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Stimulation of Callogenesis from Anthers of Chinery and Cambod Tea (*Camellia* sp.) Clones

CH Naga Pavan Kumar, E. Edwin Raj, R. Raj Kumar, P. Mohan Kumar and *K. N. Chandrashekara

Department of Plant Physiology and Biotechnology, UPASI TRF TRI,
Nirar Dam P.O., Coimbatore District, Valparai 642 127, India
*Corresponding author: Email: knchandu1@gmail.com,
Tel: +91-4253235303; Fax: +91-4253235302

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ABSTRACT:

Camellia sp., is a perennial tree belongs to the family Theaceae. Flower buds from clones UPASI-9 (Chinery, 2n=30) and TRI-2025 (Cambod, 2n=30) of size 0.1 to 1.5 cm range were collected early in the morning to ascertain growth stage of the anthers. Mid exponential phase with uninucleate cells were established from flower bud size around 0.5 to 0.6 cm. Initially, flower buds were subjected to temperature treatments at 4 ^{o}C, 25 ^{o}C and 32 ^{o}C. The anthers from these clones were used in the present investigation for callogenisis on three different basal media *viz.*, Murashige & Skoog (MS), Nitsch (N), and Nitsch & Nitsch (N&N) in full strength and half strength at pH 5.8 fortified with varying concentrations of plant growth regulators (Kn & BAP) tried individually and in combined with auxin (2,4-D). Fifteen anthers were inoculated per culture plate with 6 replicates, three plates were incubated under white fluorescent light 37 µ mol m^{-2} sec^{-1} for a photoperiod of 9 h light:15 h dark at temperature 24 ± 2 ^{o}C and three plates under 24 hours complete dark. Anthers inoculated on half strength MS media fortified with 2,4-D (9.05 µM) + Kn (4.65 µM) inducted callus under photoperiod of 9:15 h light and 24 h dark. Proliferative callus was found from anthers treated at 4^{o}C followed by dark incubation. TRI-2025 had a highly significant callusing (42.9 ± 4.9) than UPASI-9, when anthers cultured on MS medium under light condition. Production of the callus frequency was significantly affected by hormone concentrations, media types and photoperiod.

Keywords: Androgenesis, anther, callus, *Camellia* sp., haploids, uninucleate

INTRODUCTION

Camellia sp, is a perennial, evergreen tree of the family Theaceae. Tea is non-alcoholic beverage consumed by the people since 3000 B.C in the world due to its pleasant taste, attractive aroma and its medicinal property [9]. Even though Tea (*Camellia* sp.) is a monophyletic origin few higher ploidy levels, such as triploids (2*n* = 45), tetraploids (2*n* = 60), pentaploids (2*n* = 75), hexapolids (2*n* = 90), octaploids (2*n* = 120) and aneuploids (2*n* ± 1 to 29) had also been

identified [15, 17]. Tea fields are planted with seedlings, screening of desired trait will be difficult task due to its perennial nature and self-incompatibility. Poor seed set, rapid loss of seed viability and long durations for sexual cycles are the barriers for conventional breeding program, besides lack of reliable selection criteria [1].

To develop a pure homozygous breeding line it requires 15 to 20 years and labour intensive. It has been already reported vegetative propagation is standard, but limited due to slow multiplication rate, poor survivability of some clones, and need for profuse initial planting material [19]. Standard breeding of diploid plants often requires screening and backcross of a large number of plants to achieve the desired genotype [20]. One solution to resolve the problem of screening large numbers of progeny has been to produce haploid plants, the chromosomes of which can be doubled using colchicine or other means to achieve instantly homozygous doubled-haploid plants. With doubled haploid production systems, homozygosity is achieved in one generation, thus the breeder can eliminate the numerous cycles of inbreeding to achieve practical levels of homozygosity using conventional methods [3, 26].

Specialized plant tissue culture methods has enabled the production of completely homozygous breeding lines from gametic (anther) cells in a shorter time frame compared to conventional plant breeding [3, 4, 8]. The ability of plant gametic cells to produce plants is being progressively more oppressed by geneticists to obtain stable inbreed line in a single step [13, 27]. Anther culture in tea was first described by Katsuo in 1969 [7] and Okano & Fuchinone in 1970 [12], they established the technique which could develop to individual plantlets through callogenisis or via direct or indirect embryogenesis. Anther derived callus makes gateway in generating haploids in the field of genetic as well as plant breeding and allow better discrimination among the genotypes [18].

Haploid producing technique regulated by several biotic and abiotic factors. The male gamete development stage relies on anther or microspore isolation, in combination with suitable stress treatments, which determines androgenetic response.

It can be actuated within a competitively wide developmental window around the first pollen mitosis, when uninucleate microspores divide inequitably ensuing in a generative cell embedded in a vegetative cytoplasm [10, 23]. The commonly used pre-treatments are temperature, sucrose and nitrogen starvation and osmotic stress. Depending on the plant species and genotype, temperature stress can be applied by subjecting excised flower buds. By implementing stress conditions the immature anther will modify its fate of cell division to form callus and then complete organelles via organogenesis, or develop to embriod like structures which ultimately leads shoots and roots for producing a complete plantlets based on the growth supporting medium environment, growth regulators and inhibitors stimuli [22]. Based on the above background the study was formulated to obtain effective callus induction system using different medium type, lighting conditions and hormones combination using anthers of two cultivars.

MATERIALS AND METHODS

Selection of parental clone

Selection of elite parental clones or superior genotypes having high impact were considered in the present study such as drought tolerant, high yield potential, suitability for natural breeding or controlled breeding etc. UPASI-9 (Chinary, 2n=30) and TRI-2025 (Cambod, 2n=30) were selected for the studies. UPASI-9 characterized by superior quality [15], drought tolerant [20] and excellent rooter with uniform growth [25]. TRI2025 known for high anthocyanin pigmentation [20], vigorous growth, drought hardy nature and medium quality with good yield [11]. UPASI-9 and TRI-2025 are used as female and male parents respectively and being used for conventional breeding as a biclonal seed stock
[15].

Selection of explants material

Unopened flower buds (0.1 to 1.5 cm) of the tea clone UPASI-9 and TRI-2025 were collected early morning. The immature anther's which were morphologically defined were selected by anther squash technique. We have developed protocol by combing the techniques of Robert *et al*., 2013[24] for protoplast isolation and Chazotte, 2011[2] for staining pollen cells of different stages from anther of tea.

The methodology of Robert [14]was implemented with slight modification, protoplast isolation with short stretch of incubation time with cell wall degrading enzymatic solutions and followed by staining procedure. Anthers were dissected from respective flower buds of various sizes (0.1 to 1.5 cm). Ten anthers which are morphologically identical and defined by specified flower bud were separated and gently squash with solution containing 1:1 of macerozyme 10% w/v (Himedia) and cellulase 10% w/v (Himedia) incubate at 2˚C for one hour.

Squashed samples were centrifuged at 1000 g for 2 min and the pellet was washed with 0.4 M sucrose to maintain osmoticum of cells. The suspension was filtered through a sterile 45 µm nylon mesh. Staining technique as per Chazotte [2] was adapted, the cells were rinsed three times with the 1x PBS and were not allowed to dehydrate at any time during the staining. Cells were fixed for 10 min in 3.7% formaldehyde, fixative was aspirated. The cells were rinsed three times, 5 min each in 1x PBS. Permeabilize the cells by immersion in 0.2% Triton X-100 for 5 m. Triton was aspirated and cells were incubate in 0.03 M FITC (Flourescein isothiocyanate) for 5 min. The slides were visualized and photographed under inverted microscope (Nikon Eclipse TE2000-5) after staining with 0.03 M FITC (Sigma-Aldrich).

Temperature treatment

The flower buds of 0.5 cm were collected and subjected to temperature treatments at 4^{o}C, 25^{o}C and 32^{o}C for 4 days. Proper care was taken to maintain moisture of flower buds. Floral buds were placed on cotton beds packed in petridish with wet cotton using sterile distilled water.

Surface sterilization

The pre-treated floral buds were thoroughly washed under running tap water and allowed to air dry under laminar flow, surface sterilized in 0.1% mercuric chloride solution for 60 seconds, and washed with absolute alcohol. Finally the flower buds were washed thrice with sterile distilled water to remove traces of alcohol before inoculation.

Callus induction

Anthers from pre-treated floral buds of 0.5 cm were inoculated horizontally on Murashige & Skoog (MS), Nitsch (N), and Nitsch & Nitsch (N&N) in full strength and half strength media at pH 5.8 fortified with varying concentrations of plant growth regulators Kn (2.44, 4.65, 6.98 µM) & BAP at individual concentrations (2.22, 4.43, 6.65 µM) and combined with auxins 2,4-D at 9.05 µM. Fifteen anthers were inoculated per culture plate in 6 replicates, three plates were incubated under white fluorescent light (37 µ mol m^{-2} sec^{-1}) for a photoperiod of 9/15 h at temperature 24 ± 2 °C and three plates under 24 h complete dark.

RESULTS AND DISCUSSIONS

The requirement for high quality tea clones with multiple traits like drought tolerant, high yield, resistance to pest and diseases has been focus of plant breeding programs around the world. Hence the present study was initiated to standardize the procedure to generate callus from UPASI-9 and TRI-2025 which are extensively used in breeding program, to produce biclonal seed stock (BSS) in the seed bary which are widely accepted by planters in Southern India [11, 15, 20 25]. A set of experiment was carried out to optimize the callusing response of anthers from UPASI-9 and TRI-2025 as a function of varying concentrations of plant growth regulators Kn (2.44, 4.65, 6.98 µM) and BAP (2.22, 4.43, 6.65 µM) combined with 2, 4-D at 9.05 µM.

Androgenesis, haploid producing technique regulated by several biotic and abiotic factors. The male gamete development stage relies on anther or microspore isolation, in combination with suitable stress treatments, which determines

androgenic response. According to anthers tend to follow the first pollen mitosis cells when uninucleate microspore cells divide unequally and results in generative cell surrounded in a vegetative cytoplasm [10, 16, 23]. The anthers from floral buds size ranging from 0.5 to 1.5 cm were selected to monitor mitotic cells from anther lobes by Anther squash technique (Figure 1).

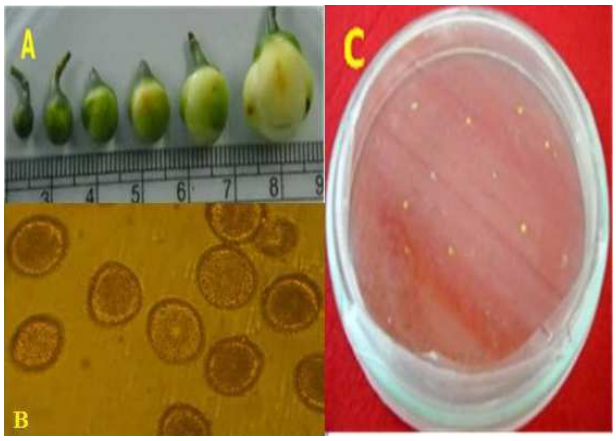

Fig: 1(A) Flower buds 0.5cm to 1.5cm screening for identifying uninucleate microspore cell stage; **(B)** Anther squash for screening uninucleate cells; **(C)** Callus initiation confirmation by puffy yellow colored anthers 10 days after inoculation.

This technique used in present study revealed that anthers from floral bud sizes (0.5 - 0.6 cm) from UPASI-9 and TRI-2025 had uninucleate cells. Hence, the floral bud size of 0.5 cm was used for temperature treatment studies. Similarly studies on white rape (2.5 - 3.0 mm) and strawberry (1.0 - 1.5 mm) revealed anthers/microspores with uninucleate stage and used for temperature treatments were appropriate for androgenesis [6] and which leads to callus formation [18, 24].

Anthers inoculated on full strength media turned brownish black color within 10 days and did not respond further. Whereas anthers showed response by enlarge in their size and started bursting within 2-3 weeks on half strength media. Our results indicated that incubation at 4 °C for 4 days had better response towards callus development on half strength MS (9.05 µM 2,4D + 2.44 µM Kn), (9.05 µM 2,4-D + 6.65 µMBAP), as same media in full strength was used previously [21]. The callus responded differently towards photoperiod incubated, anthers under 9:15h photoperiod stimulated to produce green friable calli (Figure 2A) and whereas dark incubated anthers prompted white friable calli (Figure 2B).

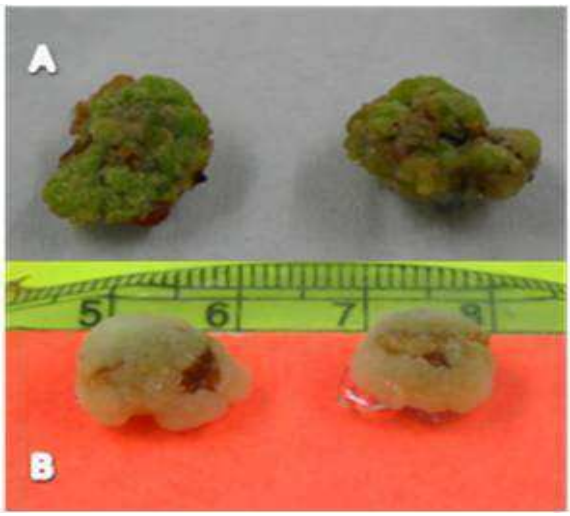

Fig: 2. Profusely growing (A) Green compact calli proliferation when incubated for 9:15 h photoperiod (B) White friable fragile calli proliferation when incubated for 24 h dark.

Response of clone to type of medium The present study involves three medium types for callus induction from anthers of UPASI-9 and TRI-2025. MS medium had a highly significant response in callogenesis (30.2 ± 2.2, Table 1) and having highly significant interaction with hormone (F=93.9, P=0.00), lighting (F=154.9, P=0.00), clone (F=311.4, P=0.00) and all other interactions with medium type. TRI-2025 had a highly significant callusing (42.9 ± 4.9) when cultured on MS medium under light condition, where as it was not significant under dark conditions, where N and N&N medium registered identical response in callusing frequency. UPASI-9 registered higher percentage of callusing on MS medium under light (26.4 ± 3.9) followed by N&N and N medium, but it is not significant. However, N & N medium induced higher percentage of callus and was significant than other two media used.

Response of clone to culture conditions Statistical analysis of data showed that when anthers of UPASI-9 and TRI-2025 cultured on both dark and light conditions did not affect the callogenesis significantly and had identical

response (Table 1). Even though the clones exhibited minor variations under different light regime, UPASI-9 responded poorly than that of TRI-2025, 9 h light accelerated callus induction to certain extent in TRI-2025 and UPASI-9 clones. In contrast, linear response in callus induction was observed with increasing concentration of 2,4-D with Kn and BAP in UPASI-9 anthers cultured under dark condition, but TRI-2025 having linear response only with BAP. Interestingly UPASI-9 and TRI-2025 clones registered linear response Kn and BAP concentrations under light and dark conditions respectively, but the response was not significant. Culture conditions had a positive and highly significant interaction with medium types, hormones (F=74.4, P=0.00) and clone (F=5.8, P=0.02).

Response of clone to Auxin vs Cytokinin ratio

Among the various hormones supplemented medium devoid of plant growth regulators showed no response in terms of callus production. Hormone types had a significant effect on callus induction (Table 2). 9.05 µM 2,4D combination with 2.44 µM Kn or 2.22 µM BAP exhibited significantly higher response than counterpart hormone free medium, and on par with all other combinations of hormones. On the other hand 2,4-D and BAP all the tested concentrations showed comparable and identical response with Kn, but not statistically significant (Table 1). Optimal hormone concentration for effective callus induction was differ in response to medium types, photoperiod condition and clones involved in the study.

CONCLUSION

The protocol has been standardized for stimulation of callogenesis from anthers of Chinery and Cambod tea clones. Anthers inoculated on half strength MS media fortified with 2,4-D (9.05 µM) + Kn (4.65 µM) produced high callus induction under photoperiod of 9:15 hours light and 24 h dark. Proliferative callus stimulation was found from anthers treated at 4^{o}C followed by dark incubation. The above given medium composition and conditions, hormone concentrations, medium types and lighting conditions produced high callus frequency.

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Table 1: Effects of different growth hormones and media on callus formation on TRI 2025 and UPASI 9 tea genotypes.

Treatments	TRI 2025		UPASI 9		Mean
	Light	Dark	Light	Dark	
MS	42.9±4.9b	33.7±4.3b	26.4±3.9a	18.1±2.4a	30.2±2.2c
Nitch	5.1±2.8a	6.4±3.5a	14.9±4.1a	11.1±3.0a	9.4±1.7a
Nitch & Nitch	14.3±4.0a	23.5±3.7b	16.8±3.5a	24.4±4.0b	19.8±1.9b

Hormone free medium	0.0a	0.0a	0.0a	0.0a	0.0a
9.05 µM2,4-D + 2.44 µM Kn	20.7±10.4a,b	10.4±5.2a,b	9.6±4.9a	11.9±6.0a,b	13.1±3.4b
9.05 µM2,4-D + 4.65 µM Kn	34.1±9.7b	31.1±7.9c	28.9±7.3b	20.0±6.6b,c	28.6±3.9c
9.05 µM2,4-D + 6.98 µM Kn	21.5±5.4a,b	22.2±5.7b,c	32.6±1.7b	25.2±2.7b,c	25.4±2.1c
9.05 µM2,4-D + 2.22µM BAP	17.0±8.5a,b	19.3±4.9b,c	31.9±3.1b	18.5±6.3b,c	21.7±3.0c
9.05 µM2,4-D + 4.43 µM BAP	34.1±8.7b	31.9±3.5c	25.9±6.6b	19.3±2.6b,c	27.8±3.0c
9.05 µM2,4-D + 6.65 µM BAP	17.8±5.0a,b	33.3±9.7c	6.7±3.3a	30.4±1.6c	22.0±3.3c
Culture condition	20.74±3.1a	21.2±2.6a	19.4±2.3a	17.9±1.9a	19.8±1.2
Clone	20.95±1.9b		18.62±1.5a		19.8±1.2

Values are percentage of responding explants (mean ± SE) followed by different letters are significantly different from each other at P <**0.05** level comparison by DMRT. Medium type and hormone concentration considered separately for the analysis.

Table 2: Interaction among the genotype, type of medium, growth condition and hormone.

Source	**df**	**SS**	**MS**	**F**	**Sig.**
Corrected Model	83	96795.7	1166.2	116.3	0.00
Intercept	1	98667.4	98667.4	9842.9	0.00
Medium (M)	2	18289.7	9144.9	912.3	0.00
Hormone (H)	6	22154.4	3692.4	368.3	0.00
Light (L)	1	17.6	17.6	1.8	0.19
Clone (C)	1	341.6	341.6	34.1	0.00
M × H	12	11299.4	941.6	93.9	0.00
M × L	2	3106.3	1553.2	154.9	0.00
M × C	2	6242.9	3121.5	311.4	0.00
H × L	6	4472.4	745.4	74.4	0.00
H × C	6	2771.5	461.9	46.1	0.00
L × C	1	57.9	57.9	5.8	0.02
M × H × L	12	20590.5	1715.9	171.2	0.00
M × H × C	12	4448.7	370.7	37.0	0.00
M × L × C	2	95.8	47.9	4.8	0.01
H × L × C	6	1268.9	211.5	21.1	0.00
M × H × L × C	12	1638.1	136.5	13.6	0.00