Mishra et al. (2017), for the very first time reported regeneration of complete haploid plantlets in TV 21, *Camellia assamica* ssp. *assamica* (Masters) or Assam type tea. Present research work is the pioneering report on in vitro androgenic haploid plantlet development, with simultaneous metabolite profiling and antioxidant activity analysis from haploid cultures of TV19, Cambod type tea, *Camellia assamica* ssp. *lasiocalyx* (Planch MS). The results obtained in this study confirm the similar metabolite profile in the in vitro regenerated haploids as that were present in the mother plant. Thereby, these haploid plants can serve as potential source for genome duplication and development of genetically stable homozygous pure lines for breeding.

Materials And Methods

2.1. Explant selection and initiation of anther cultures

The flower buds of Cambod variety, TV19 cultivar of tea growing in experimental garden of Tocklai Experimental Station, Tea Research Association, Jorhat, Assam, India (94°12'E and 26°47'N), were collected between 6 AM - 7AM during October to December to conduct the present study. The selection of buds, was made such that they contain pollen bearing anthers at early-to-late uni-nucleate stage of microspores (Chaturvedi et al. 2003). Surface sterilization of the flower buds was performed using 0.8% sodium hypochlorite (NaClO, Merck, India) as described (Mishra et al. 2017). The correct stage of microspore was determined by squash preparation of anthers using aceto-carmine stain (Sigma-Aldrich, USA) (Srivastava and Chaturvedi 2011). The anthers were carefully isolated from buds with the aid of stereo microscope (Nikon, Japan) using pre-sterilized petri plates, forceps and fine needles. Twenty anthers were cultured in each 60 mm x 15 mm pre-sterilized, disposable petri plate (Tarsons, India) containing 10 ml of MS (Murashige and Skoog 1962) basal medium. Damaged anthers, if any, were discarded. The basal medium was supplemented with different combinations and concentrations of growth regulators like, 2,4-D, BAP, IAA, IBA, kinetin, NAA, TDZ, zeatin, and amino acids like, L-glutamine

and L-serine. All plant growth regulators and additives were procured from Sigma Aldrich Co. (Sigma , USA). After inoculation of anthers, petri plates were sealed with Parafilm (Pechiney, USA). Cultures were incubated either in the presence of light or in complete darkness until callus induction. Anthers were subjected to either cold temperature pre-treatment (5° C in the dark for 0, 5 and 10 days) or hot temperature pre-treatment (33° C in dark for 0, 5 and 10 days). The cultures incubated either in dark or in diffused light at 25° C served as the controls for these experiments. Best responding medium was tested with variable concentrations of glucose and sucrose at concentration range of 3%, 6%, 9% and 12% (w/v). A total of 100 anthers were used for each treatment and every experiment was repeated at least three times. The cultures were periodically scrutinized and all changes in pattern of growth were recorded.

2.2. Callus multiplication and regeneration

Only those calluses emerged from inside the anther locules (microspore) were considered for further experiments. After the passage of two subcultures, each of 6 weeks duration on the callus induction medium, the calluses were shifted to diffused light conditions of 16-hour photoperiod (irradiance 1000-2000 lux) provided by cool day light fluorescent tubes (Philips TL 40W) at 25±2º C with relative humidity of 50-60 %. After third subculture cycle on callus induction medium, the calluses were transferred to multiplication medium with a different sets of growth regulators. Regeneration from calluses via embryogenesis occurred when these calluses exhibiting nodular structures on multiplication medium were transferred to MS medium consisting of BAP and GA_3 as growth regulators and L-glutamine and L-serine as amino acids supplements.

2.3. Embryo maturation and germination

Maturation of embryos was attained when the fully developed embryos from embryo induction medium were transferred to MS medium consisting of 10 times reduced concentrations of the growth regulators; BAP, GA_3 as well as additives, L-glutamine and L-serine. After a period of 6 weeks incubation into maturation medium, the embryos were transferred to germination medium consisting of ½ MS medium (only major salts reduced to half strength) supplemented with BAP, GA_3 and IBA in combination with, L-glutamine and L-serine. All the cultures were maintained at standard culture room conditions with 16 h photoperiod. Medium containing 3% sucrose was used unless mentioned otherwise. Each experiment was repeated three times and 54 cultures were raised during the treatment. Weekly observation of cultures was made to visualize the pattern of development.

2.4. Histological studies and scanning electron microscopy

Histological analysis was done to determine the anatomical growth of in vitro obtained haploid cultures. The protocol for sample preparation and processing was adopted from Mishra et al. (2017). The processed samples were mounted on wooden stubs, and sections of 8-10 µm thickness were made using a manual rotary microtome (Leica, Germany). The sections retrieved were mounted on glass slides, dewaxed and stained, following double staining method as described by Chaturvedi et al. (2003). Stained sections were finally observed under the microscope (Nikon, Japan). Anther derived calluses showing embryogenesis on embryo induction medium were analyzed via scanning electron microscopy analysis. The samples were pre-fixed in 2.5 % glutaraldehyde and passed through ascending series of alcohol dehydration (30 %, 50 %, 70 %, 90 % and pure alcohol), followed by drying in desiccators. The samples were sputter-coated with gold and observed under a scanning electron microscope (Leo 1430vp, Carl Zeiss, Germany).

2.5. Ploidy analysis

The ploidy level of the in vitro regenerated haploid plants and field-grown donor plant (control) was determined via cytological squash preparation and was further confirmed through flow cytometry as described below.

2.5.1. Cytology

For cytological analysis root-tips of in vitro grown haploid plants and shoot-tips from field-grown donor plant (control) were washed and fixed in 0.02 % 8-hydroxyquinoline for 4 hours at 4º C (harvested around 10.00-10:30 AM), followed by washing and transfer to modified Carnoy's fixative (7:3:1:1v/v/v/v) absolute alcohol: chloroform: methanol: glacial acetic acid) under refrigeration for 48 hours. The root-tips were then stored in 70% ethanol (Chaturvedi et al. 2003). The root-tip squash preparation was performed following the procedure for chromosomal count analysis described by Mishra et al. (2017). The number of chromosomes per cell in the root-tip squash preparations were counted under the ×100 objective lens of a 80i microscope (Nikon, Japan)

2.5.2. Flow cytometry analysis

Ploidy analysis of in vitro regenerated haploid plants and donor plants was assessed through BD accurie C6 flow cytometer (Beckton–Dickinson, USA), equipped with an argon laser (15mV) at 488 nm encompassing an emission range of greater than 590 nm. Fresh, young leaves from in vitro regenerated haploid plants and the donor plant leaf (as an external standard) were chopped in modified woody plant buffer. The buffer composition and the process for sample preparation was adopted from Mishra et al. (2017). The filtered nuclear suspensions obtained by chopping leaf samples, were treated with 50 µg/ml concentration of RNase (Sigma, USA) and stained simultaneously with 50 µg/ml concentration of propidium iodide (Sigma, USA).

2.6. Extraction Procedure

The calluses from multiplication medium and embryogenic cultures from embryo maturation medium, were harvested after 8 weeks, washed with distilled water and vacuum filtered. Subsequently, vacuum filtered in vitro cultures, and thoroughly washed leaf samples from donor plant (control) were all dried in an oven at 35±2° C until constant weight was achieved. The dried samples were powdered, and 10g (dry weight) of each powdered samples, were soaked for 12 h either in 400 ml of hot-water or analytical grade organic solvents, like methanol, ethyl acetate and hexane. Samples soaked in water were heated at 80º C for 20 min and then cooled down to room temperature. The various extracts were individually filtered through Whatman filter paper no.-1 (GE Healthcare, England). The filtrate obtained was centrifuged at 10,000 rpm for 10 min. The aqueous extract was lyophilized in freeze dryer (Chaist Model alfa 1-4, Osterode am Harz, Germany) while the organic solvent based supernatants were evaporated until completely dried in rotary evaporator (Buchi Rotavapor R-200, Japan) at 40 ºC . The samples were stored at 4 ºC for further studies. The percentage yield of extracts was calculated using the following formula:

$$\text{Extraction yield (\%)} = \frac{\text{Weight of dried extract (in g) X 100}}{\text{Weight of dried callus or leaves (in g)}}$$

2.7. Total phenolics estimation

Total phenolic content in the extracts was determined as described previously (Singh et al. 2013; Hazarika and Chaturvedi 2013) involving Folin–Ciocalteau reagent and gallic acid standard (Sigma, USA). The total phenolics concentration is expressed as gallic acid equivalents (GAE) (mg/g dry weight). The method was repeated using standard gallic acid solutions, and a standard curve was obtained. All the samples were analyzed in triplicate.

2.8. Determination of antioxidant activity

2.8.1. DPPH free-radical scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) free-radical scavenging activity of calluses, embryogenic cultures and leaves from donor plants (control) was evaluated following the method used by Leong and Shui (2002) and Chan et al. (2007) with some modifications. Stock solution of DPPH (Sigma, USA) was prepared at concentration of 5.9 mg/100 ml

methanol. Ascorbic acid, catechin, epicatechin, epigallocatechin gallate, gallic acid and vanillic acid (all purchased from Sigma, USA) were used as reference standards and their stock solutions (1 mg/ml) were prepared in methanol. The stock solutions of the crude extracts were prepared at (1mg/ml) concentration in their respective solvents. Working solutions of each reference standard and crude extracts were prepared in separate test tubes, at different dilutions (in the range of 1 µg/ml -180 µg/ml). Final sample consisted of both the standard and sample amounting to a total of 1 ml. Thereafter, 2 ml of DPPH solution was added to the above solution, mixed gently and allowed to stand for 30 min at room temperature. The absorbance of each mixture was measured at 517 nm with spectrophotometer (Carry 100, Netherlands). The radical scavenging activity was expressed as the percentage inhibition (I %) of free-radical and was calculated using the following formula:

$$\text{Percentage inhibition (I \%)} = \frac{(A_{sample} - A_{blank})\ X\ 100}{A_{blank}}$$

Where A_{blank} is the absorbance for control containing 1 ml of respective solvents (water, methanol, ethyl acetate and hexane) along with 2 ml of DPPH without extract. $A_{sample,}$ on the other hand is the absorbance value of the tested sample. The antioxidant activity of each sample is expressed as IC_{50} value (concentration in µg/ml required to inhibit DPPH radical formation by 50 %) and is calculated from the dose inhibition curve.

2.8.2. Ferric-reducing antioxidant power

Ferric-reducing antioxidant power (FRAP) assay was performed for only those samples which possessed high free-radical scavenging activity. The methodology for FRAP assay was adopted from Chu et al. (2000) and Chan et al. (2007) with minor modifications. Stock solution (1 mg/ml) of all three samples; calluses, embryogenic cultures and leaves from donor plants (control) was prepared. Each stock solution measuring 1 ml was collected, and 2.5 ml of both phosphate buffer (0.2 M, pH 6.6) and potassium ferricyanide (1%, w/v) were added to it and, mixed properly and incubated at 50°C for 20 min. Trichloroacetic acid solution (10%) measuring 2.5 ml (w/v) was added to the above solution to stop the reaction. The mixture was separated into aliquots of 2.5 ml each and diluted with 2.5 ml water. To each diluted sample, 500 µl of 0.1% ferric chloride solution (w/v) was added and it was incubated for 30 min. The absorbance was recorded using a spectrophotometer (Carry 100, Netherlands) at 700 nm. The FRAP of extracts was expressed as mg GAE/g. A standard curve was generated by using gallic acid for estimating the reducing power. The calibration equation for gallic acid was y = 15.91x (R^2 = 0.9974), where y is the absorbance and x is the gallic acid concentration in mg/ml.

2.9. Detection and quantification of (+)-catechin, (-)-epicatechin, (-)-epigallocatechin gallate, caffeine and theophylline

2.9.1. Method for preparation of standards

Individual stock solutions at a concentration of 5 mg/ml for (+)-catechin (C), (-)-epicatechin (EC), (-)-epigallocatechin gallate (EGCG), caffeine (CAF) and theophylline (T) standards were prepared in Milli Q water and stored at 4° C. Five serial dilutions of stock solutions, in a range of 25 µg/ml to 500 µg/ml were prepared and external standards were used during quantification. Each standard was run for at least three times. Confirmation of the compounds was done through spiking using both external and internal standards.

2.9.2. Conditions for High Performance Liquid Chromatography (HPLC)

Identification and estimation of (+)-catechin, (-)-epicatechin, (-)-epigallocatechin gallate, caffeine and theophylline was performed using Varian Prostar HPLC system (Varian, USA) consisting of binary pump, UV detector and a 20 µl injection loop. Hypersil BDS RP-C_{18} column (Thermo, USA) with dimensions 250 x 4.6 mm, 5 µm particle size, was used during analysis. Theophylline was analyzed using acetonitrile: water (10:90) (v/v) ratio at a flow rate of 1 ml/min. The eluted

samples were detected at a wavelength of 273 nm. Analysis of (+)-catechin, (-)-epicatechin, (-)-epigallocatechin gallate and caffeine was performed using acetonitrile: ethyl acetate: 0.05% H_3PO_4 (12: 2: 86 v/v) as mobile phase at a flow rate of 0.5 ml/min. The eluted samples were detected at wavelength of 280 nm. Retention time of all five compounds was calculated by comparing it with the retention time of the commercially available standards. All samples including standard and crude were filtered using nylon membrane filters 0.22 mm (Millipore, USA) and 20 ml of each sample was injected for detection into the HPLC system. Linearity, precision and recovery of compounds was performed for system suitability tests. The linearity of the procedure considered for experiment was determined by running standards of all compounds on five different concentrations. Peak area and concentration were plotted against each other on sigma plot 11.0 and a calibration curve was obtained. Quantification of all compounds in the unknown sample was performed using the equation obtained from the calibration curve. The correlation coefficients (R^2) was generated using the same software by putting the linear trendlines into the standard curves attained for all the five compounds.

Accuracy of the method developed was estimated by running sole concentration of each standard compound thrice on very day (intraday) and also on a day's interval (interday). Values for relative standard deviation (RSD) were calculated using below mentioned formula.

RSD= (SD/average) x 100%

Experiments for recovery of all the five compounds was done by addition of known amount of C, EC, EGCG, CAF and T standards to the extracted sample. The percentages of recovery for compounds was calculated using the formula

Recovery (%) = (*A − B / C) × 100*

Where, *A* is the quantity of C, EC, EGCG, CAF and T in the spiked powder, *B* is the quantity of C, EC, EGCG, CAF and T in the powder without added standards and *C* is the quantity of added C, EC, EGCG, CAF and T.

2.9.3. Instrument condition for mass spectrometry (MS) analysis

Identification of compounds was performed using Waters quadrupole-Tof premier mass spectrometer equipped with a micro channel plate detector (Waters, USA). Mass spectrometer was used in the positive ion mode, where the collision energy was 5V. Voltage on ESI probe tip was 3 kV to 3.5 kV in positive mode. Data were collected in complete scan mode (mass range 100–1000 amu). A comparative assessment of mass spectra obtained from HPLC eluted standards of all five compounds purchased from Sigma-Aldrich was done by matching it with the mass spectra of each corresponding sample isolated from HPLC. Mass spectrometric analysis confirmed the occurrence of all the five compounds.

2.10. Statistical analysis

For all experiments, 3 replicates were used and each experiment was repeated a total of three times. Data analysis was performed using SPSS (version 16) software and significant differences among the mean values was assessed on the basis of the Duncan's multiple range test. All the readings were mean of three independent experiments. Significance of data was determined at $P < 0.05$. For antioxidant activity, reducing power assay and phenolic content estimation, observations are an average of three independent analyses.

Results

3.1. Callus induction

For androgenic callus induction, anthers, bearing early-to-late uni-nucleate stages of microspores from 4.0 mm size flower buds of TV 19 cultivar of tea **(Fig. 1A)** were utilized. Surface sterilization of flower buds with 0.8% sodium hypochlorite for 7 minutes yielded more than 93% aseptic cultures. MS medium in combination with various growth regulator

combinations and the carbon sources were tested, callus induction was observed in the four media combinations listed below.

MS (60 g/l sucrose) + 2,4-D (1 µM) + NAA (1 µM) + BAP (5 µM).

MS (40 g/l sucrose) + 2,4-D (3 µM) + TDZ (18 µM).

MS (30 g/l sucrose) + 2, 4-D (5 µM) + kinetin (5 µM) + L-glutamine (800 mg/l) + L-serine (200 mg/l).

MS (60 g/l glucose) + 2, 4-D (5 µM) + kinetin (5 µM) + L-glutamine (800 mg/) + L-serine (200 mg/l).

Among the four media combinations tried, the highest percentage callusing was observed on MS1 medium **(Table 1).** Percentage response on callus induction medium was higher for the cultures incubated continuously in dark, compared to those incubated in light, at 25±2ºC **(Table 1).**

The anthers were exposed to two different temperature pre-treatments before being transferred to 25±2ºC light conditions. Maximum callusing was observed at 25ºC under dark incubation (Control), followed by cold pre-treatments at 5ºC for 5 days in the dark. Pre-treatment of anther cultures at 33ºC for 5 days in the dark was not effective for callus induction, rather it showed adverse effect **(Table 1).** Two-way ANOVA was done to study the effect of responding media and temperature pre-treatments on anther cultures. Both the factors, media and temperature pre-treatments were found to be significant factors with p-value less than 0.05. Cold or heat treatment beyond 5 days was found to be inhibitory for callus induction.

Although incubation of anthers in the dark at 25ºC (control) showed highest percentage of callusing, a better response in terms of induction of calluses from inside the anther locules was recorded when anthers were subjected to cold pre-treatment at 5ºC for 5 days in the dark **(Table 1)**. Maximum callusing 41% was induced from bursting of anthers at 5ºC for 5 days in the dark on MS1 medium while cultures incubated in the dark at 25ºC temperature on same medium exhibited only 35 % of callusing **(Fig. 2)**.

Further, the effect of addition of two carbon sources, at varying concentrations, on anther culture was studied. It was observed that sucrose was significantly better ($p<0.05$) than glucose **(Fig. 3).** But, the percentage of anthers that showed callusing and the extent of callusing differed with varying concentration of sucrose (30 - 120 g/l). Distinctly better callus induction was found with (60 g/l) sucrose, followed by 30, 90 and 120 g/l. The concentrations of sucrose above 9%, however inhibited callusing **(Fig. 3)**.

The anthers enlarged to almost double of their original size on best responding medium within two weeks of culture initiation and a complete longitudinal furrow appeared on the anthers after 6 weeks from culture initiation time **(Fig. 1 B).** After seven weeks, anther sacs burst open, oozing off small calluses from inside the locules, which later showed shiny, white, transparent appearance after 8 weeks **(Fig.1 C)**. After 10 weeks, distinct white, shiny, profuse callus was seen from each anther locule **(Fig.1D)** and, entire anthers were covered with cream and brown callus later. Therefore, after 10 weeks, the anthers were subcultured into a new medium with similar composition and maintained in the dark. Some callusing was also observed from walls of anthers, however, only the calluses that originated from inside anther locules were considered for further experiments on regeneration.

3.2. Callus multiplication and regeneration

Callus was subcultured twice at six weeks intervals on callus induction medium, with dark incubation. Though rate of callus multiplication was slow on callus induction medium, the calluses grew into white hard callus without any sign of regeneration **(Fig. 1E)** until after the first two subcultures. During the third subculture, the calluses were shifted to diffused light conditions at 25 ± 2ºC, where it turned green, hard and compact nodular **(Fig. 1F)** on the induction medium. The rate

of callus multiplication was low, thus, to obtain profusely growing calluses, a range of growth regulator combinations were tested. Best callus proliferation in terms of fresh cell biomass increase was observed on MS + NAA (5 µM) + BAP (10 µM) medium in single growth cycle of 8 weeks. Initially the calluses were soft and fast growing. However, at the end of 8 weeks, texture of the calluses turned into hard, compact and nodular callus **(Fig. 1G).** Later histological examination of the nodular callus confirmed the presence of meristemoids within the calluses **(Fig. 1H)**.

3.3. Embryo regeneration, maturation and germination

Regeneration in the calluses was observed via embryogenesis when the nodular calluses from callus multiplication medium were transferred to the regeneration medium that consisted of MS + BAP (10 µM) + GA_3 (3 µM) + L-glutamine (800 mg/l) + L-serine (200 mg/l). Asynchronous embryogenesis was observed on regeneration medium after two subcultures of 8 weeks each. More than 18 embryos per culture were developed in clusters. All the stages of embryos development, globular, heart, torpedo and dicotyledonous, were seen within a single cluster in single passage and the embryos were attached loosely to the surface of calluses **(Fig. 4A, B)**. The SEM images also confirmed asynchronous embryogenesis **(Fig. 4 C, D).**

Full and ½ MS basal medium (only major salts reduced to half) were used as controls for maturation and germination of embryos, but did not support growth. Maturation and germination of embryos occurred, after 8 weeks, only when cultures were transferred to ten times reduced concentration of growth regulators and nitrogen sources present in embryo differentiation medium. On MS + BAP (1 µM) + GA_3 (0.3 µM) + L-glutamine (80 mg/l) + L-serine (20 mg/l), complete bipolar embryos were observed showing long radicular end and green plumular end **(Fig.4 E).** Fully developed bipolar embryos germinated into complete plantlets when transferred to ½ MS medium (major salts reduced to half strength) containing BAP (10 µM), GA_3 (0.5 µM), IBA (1 µM), L-glutamine (80 mg/l) and L-serine (20 mg/l) **(Fig.4 F)** within 10 weeks.

3.4. Ploidy analysis

3.4.1. Cytological analysis

Cytological analysis of shoot-tips from field-grown plants (Control) showed the diploid number of chromosomes (2n=2x=30) **(Fig. 5 A),** while root-tips of in vitro developed plantlets revealed that the majority of the cells were in haploid state (2n=x=15) confirming androgenic haploid induction **(Fig. 5 C)**.

3.4.2. Flow cytometry analysis

The linear fluorescence intensity histograms of relative nuclear DNA content of leaves from field-grown plant (control) and in vitro derived plantlets showed distinct G0/G1 peaks with coefficients of variation (CV) less than 5.0% for leaves from field-grown plant and in vitro derived plantlets. The representative histograms from leaves of field grown donor plants (control) is seen in (**Fig. 5 B),** where G1 peak was observed at channel position 699 and G2 phase at 1397. In contrast to control plants, the ploidy analysis of in vitro regenerated haploid plant leaves showing G1 peak at channel position 353 and G2 phase at 712 channel position **(Fig. 5 D)**. The results of flow cytometry confirm that regenerated plantlets have half the DNA content of that found in control diploid plants. With this it can be concluded that in vitro grown cultures have maintained their haploidy status.

3.5. Total phenolic content of androgenic lines

The total phenolic contents from various extracts of TV19 cultivar of tea, i.e. hot water, methanol, ethyl acetate and hexane, was determined through a linear standard curve ($y = 0.002x+0.074$; $r^2 = 0.986$). The total phenolic contents was mentioned in order of young leaves from donor plants > embryos > calluses. Hot water extract was found to be the most

suitable solvent for extraction of phenolics **(Fig. 6)**. Among the androgenic cultures, embryo extract contained the highest amount of phenolics as 43.12 ±2.21 mg GAE/g dry weight when compared to the content in the callus.

3.6. Antioxidant activity of androgenic cultures

3.6.1. DPPH radical scavenging activity of various extracts

The percent scavenging activity of various solvent extracts, obtained from control and androgenic lines (calluses and embryos) of TV19 cultivar (percentage inhibition i.e. I%) are represented in **(Fig. 7)**. The slope of DPPH inhibition curve was greater in extracts prepared from leaves of donor plant (control) **(Fig. 7A)** followed by embryos **(Fig. 7 B)** and then calluses **(Fig. 7C)**. Solvent wise DPPH inhibition pattern could also be seen in the order of hot water > methanol > ethyl acetate > hexane **(Fig. 7 A, B, C).** IC_{50} values of reference standards and each extract was calculated from the linear equations of the DPPH inhibition curves, respectively. Lower the IC_{50} value, higher is the antioxidant activity. IC_{50} values were in the order of young leaves from donor plant < embryos < calluses. Among solvents, maximum antioxidant activity was found in hot water extracts, followed by methanol, ethyl acetate and hexane **(Fig. 7)**. The hot water extracts of TV 19 leaves (control), embryos and calluses exhibited DPPH inhibition with IC_{50} values of 29.98±1.34 µg/ml, 46.21±2.21 µg/ml and 53.37 ±2.15 µg/ml, respectively. In order to understand the free radical scavenging potential of sample extracts, their activities were compared with relative activities of standard antioxidant compounds (ascorbic acid, catechin (C), epicatechin (EC), epigallocatechin gallate (EGCG), gallic acid and vanillic acid). The IC_{50} values of these reference standards has been added as a supplementary data.

3.6.2. Ferric-reducing antioxidant power (FRAP) of androgenic cultures

Maximum antioxidant activity in above section was found in hot water extracts, therefore, ferric-reducing antioxidant power (FRAP) analysis was performed with the hot water extracts only. The FRAP measures the capability of compounds to act as an electron donor while DPPH measures their capability to act as hydrogen donor. The FRAP values obtained for hot water extracts from all three sources (leaf of control plant, embryos and calluses) of TV19 cultivar are 43.96 mg GAE/g, 26.19 mg GAE/g and 24.86 mg GAE/g respectively **(Fig. 8).**

3.7. Standardization of a methodology for separation of acid (+)-catechin, (-)-epicatechin, (-)-epigallocatechin gallate, caffeine and theophylline

Absorption maxima of the C, EC, EGCG and caffeine (CAF) standards, dissolved in water, was recorded using UV-Visible spectrophotometer (Cary, USA) which was later utilized as optimized wavelength for detection of these compounds through HPLC. For theophylline (T) standard compound, 273 nm was found as the absorption maximum. Initially, methanol was used as the mobile phase for HPLC, as it has higher absorption and gives higher interference at 280 nm. But, separation of catechins (C & EC) which are structural isomers, requires a solvent with low absorbance which would not obstruct process of peak separation. Hence, separation of all catechins, was carried out using acidic water along with acetonitrile and ethyl acetate i.e. acetonitrile: ethyl acetate: 0.05 % H_3PO_4 (12: 2: 86 v/v) at a flow rate of 0.5 ml/min, which provided clear separation of all four catechins. The chromatograms of standards C, EC, EGCG and CAF obtained by the same method is shown in **Fig. 9 A-D.** Acquisition time of all these compounds was less than 25 min. Separation of T standard was carried out using acetonitrile and water in ratio of (10:90) (v/v) as mobile phase with flow rate of 1 ml/min. The chromatogram of T is shown in **(Fig. 9 E).** An acquisition time of 20 min was required for its separation. The retention times (in minute) for all five compounds are mentioned in **Fig. 9.**

3.8.1 Linearity and precision

Calibration curves obtained for each of the five standard compounds exhibited clear linearity at tested concentrations in range of (0.025 mg/ml to 1 mg/ml) having the correlation coefficients (R^2) of 0.992, 0.996, 0.991, 0.999 and 0.987 for C,

EC, EGCG, CAF and T, respectively. The equation achieved from the calibration curve using the external standard was utilized for the estimation of the quantity of compounds that exist in the crude extract. Existence of similar compounds in the crude extracts was assured by co-injecting internal standards of all concerned compounds along with the crude extract in HPLC **(Fig. 10 A, B)**. The accuracy of the method adopted during analysis, as described in materials and methods section, by running the uniform concentration of the standard samples, thrice on same day, RSD values obtained were 1.3 %, 0.9 %, 0.5 %, 1.2 % and 0.3 % for C, EC, EGCG, CAF and T, respectively. The variability in values obtained for inter-day run using similar concentration of all five standards were 1.1 %, 1.2 %, 0.8 %, 1.4 %, and 0.2 % for C, EC, EGCG, CAF and T, respectively **(Fig. 9).**

3.8.2. Quantification of compounds in crude extract

The amount of C, EC, EGCG, CAF and T in samples (callus and embryo) was calculated using the standard equations and are listed in **(Table 2).** The pattern of peaks was similar for both standards and sample compounds, where almost all the peaks present in the standard were present in the in vitro haploid callus and embryo samples as well **(Fig.10 A-H).** Hot water extract from leaves of field-grown donor plant (control) contained highest amount of metabolites. However, among in vitro cultures, embryogenic cultures possessed highest content of all metabolites tested in this study as compared to that in the dedifferentiated (callus) cultures.

3.8.3. Analysis of mass spectra

The HPLC eluted fractions of crude sample were collected at respective retention time, analyzed by mass spectrometry and the fragmentation pattern achieved was compared with that of HPLC eluted standards of respective compounds, procured from Sigma, Aldrich. All the five compounds were analyzed using, positive mode electrospray ionization (+ESI) conditions. Spectra was obtained in full scan mode. Base peak for caffeine was obtained at m/z 195 due to the addition of hydrogen ion in positive mode electrospray ionization [MH^+], m/z 217 formed due to formation of sodium adduct [MH^++Na^+] in HPLC eluted crude sample as well as in standard **(Fig. 11 A, B).** For (+)-catechin m/z fragments in standard compound and HPLC eluted crude fraction are shown in **(Fig. 11 C , D),** where the base peak of m/z 291 was obtained due to the addition of hydrogen ion in positive mode electrospray ionization [MH^+] and fragments peaks are m/z 139 and m/z 183 due to fragmentation of compound. Similarly in (-)-epicatechin, base peak of m/z 291 was seen due to the addition of hydrogen ion [MH^+] and it's fragment peak was seen as m/z 139 **(Fig. 11 E, F).** Mass spectra of HPLC eluted (-)-epigallocatechin gallate standard and crude samples is shown in **(Fig. 11 G, H)**, base peak is observed at m/z 459 of [MH^+] and two characteristic fragment peaks at m/z 139 and m/z 289, thus, confirming presence of EGCG; m/z 497 formed due to formation of potassium adduct [MH^++K^+] in HPLC eluted crude samples as well as in standards (Fig. 8 G, H). Mass spectra of HPLC eluted standard and crude samples of theophylline are presented in **(Fig. 11 I , J)** where base peak is m/z 181 due to addition of hydrogen ion in positive mode electrospray ionization [MH^+] and m/z 200 is due to adduct with water [MH^++ H_2O].

Discussion

In vitro production of haploid plants is a much needed technique for the varietal improvement of tree species having lengthy juvenile phase, high heterozygosity, and extensive inbreeding depression (Srivastava and Chaturvedi, 2011; Ying et al., 2013; Silva et al., 2019). Haploid induction mediates production of pure breeding homozygous lines in a relatively short span of time as compared to traditional breeding methods, which requires several generations of self fertilization. Traditional methods are labour intensive and often unpredictable (Islam and Tuteja, 2012; Bajpai and Chaturvedi, 2018). Tea is a commercially important cash crop, but it has not been easy to produce haploids for this crop owing to its recalcitrant nature. Considerable attempts were made in the past to produce haploid plants in tea, but success remained confined up to the regeneration of micro-calluses, which was either organogenic or embryogenic but, did not grow any

further. Thus complete haploid plantlets were not reported in any of the published reports (Saha and Bhattacharya 1992; Kumar et al., 2014; 2019) until before 2017. Although Mishra et al. (2017), obtained haploid plantlets of *Camellia assamica* ssp. *assamica* (TV21) (Assam type tea) from androgenic embryos attained via callusing in anther cultures, metabolite content and antioxidant activity analysis of the regenerants was not recorded in the report. The present investigation is a self-contained study illustrating development of haploid plantlets in *Camellia assamica* ssp. *lasiocalyx* (TV19) (Cambod type Tea). The results obtained in this research represent uniformness in terms of the metabolite profile, total phenolic content and antioxidant activity in the regenerated haploid plants. Moreover, the haploid plants also will serve as raw material for the development of genetically uniform homozygous diploid lines in the future, which would be a source for consistent metabolite production.

The medicinal and commercial significance of tea is predominantly attributed to the reservoir of potentially active metabolites including flavonoids, alkaloids and aminoacids (Hajimahmoodi, 2008; Li et al., 2017). The pool of polyphenols in tea has been reported to possess high antioxidant property, restricting generation of reactive oxygen species (ROS) such as O_2^-, OH^-, and H_2O_2. The reactive oxygen behave as a two-way sword, acting a secondary messenger in different physiological processes facilitating free radical scavenging activity and oxidative damage as a result of immune response to microbial infection (Khorasani et al., 2015; Yan et al., 2020). High antioxidant ability mediates inhibition of apoptosis regulating occurrence of cancer (Khorasani et al., 2015). Antioxidant activity of tea is influenced by the amount of natural polyphenols, like catechins, oxyaromatic acids, tannins, flavonols, thearubigins, theaflavins, etc. (Hajimahmoodi, 2008), which could vary depending on the genotype and environmental variations (Ahmed et al., 2019). In the present study, antioxidant activity of the extracts from the above androgenic lines (embryos and calluses) and leaf samples (control) of *Camellia assamica* ssp. *lasiocalyx* (TV19) (Cambod Tea) was determined using DPPH and FRAP assays. The DPPH assay describes the ability of a compounds to act as hydrogen donor while FRAP acts as an electron donor (Chan et al., 2007). IC_{50} is a measure of DPPH inhibitory concentration, thus the sample with a lower IC_{50} value reflects higher antioxidant activity. The results shown in this study are in agreement with earlier reports, which suggest that the dedifferentiated cultures have lower anti-oxidant activity as compared to organized structures (Grzegorczyk et al., 2007; Matkowski 2008). Our results reveal that maximum phenolic content and lowest IC_{50} value was found in young leaves from donor plants > embryos > calluses (29.98±1.34 µg/ml, 46.21±2.21 µg/ml and 53.37.0±2.15 µg/ml).

Green tea extracts usually consist of 10–15% EGCG, 6–10% EGC, 2–3% ECG, and 2% EC (Shi et al., 2009; Vuong et al., 2010; Singhal et al., 2017). A few reports have been published till date on metabolite extraction from in vitro cultures of tea obtained using different somatic tissues (explants). However, no reports for metabolite content analysis from the androgenic cultures in tea is available so far. In the current investigation, high content of EC and C from free catechin and EGCG from esterified catechin was selected for the analysis of production in androgenic cultures of *Camellia assamica* ssp. *lasiocalyx* (TV19) (Cambod Tea) and was compared with in vivo donor plant.

In the present study in vitro cultures (calluses as well as embryos both) along with the donor plant had higher EGCG content. The fragmentation pattern obtained from various fractions eluted by HPLC resembled the fragmentation pattern of their respective standards, this further ascertained the presence of C, EC, EGCG, CAF and T in the haploid cell lines of tea. Fragmentation pattern with base peak at 139 m/z is the most preferred pattern for catechins but this pattern is achieved only when gallate is not present. This fragment is of utmost importance and can be obtained by breaking two bonds in the ring C and sits origin lies in: (i) phenolic ring A of all eight catechins or (ii) phenolic ring B of galocatechins (Spáčil et al., 2010). We also obtained mass spectra of C, EC and EGCG showed in a positive ESI mode with a base m/z 139 characteristic fragment, which is due to a retro Diels-Alder (RDA) fragmentation of the nonviable portion of the catechin ring structure (Zeeb et al., 2000). The pathway for this fragmentation is provided as supplementary data.

Conclusion

The findings of our study illustrate an efficient methodology for regeneration of complete haploid plantlets in *Camellia assamica* ssp. *lasiocalyx* (TV19) (Cambod type) cultivar of tea coupled with a study of its antioxidant efficacy and targeted metabolite profile determination. The study reveals similar pattern of metabolite profile in the haploid regenerates as found in the in vivo plants, thereby suggesting that the in vitro developed haploid plants could serve as potential source for the development of true breeding lines and would also facilitate consistent production of medicinally important high value metabolites independent of seasonal variation.

Abbreviations

2,4-D – 2,4-dichlorophenoxyacetic acid; BAP- 6-benzylaminopurine; GA_3 - Gibberellic acid; IBA – Indole-3-butyric acid; Kinetin – 6-furfurylaminopurine; MS – Murashige and Skoog (1962) medium; NAA – α-naphthaleneacetic acid; TDZ - Thidiazuron; TV – Tocklai vegetative

Declarations

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The authors declare that they have no known competing financial/non-financial interests or personal relationships that could have appeared to influence the work reported in this paper. It is to further confirm that all authors agree to submit the work for publication.

Data availability statement

All data generated or analysed during this study are included in this article and its supplementary information files. The additional information relevant to the current article, if needed, will be available from the corresponding author on reasonable request.

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Tables

Table 1: Effect of temperature pre-treatments on callus induction in anther cultures of TV19 cultivar of tea

Media	Number of anthers cultured per treatment	Percentage anther response incubated in			
		25ºC dark (control)	5ºC dark for 5 days	33ºC dark for 5 days	25ºC in light
MS1	100	91 ± 4^{a}	69 ± 4^{b}	25 ± 3^{ef}	21 ± 4^{fg}
MS2	100	69 ± 4^{b}	40 ± 5^{d}	19 ± 4^{fg}	18 ± 5^{g}
MS3	100	52 ± 2^{c}	29 ± 4^{e}	17 ± 5^{g}	6 ± 4^{h}
MS4	100	41 ± 4^{d}	21 ± 4^{fg}	4 ± 4^{h}	4 ± 6^{h}

Values are mean ± standard deviation

Same letter after the value are not significantly different ($p<0.05$) according to Duncan's multiple range

Table 2: (+)-Catechin, (-)-Epicatechin, (-)-Epigallocatechin gallate, Caffeine and Theophylline content in in vitro and in vivo sample of TV19 cultivar of tea.

Sample	(+)-Catechin concentration	(-)-Epicatechin concentration	(-)-Epigallocatechin gallate concentration	Caffeine	Theophylline
In vitro callus	1.6±0.1	5.3±0.2	65.1±0.9	21.1±0.4	1.1±0.1
In vitro embryos	2.1±0.2	8.4±0.4	83.8±1.2	28.1±0.5	1.6±0.1
Donor plant leaf	4.8±0.2	12.5±0.3	112.8±1.0	31.4±0.8	2.3±0.2

Values are mean ± standard and mentioned in mg/g DW

Figures

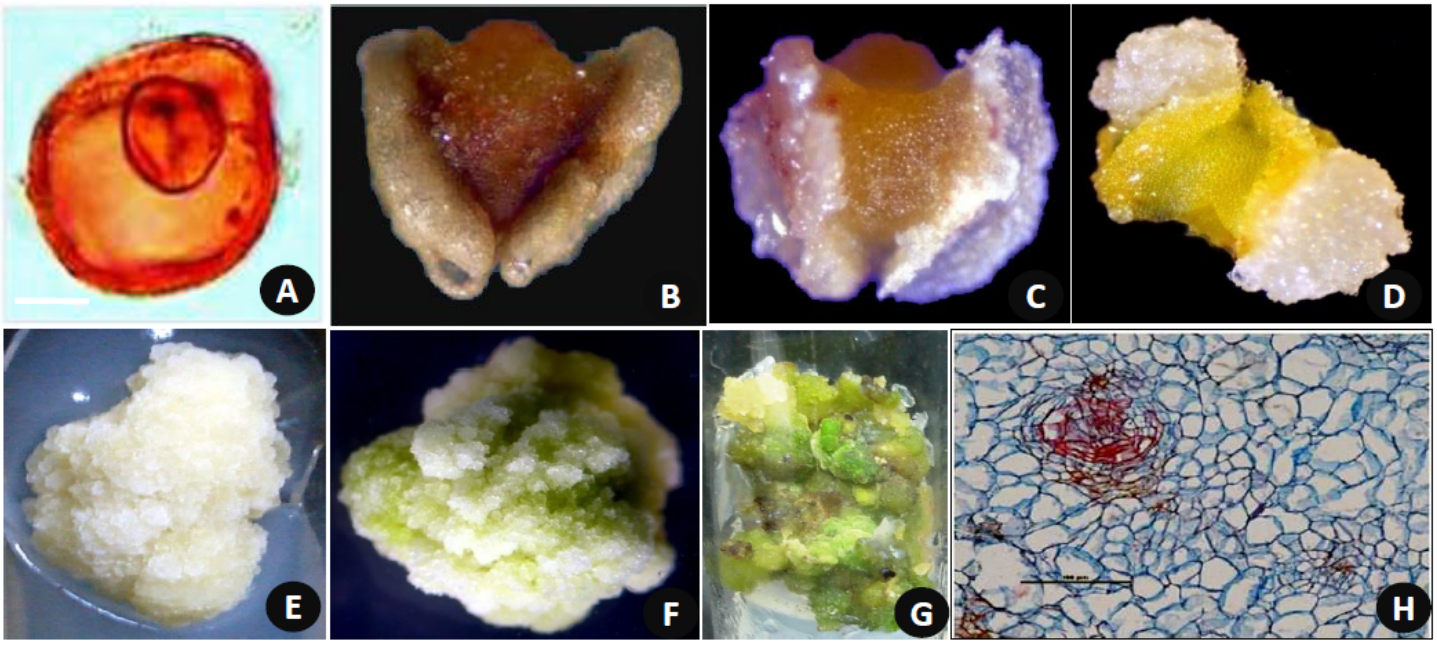


Figure 1

Establishment of anther cultures, callus initiation and multiplication in TV19 cultivar of tea A- A microspore at uni-nucleate stage (Bar = 5 µm) B- Anthers showing longitudinal rupturing of anther walls after 6 weeks of culture on MS (60 g/l sucrose) + 2,4-D (1 µM) + NAA (1 µM) + BAP (5 µM) (Bar = 400 µm) C- Same as B, shiny, white callus appearing from inside the anther locules after 8 weeks (Bar = 400 µm) D- Emergence of profusely growing white, shiny calluses from each anther locules after 10-weeks (Bar = 0.7 mm) E- 6-week-old callus subculture in dark on callus induction medium. The calluses grew well as white, hard callus after second subculture (Bar = 4 mm) F- Green hard calluses on induction medium when shifted to diffused light condition, growing without any sign of regeneration after 12 weeks (Bar = 3.5 mm) G- 8-week-old cultures on callus multiplication medium, MS (3% sucrose) + NAA (5 µM) + BAP (10 µM). The calluses turned hard, compact and nodular (Bar = 3.5 mm) H- Same as G, histological sections revealed the presence of meristemoids within the nodular callus (Bar = 100 µm)

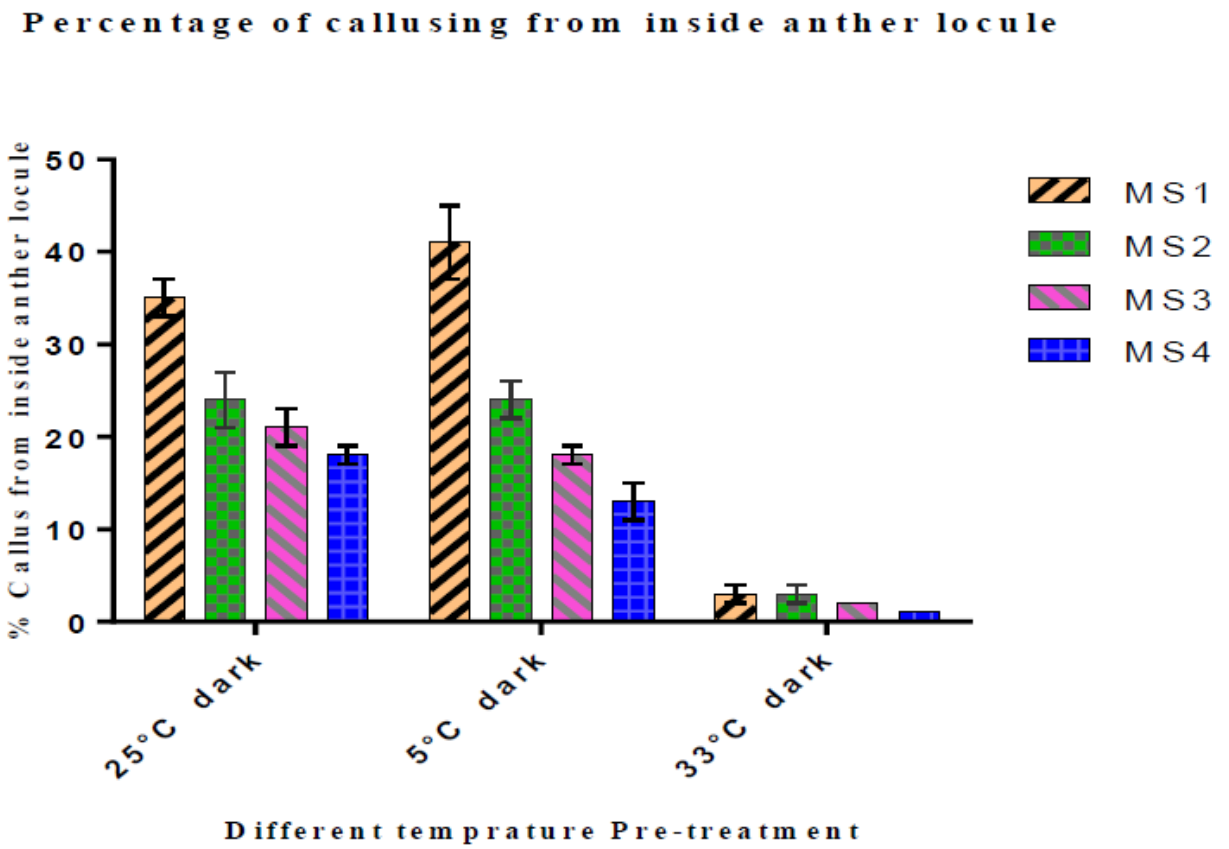


Figure 2

Percentage of anther cultures showing callusing from inside the anther locules. Cultures were incubated continuously in the dark. MS medium (with 60 g/l sucrose) + BAP (5 µM) + 2,4-D (1 µM) + NAA (1 µM) and temperature pre-treatments at 5°C for five days before shifting to 25°C, supported maximum microspore callusing in 41% cultures.

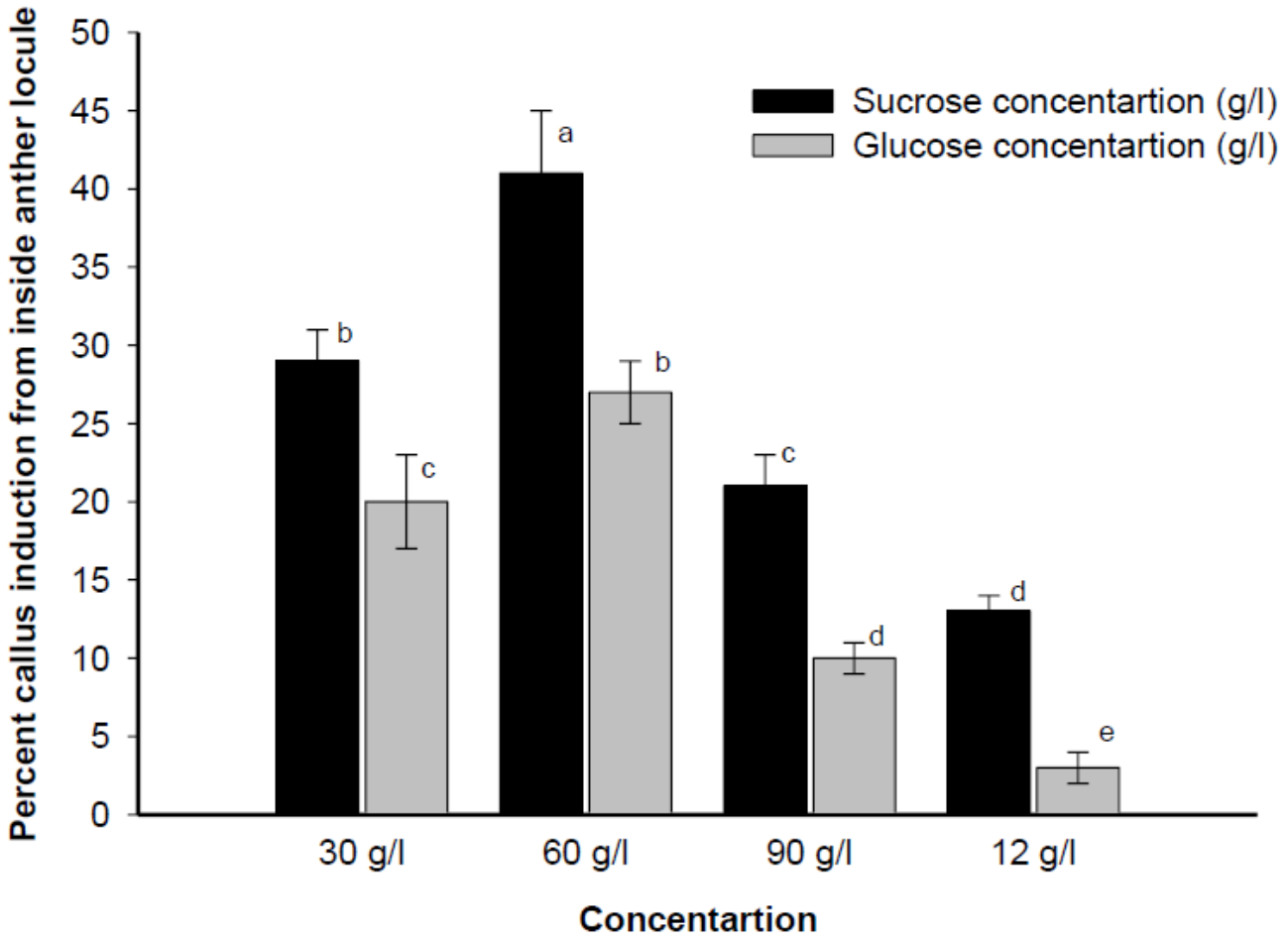


Figure 3

Same as Figure 2, subjected to sucrose and glucose treatments at various concentration range. Sucrose at 60 g/l was observed to be the best.

Figure 4

Embryogenesis on MS (3% sucrose) + BAP (10 µM) + GA3 (3 µM) +L-glutamine (800 mg/l) + L-serine (200 mg/l). A & B- 8-week-old culture showing clusters of asynchronously developed embryos. G- globular shape; H- heart shape; T- torpedo shape; C- cotyledenous stage. Note the presence of shiny, green and white embryos (Bar = 1.5 mm). C- The SEM image showing clusture of embryos in globular shape, heart shape and torpedo shape stages (Bar = 100 µm). D- The SEM image showing dicotyledonus stage of embryo (Bar = 20 µm). E- Showing fully mature embryo with long radicular end and small green plumular end on MS + BAP (1µM) + GA3 (0.3 µM) + L-glutamine (80 mg/l) + L-serine (20 mg/l) (Bar = 0.5 mm) (Bar = 0.15 mm). F- 10-week-old culture showing complete plantlet on ½MS (major salts reduced to half strength) + BAP (10 µM) + GA3¬¬ (0.5 µM) + IBA (1 µM) + L-glutamine (80 mg/l) + L-serine (20 mg/l) (Bar = 0.6 mm).

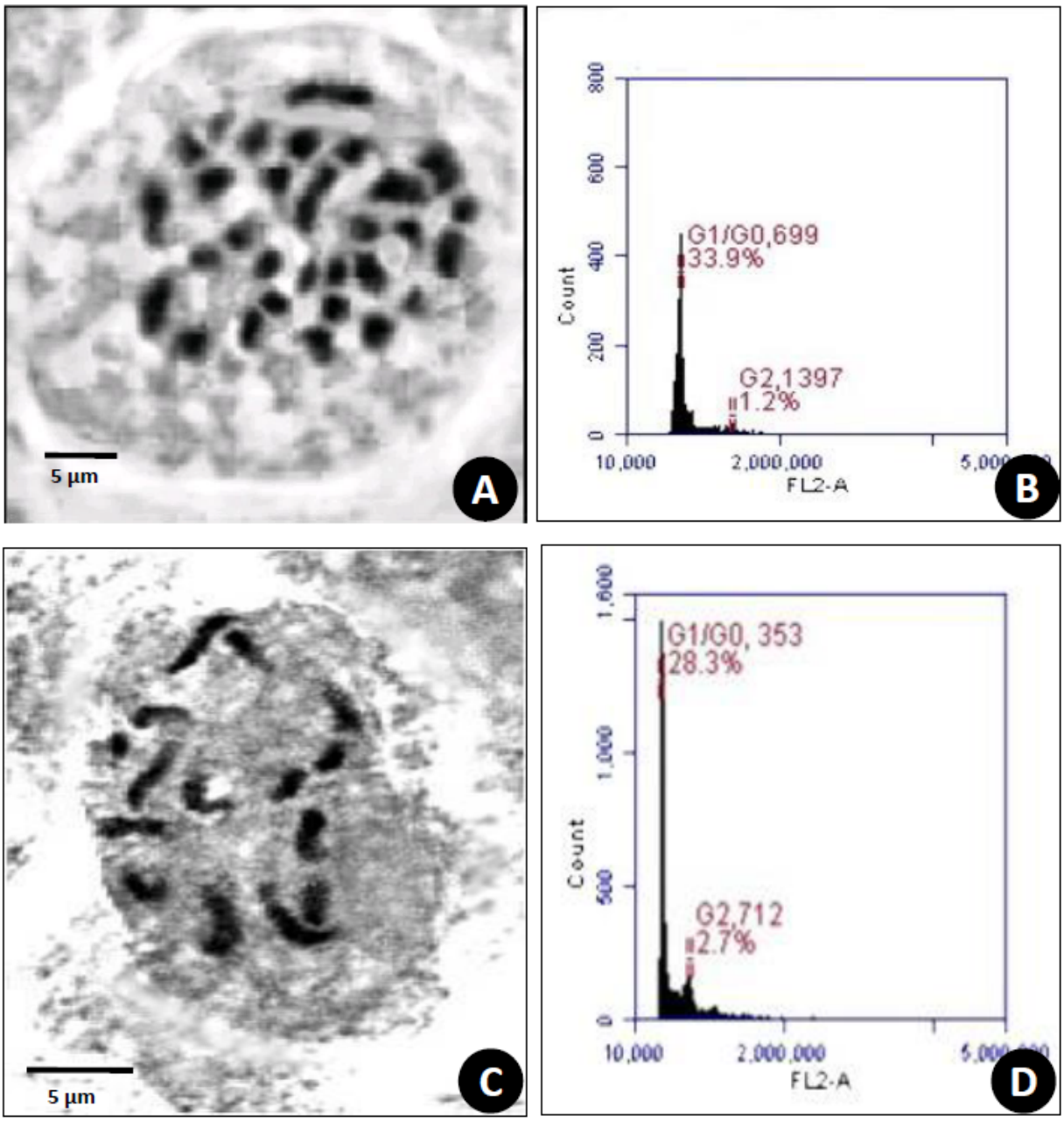


Figure 5

Ploidy analysis of androgenic cultures by cytological squash preparation and flow cytometry. A- Squash preparation of shoot-tip from field grown donor plant of TV19 cultivar showing diploid number of chromosome as 2n=2x=30 (Bar = 5 µm). B- Flow cytometry histogram of leaf of TV19 cultivar, grown in the field as a control. Note G1 peak at position 699 and G2 at 1397 C- Ploidy analysis of in vitro regenerated haploid TV19 cultivar by root-tip squash preparation showing haploid number of chromosome as 2n=x=15 (Bar = 5 µm). D- Flow cytometry histogram of leaves from in vitro regenerated plants of TV19 cultivar showing G1 peak at 353 channel position and G2 at 712 channel position.

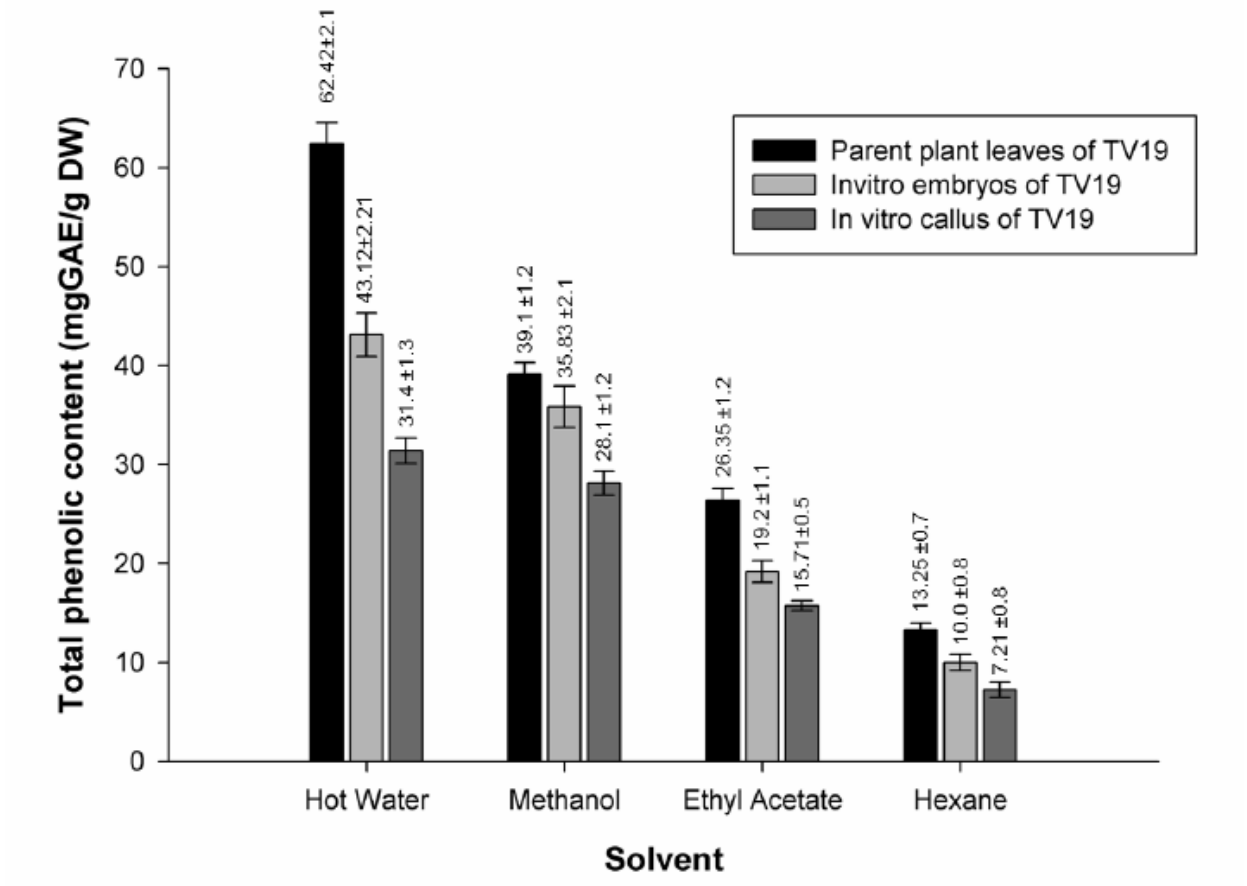
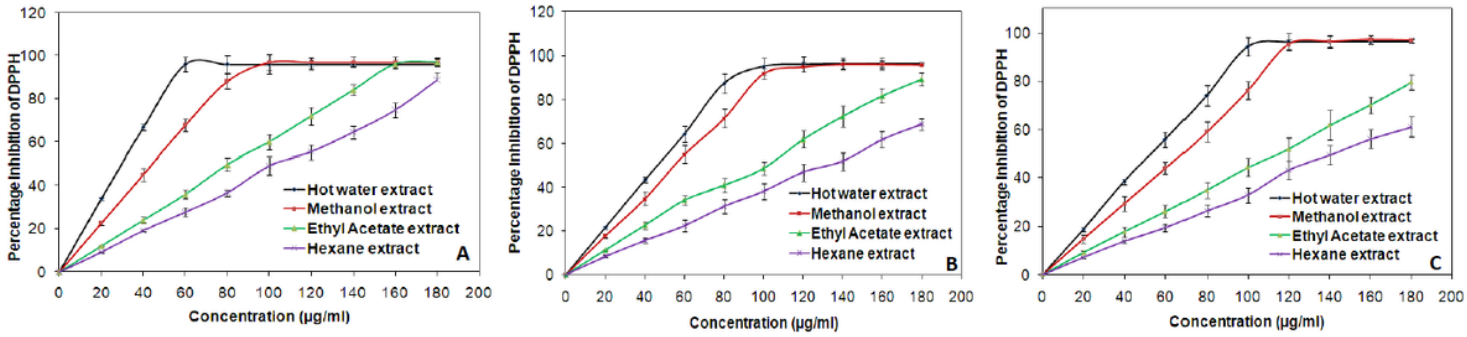


Figure 6

Total phenolic content from leaves of donor plant and in vitro androgenic cultures, embryos and calluses



Solvents	Donor plant leaves (control)	Haploid embryos	Haploid calli
Hot water	29.98±1.34	46.21±2.21	53.37±2.15
Methanol	44.21±1.82	56.18±2.42	68.25±3.12
Ethyl acetate	83.21±3.33	98.13±3.64	110.72±4.12
Hexane	108.31±5.41	120.01±3.41	140.41±5.41

Concentration are in µg/ml. Values are mean ± standard deviation

Figure 7

DPPH inhibition pattern in various extracts of tea A- Extract from young leaves of donor TV19 plant B- Extract from androgenic embryos C- Extracts from androgenic calluses.

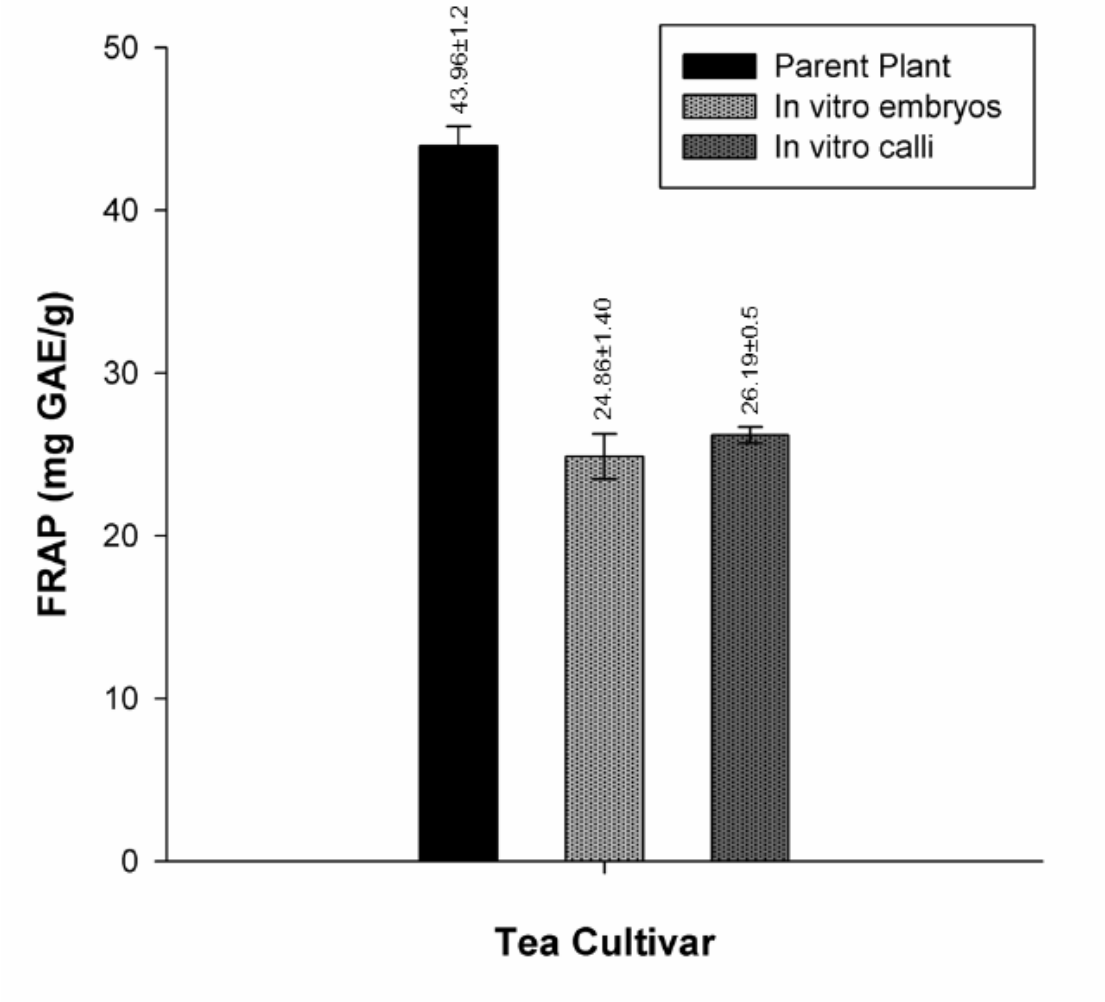
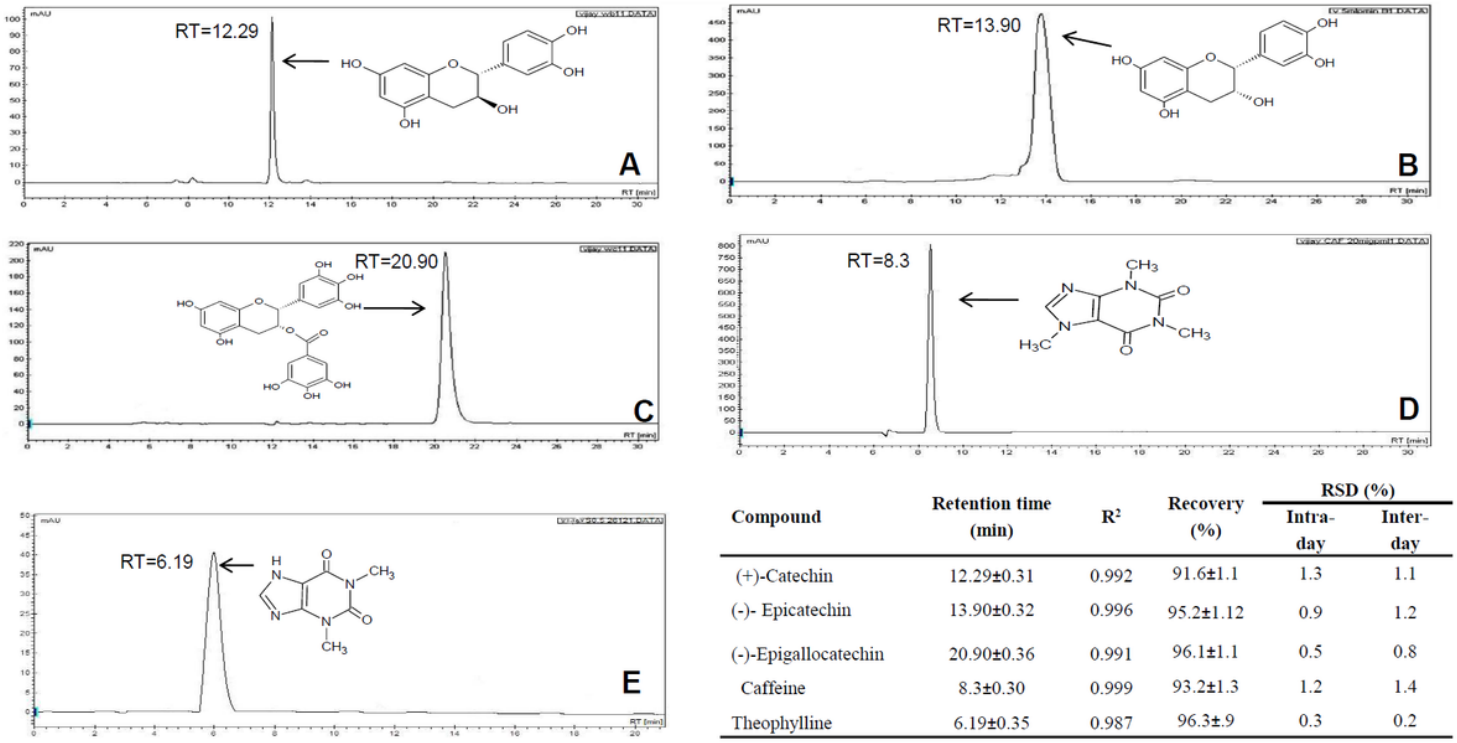


Figure 8

Ferric-reducing antioxidant power (FRAP) assay of hot-water extracts of leaves from field grown donor plants (control) androgenic cultures of TV19 cultivar



Compound	Retention time (min)	R^2	Recovery (%)	RSD (%)	
				Intra-day	Inter-day
(+)-Catechin	12.29±0.31	0.992	91.6±1.1	1.3	1.1
(-)- Epicatechin	13.90±0.32	0.996	95.2±1.12	0.9	1.2
(-)-Epigallocatechin	20.90±0.36	0.991	96.1±1.1	0.5	0.8
Caffeine	8.3±0.30	0.999	93.2±1.3	1.2	1.4
Theophylline	6.19±0.35	0.987	96.3±.9	0.3	0.2

Figure 9

High Performance Liquid Chromatography of standards A- A chromatogram of (+)-catechin (C), eluted at retention time of 12.29±0.31 min. B- A chromatogram of (-)-epicatechin (EC), eluted at retention time of 13.9 ± 0.32 min. C- A chromatogram of (-)-Epigallocatechin gallate (EGCG), eluted at retention time of 20.9 ± 0.36 min. D- A chromatogram of caffeine (CAF), eluted at retention time of 8.3±0.31 min. E- A chromatogram of theophylline (T), eluted at retention time of 6.19±0.35 min.

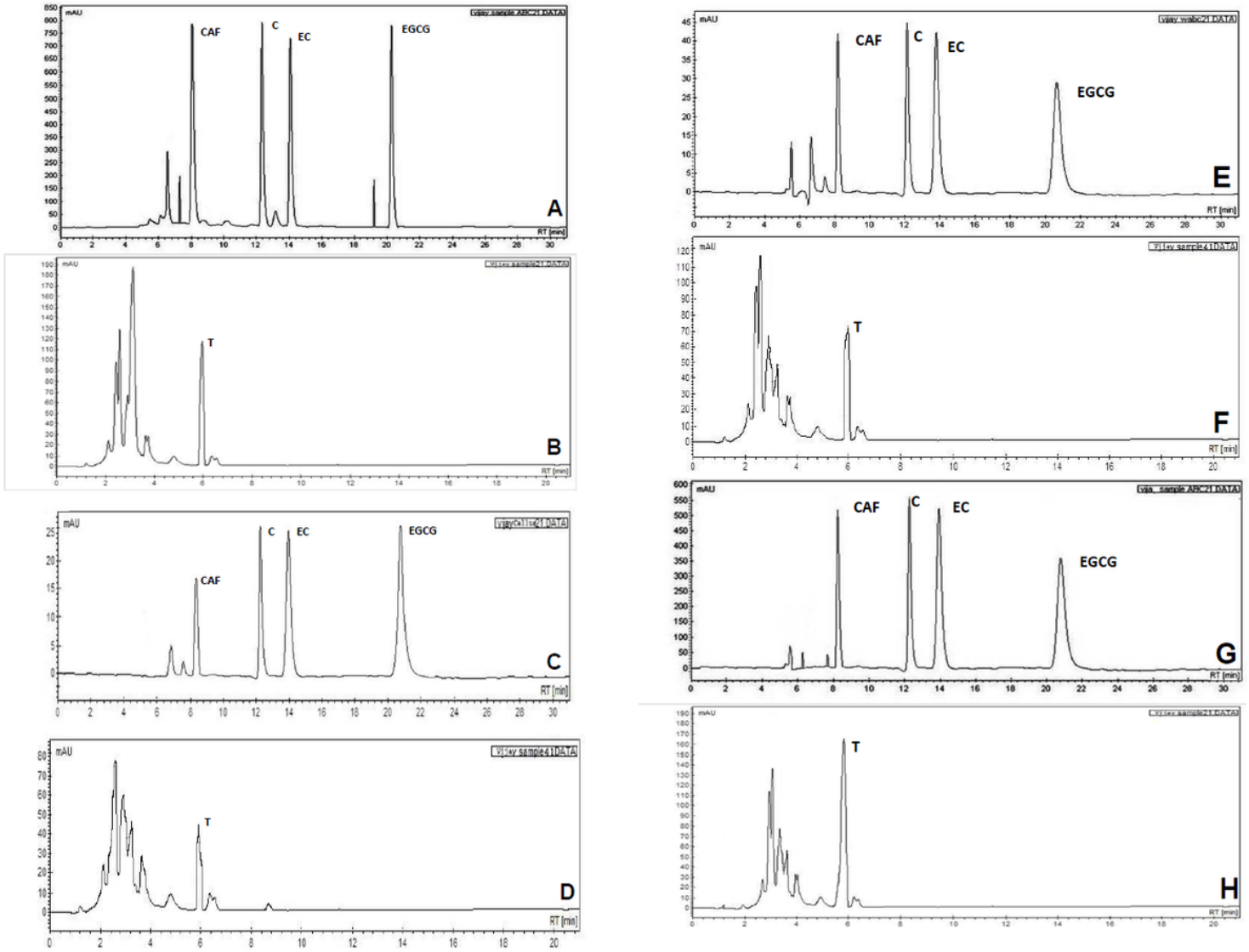


Figure 10

HPLC Chromatogram of hot water extracts from TV19 cultivar A- From leaves of field grown donor plant showing elution of the four compounds CAF, C, EC and EGCG. B- From leaves of field grown donor plant showing elution of T. C- From haploid calluses showing presence of CAF, C, EC and EGCG D- From haploid calluses showing presence of theophylline. E- From haploid embryos showing presence of CAF, C, EC and EGCG. F- From haploid embryos showing presence of theophylline. G- Spiked with the standards of CAF, C, EC and EGCG. The samples and standards were co-eluted at their respective retention times H- Spiked with the standard of theophylline. The samples and standards were co-eluted at the same retention time.

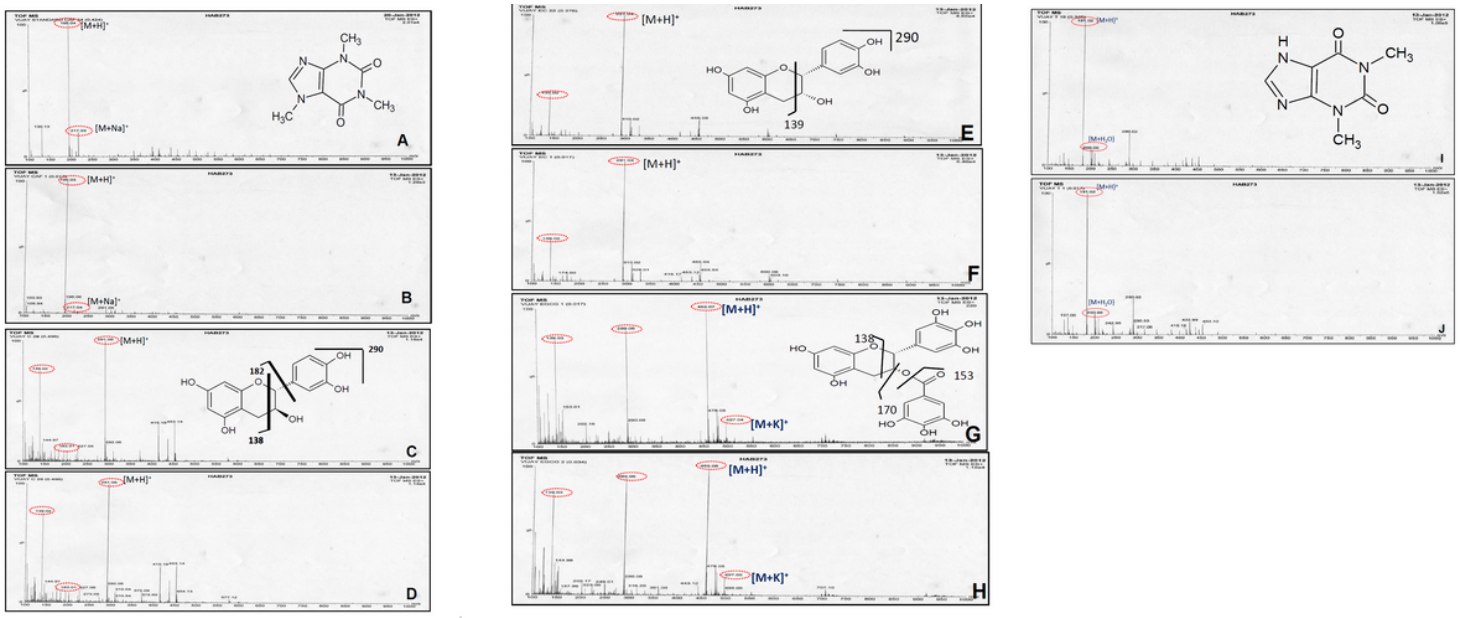


Figure 11

Comparative positive mode electrospray ionization (+ESI) mass spectra of standard compounds and the purified samples A- HPLC eluted caffeine standard. B- HPLC eluted crude hot-water extract. C- HPLC eluted (+)-catechin standard. D- HPLC eluted crude hot-water extract. E- HPLC eluted (+)-epicatechin standard. F- HPLC eluted crude hot-water extract. G- HPLC eluted (+)-epigallocatechin gallate standard. H- HPLC eluted crude hot-water extract. I- HPLC eluted Theophylline standard. J- HPLC eluted crude hot-water extract

Supplementary Files

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- SupplementaryDataTV19PCTOC.docx