Research Article

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# Influence of genotypes, stages of microspore, pre-treatments and media factors on induction of callus from anthers of cocoa *(Theobroma cacao L.)*

**R. Sivachandran*, R. Gnanam, D. Sudhakar, J. Suresh and S. Ganesh Ram**

*Tamil Nadu Agricultural University, Coimbatore-641 003, Tamil Nadu, India*

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## Abstract

*In vitro* anther culture in cocoa will be useful for the production of homozygous line to accelerate breeding programs. The efficiency of callus induction and plant regeneration are highly dependent on genotypes and other physiological factors. The influence of genotypes, pre-treatments and medium combinations were examined with anthers isolated from two different flower bud sizes (2 to 3 and 4 to 6 mm long representing uni-nucleate and bi-nucleate microspore development stages, respectively) during androgenesis. Anthers isolated from 2 to 3 mm long flower buds, both fresh and cold pre-treated, were found to have good callus induction than gamma irradiated. Anthers of CCRP 1 genotype recorded highest percentage of callus induction in CIM 4 medium compared to other genotypes and medium combinations with both flower bud sizes. Anthers from 4 to 6 mm long flower buds showed better callus induction response in fresh and gamma irradiated than cold pre-treated flower buds. However, 2 to 3 mm long flower buds were superior to 4 to 6 mm long flower buds for callus induction. Maximum callus induction frequencies for CCRP 1 and CCRP 5 were obtained from 2 to 3 mm long flower bud in DKW medium supplemented with glutamine (250 $mgL^{-1}$), myo-inositol (200 mg $L^{-1}$), 2,4-D (2 mg $L^{-1}$), TDZ (10 µg $L^{-1}$), and glucose (20 g $L^{-1}$) in pre-treated flower buds (either with cold or gamma irradiated). However, the combined interaction effect between genotypes, pre-treatments and medium combinations had no significant influence on callus induction when 2 to 3 mm long flower buds were used. The development of different callus morphotypes *viz.*, friable/embryogenic, watery, compact, hairy and rhizogenic has been observed in this study. In addition, greening of calli was also noticed.These findings would be valuable for regeneration and development of haploid cocoa plants in future.

*Keywords:* Androgenesis, cocoa, media, microspore stage, pre-treatment

## Introduction

In India, cocoa (*Theobroma cacao* L.) is being cultivated in various states including Tamil Nadu, covering an area of 78000 ha and 16,050 metric tonnes of production annually (DCCD, 2017). There is tremendous scope for area expansion of cocoa because of heavy demand in Indian chocolate industry and confectionaries, which is portrayed as 60,000 metric tonnes for the year 2025 (Jaganathan *et al.,* 2015). Since, cocoa beans and butter are widely used in chocolate, drug and cosmetic derivatives; it fetches high commercial value (Silva *et al.,* 2009). Considering the health benefits due to the presence of polyphenolic compounds in cocoa, a prophylactic agent for brain and cardiovascular disorders, there has been significant demand on growing cocoa in large scale (Noah *et al.,* 2013).

In cross pollinated crop like cocoa, availability of homozygous inbred lines suitable for breeding purpose is a limiting factor (Falque *et al.,* 1992). Development haploids through anther culture followed by converting into di-haploids will be a promising approach in the development of inbreds suitable for hybridization programme (Srivastava and Chaturvedi, 2008). Even though spontaneous occurrence of *in vivo* haploid cocoa plants has been reported (Dublin, 1972), the natural occurrence of haploid in the field is very low with a maximum of

*Corresponding Author: siva.biotek@gmail.com

0.1 per cent. Hence, *in vitro* production of haploid plants through anther culture will be an effective and preferable method for the development of haploid cocoa plant (Ramawat *et al.*, 2014). Besides *in vitro* production, haploid plants has an additional advantage of shortening breeding cycle through development of novel dihaploid inbred lines to be used in hybridization programmes (Germana, 2006).

Immature anthers or enriched microspores are used as explants for *in vitro* production of haploids through anther culture (Ramawat *et al.*, 2014). Since this technique is more specific to species, genotype, environment and climatic conditions, an optimized genotype specific protocol is required (Custodio *et al.*, 2005; Germana, 2011). There are only a few reports on production of haploids in woody plants through *in vitro* androgenesis, also owing to their recalcitrant nature (Srivastava and Chaturvedi, 2011). In a recalcitrant plant like cocoa, the factors like size of flower bud, stage of microspores, culture media, growth regulators, growth additives, carbon source, stress pre-treatments and anther wall thickness, severely affects anther culture efficiency (Germana, 2011; Mishra and Gowswami, 2014). A few attempts have been made for the development of callus from cocoa anthers, but successful regeneration of cocoa haploid plants through anther culture has not been reported (Santana *et al.*, 2010).

This study mainly focused on the influence of genotypic variations, appropriate flower bud size (microspore stage), pre-treatments and culture medium combinations in cocoa anther culture and a protocol for callus induction and maintenance has been standardized.

## Materials and methods

### Plant materials

Five to six year old healthy cocoa genotypes (CCRP 1, CCRP 2, CCRP 3 and CCRP 5) intercropped in coconut plantation maintained at the Department of Spices and Plantation Crops (DSPC), Tamil Nadu Agricultural University (TNAU), Coimbatore were selected as donor plants for flower bud collection. *In vitro* anther culture and related investigations were carried out in the Department of Plant Biotechnology (DPB) at TNAU, Coimbatore.

### Evaluation of flower buds and microspore development

Flowers buds with various lengths ranging from 1.5 to 2 mm, 2 to 3 mm and 4 to 6 mm were chosen to investigate the stages of microspores. Microspore stages were assessed by squashing the anthers in one per cent acetocarmine stain, and examined with a bright field microscope to characterise the microspore development stages.

### Collection and preparation of explants for *in vitro* culture

For anther culture studies, flower buds of two different sizes (2-3 and 4-6 mm in length) were collected, followed by cytological confirmation. The buds were used either fresh or subjected to pre-treatments (cold shock at 4 °C for four days in dark or gamma irradiations at 4 Gy for 24 s). The buds (either fresh or pre-treated) were surface sterilized 30 seconds in 70 per cent alcohol followed by 10 minutes in one per cent sodium hypochlorite and washed three times in sterile water. A cut was made in bottom ¼$^{th}$ position of the flower bud and, petals and sepals were removed carefully. Anthers were separated using fine needle and cultured in sterile disposable Petridishes containing callus induction medium (CIM).

### Culture media

Initially, experiments were carried out with 26 medium combinations derived based on MS (Murashige and Skoog, 1962; Gamborg, 1968), WPM (Lloyd and McCown, 1980) and DKW (Driver and Kuniyuki, 1984) media composition by adding or modifying different growth regulator combinations and additives to establish the callus from anther. Out of 26 media combinations, four combinations showed more than 40 per cent callus induction and frequency (Table 1). Proliferative callus morphotypes were shortlisted for further experiments.

### Culture conditions

The anthers were inoculated in a plate containing callus induction culture medium and incubated without light for 30 days at 25±2 °C for callus initiation (Fig. 2a). For maintenance of the callus, they were sub-cultured in a same media composition plate once in 30 days. During

**Table 1. Media combinations that showed better callus induction and frequency**

| CIM | Proportion of different constituents in each media |
|---|---|
| CIM 1 | WPM + kinetin (0.5 $mgL^{-1}$) + NAA (1 $mgL^{-1}$) + casein hydrolysate (500 $mgL^{-1}$) + proline (50 $mgL^{-1}$) + sucrose (30 $gL^{-1}$) |
| CIM 2 | MS + B5 vitamins + kinetin (0.5 $mgL^{-1}$) + NAA (1 $mgL^{-1}$) + sucrose (30 $gL^{-1}$) |
| CIM 3 | MS + B5 vitamins + kinetin (0.5 $mgL^{-1}$) + NAA (1 $mgL^{-1}$) + casein hydrolysate (500 $mgL^{-1}$) + proline (50 $mgL^{-1}$) + sucrose (30 $gL^{-1}$) |
| CIM 4 | DKW + glutamine (250 $mgL^{-1}$) + myo-inositol (200 $mgL^{-1}$) + 2, 4-D (2 $mgL^{-1}$) + TDZ (10 $\mu gL^{-1}$) + glucose (20 $gL^{-1}$) |

sub culturing, the residual pieces of inoculum and the brown part from the calli were removed before transferring to the fresh medium. In case of CIM 4 medium, the anthers were cultured according to Li *et al.* (1998). Anthers were first inoculated in CIM 4 medium. Plates were maintained without light at 25 °C for 14 days. Explants of 14 days old on CIM 4 medium were transferred onto new plates which contained WPM salts supplemented with B5 vitamin solution, glucose (20 g $L^{-1}$), 2,4-D (2 mg $L^{-1}$), kinetin (0.3 mg $L^{-1}$), coconut water (50 mL $L^{-1}$) and phytagel (2.2 g $L^{-1}$) and maintained for next 14 days in dark. After 14 days anther derived calli were transferred to embryo development (ED) culture medium composed of DKW salts, sucrose (30 g $L^{-1}$), glucose (1 g $L^{-1}$) and phytagel (2 g $L^{-1}$). The dark condition at 25 °C was used to maintain the culture and sub-cultured at 14 days interval until enough somatic embryos were obtained. The cultured plates were observed at weekly intervals under a stereo-microscope (Leica-S8APO) to monitor the callus development. Proliferative callus (90 days old calli) was incubated at 25±2 °C with a light intensity of 2000-4000 lux under 16h/8h light/dark period for regeneration. Fourteen medium combinations (MS and WPM formulations with different growth regulator combinations and additives) were used for the regeneration study.

### Experimental layout and data analysis

Factorial completely randomized design (FCRD) was utilized to perform the experiment. Two flower bud size ranges (2 to 3 and 4 to 6 mm in length), four genotypes (CCRP 1, CCRP 2, CCRP 3 and CCRP 5), three pre-treatment conditions (fresh flower bud, cold pre-treated at 4 °C for four days and gamma irradiated at 4 Gy for 24 s) and four medium combinations (CIM 1, CIM 2, CIM 3 and CIM 4) were included in the FCRD. Each treatment had three replications with 30 anthers per plate. Thirty days after inoculation, the callus induction frequency was derived from the ratio of callus producing anthers against total anthers cultured. SAS software v 9.3 was used to calculate means to perform statistical analysis and utilized to analyse the significance among the observed values through analysis of variance (SAS 2011).

## Results

### Flower bud length and determination of stage of microspore

To simplify the explant selection process correlation between selected genotype flower bud size and their respective microspore developmental stage was established (Fig. 1A). Cytological study revealed that, flower buds of 1.8 mm long showed tetrad stage of microspore (Fig. 1B). The flower buds length of 2-3 mm displayed uninucleate microspore stage (Fig. 1C) and 4-6 mm had microspores in the binucleate stage (Fig. 1D). FDA staining test showed the viability and non-viability nature of microspores (Fig. 1E).

### Influence of medium combinations on the induction of callus

The response of the anthers to four different medium combinations was variable according to flower bud size, genotypes and pre-treatment conditions. No callus induction was observed for all the three basal media (MS modified, WPM and DKW). The callus induction in the 2 to 3 mm long flower buds ranged from 6.7 to 94.4 per cent and for 4 to 6 mm long flower buds ranged from 0 to 90 per cent (Table 2). Inoculated anther produced calli within 20-25 days. Different types of callus morphotypes were observed in callus induction, embryo development and regeneration medium. Anther bursting and callus emerging from microspore was observed in CIM 4 medium (Fig. 2b).

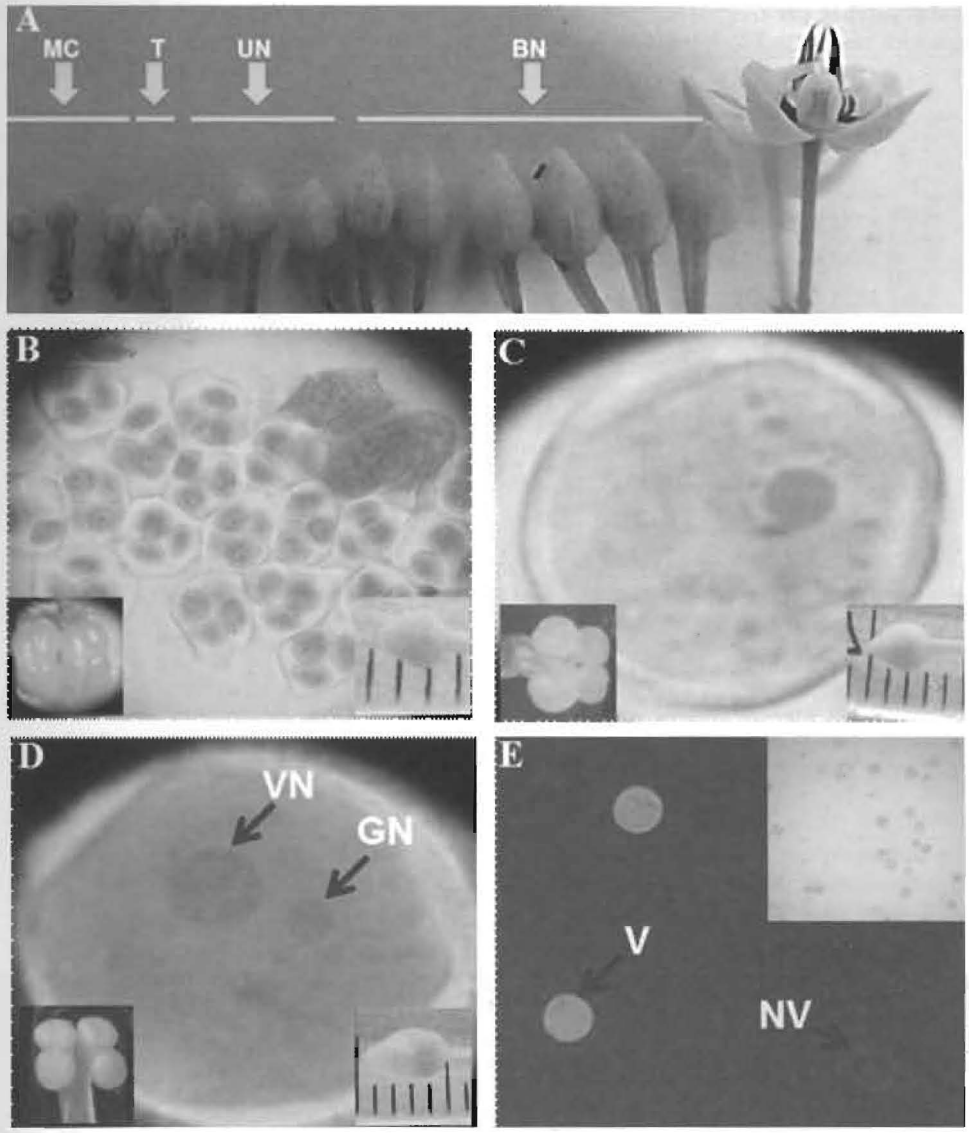


**Fig. 1. Microspore developmental stages of *Theobroma cacao* L. from different size flower bud. A) Based on anther morphology and microspore stage of development the flower buds collected were divided into four different groups (PC: pollen mother cells;T: tetrad; UN: uninucleate; BN: binucleate); B) Tetrad stage from a 1.8 mm long flower bud; C) Uninucleate microspore from a 2 to 3 mm long flower bud; D) Binucleate microspore from 4.0 to 6.0 mm long flower bud (*arrow* VN: vegetative nucleous; GN: generative nucleous); E) FDA stained microspore (*arrow* v: viable; NV: non-viable)**

Creamy yellow, compact and friable callus was observed in CIM 4 medium with all genotypes (Fig. 2c). White compact hairy callus with spot of friable areas was found in CIM 2 medium (Fig. 2d). The initial callus was friable in the CIM 3 medium but after first sub-culturing, it turned compact and at later stage, friable calli was noticed from the sides of compact calli (Fig. 2e). In CIM 1 medium, mixed types of callus was observed *viz.*, compact with watery calli; compact with friable periphery and friable callus even from dead callus (Fig. 2f) with all the genotypes. Compact rhizogenic callus with small root like structure and somatic embryos formation was observed in embryo development medium (Fig. 2g). Callus induction from 90 inoculated anthers was assessed and analysed.

**Table 2. Effect of genotypes, pre-treatments, media combinations and their interaction for induction of callus from anther of cocoa with respect to 2 to 3 and 4 to 6 mm long flower bud sizes**

| Pre-treatments | Media | 2 to 3 mm long flower buds | | | | Total mean | 4 to 6 mm long flower buds | | | | Total mean |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Genotypes | | | | | Genotypes | | | | |
| | | CCRP 1 | CCRP 2 | CCRP 3 | CCRP 5 | | CCRP 1 | CCRP 2 | CCRP 3 | CCRP 5 | |
| | | Mean callus induction frequency % | | | | | Mean callus induction frequency % | | | | |
| Fresh (control) | CIM 1 | 52.2 | 37.8 | 62.2 | 63.3 | 53.9 | 87.8 | 90.0 | 60.0 | 64.4 | 75.6 |
| | CIM 2 | 82.2 | 82.2 | 68.9 | 64.4 | 74.4 | 56.7 | 48.9 | 68.9 | 22.2 | 49.2 |
| | CIM 3 | 78.9 | 75.6 | 63.3 | 82.2 | 75.0 | 50.0 | 60.0 | 57.8 | 44.4 | 53.1 |
| | CIM 4 | 83.3 | 81.1 | 85.6 | 90.0 | 85.0 | 50.0 | 66.7 | 50.0 | 90.0 | 64.2 |
| Total mean | | 74.2 | 69.2 | 70.0 | 75.0 | 72.1 [a] | 61.1 | 66.4 | 59.2 | 55.3 | 60.5 [a] |
| Cold pre-treatment | CIM 1 | 66.7 | 31.1 | 67.8 | 64.4 | 57.5 | 64.4 | 0.0 | 0.0 | 66.7 | 32.8 |
| at 4 °C for | CIM 2 | 87.8 | 85.6 | 80.0 | 73.3 | 81.7 | 5.6 | 80.0 | 0.0 | 0.0 | 21.4 |
| four days | CIM 3 | 88.9 | 67.8 | 58.9 | 75.6 | 72.8 | 44.4 | 60.0 | 17.8 | 5.6 | 31.9 |
| | CIM 4 | 93.3 | 72.2 | 87.8 | 90.0 | 85.8 | 62.2 | 43.3 | 11.1 | 0.0 | 29.2 |
| Total mean | | 84.2 | 64.2 | 73.6 | 75.8 | 74.5 [a] | 44.2 | 45.8 | 7.2 | 18.1 | 28.8 [b] |
| Gamma irradiation | CIM 1 | 62.2 | 45.6 | 60.0 | 63.3 | 57.8 | 82.2 | 67.8 | 61.1 | 56.7 | 66.9 |
| at 4 Gy for 24 sec. | CIM 2 | 62.2 | 50.0 | 45.6 | 6.7 | 41.1 | 55.6 | 42.2 | 26.7 | 62.2 | 46.7 |
| | CIM 3 | 54.4 | 44.4 | 37.8 | 33.3 | 42.5 | 62.2 | 70.0 | 53.3 | 45.6 | 57.8 |
| | CIM 4 | 94.4 | 74.4 | 83.3 | 90.0 | 85.6 | 85.6 | 53.3 | 64.4 | 84.4 | 71.9 |
| Total mean | | 68.3 | 53.6 | 56.7 | 48.3 | 56.7 [b] | 71.4 | 58.3 | 51.4 | 62.2 | 60.8 [a] |
| Grand mean | | | | | | | | | | | |
| (Genotypes mean) | | 75.6 [a] | 62.3 [b] | 66.7 [b] | 66.4 [b] | 67.8 | 58.9 [a] | 56.9 [a] | 39.3 [b] | 45.2 [b] | 50.1 |

Analyses were performed on arcsine transformed data. Table shows untransformed data. Means followed by the same letter are not significantly different at the 0.05 probability level using LSD test

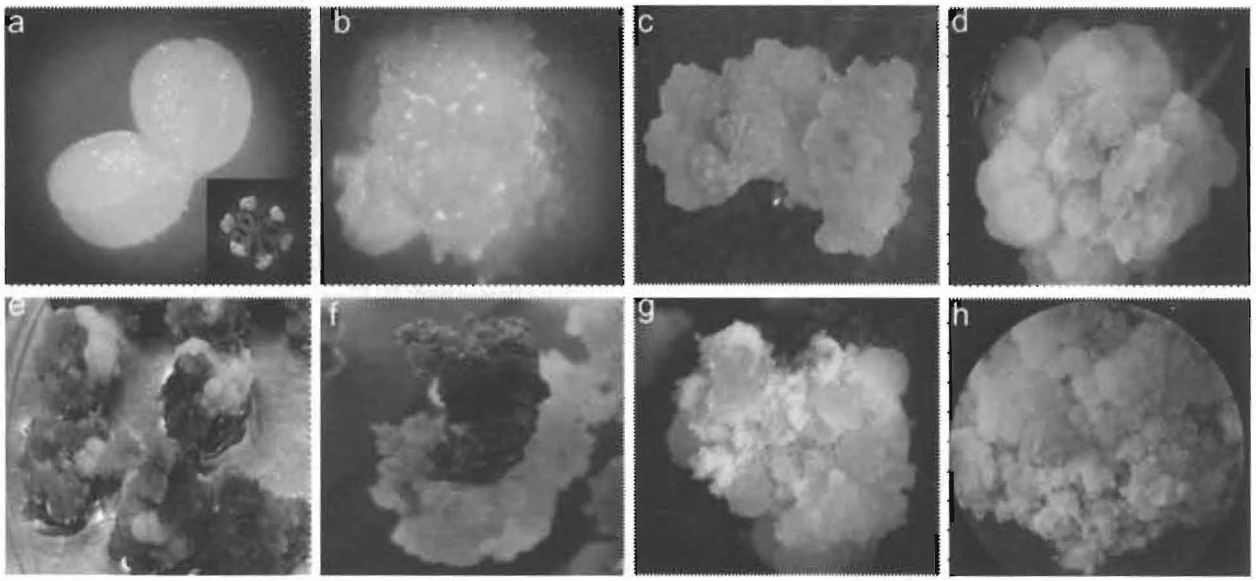


**Fig. 2. Types of callus response during cocoa anther culture. a) Anthers on callus induction medium; b) Anther bursting and callus emerging from microspore; c) Creamy yellow friable callus; d) White compact hairy callus with spot of friable callus; e) White compact hairy and light brown friable callus; f) Light brown friable callus emerging from dead callus; g) Compact rhizogenic callus and embryo formation; h) Greening of callus during regeneration**

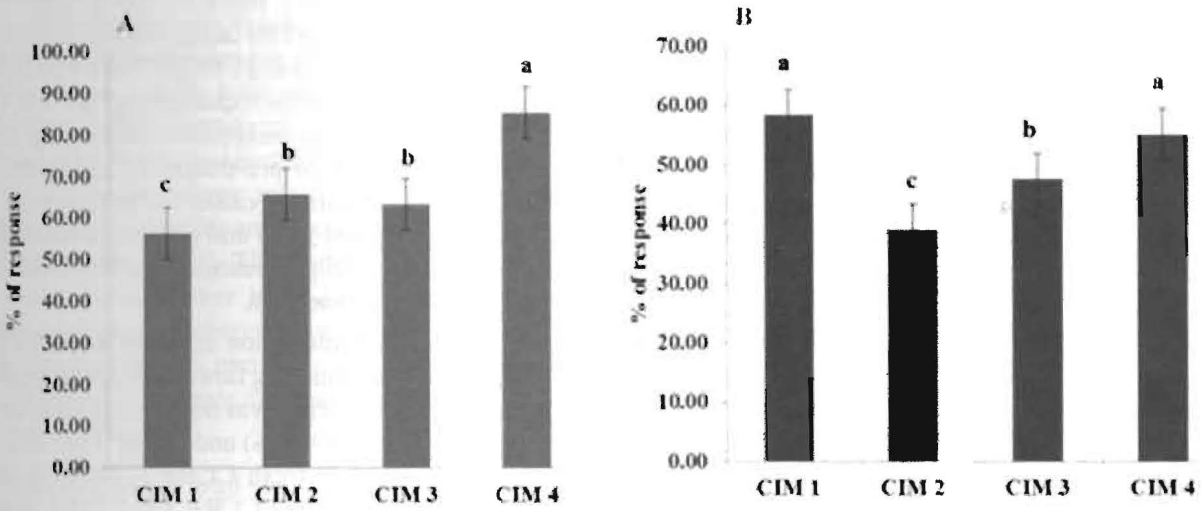


**Fig. 3. Effect of media combinations on percentage callus induction with respect to 2 to 3 mm (A) and 4 to 6 mm (B) long flower buds. Means followed by the same letter are not significantly different at the 0.05 probability level using LSD test**

Table 3 shows summary of analysis of variance and CD value at $P = 0.05$ level of probability.

In case of regeneration studies, proliferative white compact calli turned light green in MS basal medium with CCRP 1 genotype (Fig. 2h). White compact calli was found in WPM + Kinetin (3 mg $L^{-1}$) + NAA (0.2 mg $L^{-1}$) medium with CCRP 5 genotype and white compact calli with scattered green points was noticed in WPM + zeatin (3 mg $L^{-1}$) + glutamine (250 mg $L^{-1}$) + 1000 X amino acid (1 mL $L^{-1}$) + glucose (20 g $L^{-1}$) + sucrose (10 g $L^{-1}$) medium with CCRP 1 genotype. Hence, no regeneration was obtained even with proliferative callus.

## Effect of 2 to 3 mm long flower bud on callus induction

Analysis of variance on the callus induction data for 2 to 3 mm long flower bud anthers containing uni-nucleate microspore stage revealed significant effects from genotypes, pre-treatments and medium combinations alone; and factorial interactions such as pre-treatments x genotypes, genotypes x medium combinations and pre-treatments x medium combinations (Table 3).

Among all genotype and medium combinations, cold pre-treatment of flower buds was significantly better than other pre-treatments (Table 2). However, our analysis revealed that either fresh or cold

**Table 3. Summary of analysis of variance (ANOVA) for effect of genotypes, pre-treatments, medium combinations and their interaction for induction of anther callus in cocoa with respect to 2 to 3 mm and 4 to 6 mm long flower buds**

| Source of variance | df | 2 to 3 mm long flower buds | | | 4 to 6 mm long flower buds | | |
|---|---|---|---|---|---|---|---|
| | | MS | *F* value | CD (p=0.05) | MS | *F* value | CD (p=0.05) |
| Pre-treatments (P) | 2 | 1857.9 | 36.82 ** | 2.877 | 9043.5 | 101.23 ** | 3.829 |
| Genotypes (G) | 3 | 530.3 | 10.51 ** | 3.323 | 1675.5 | 18.75 ** | 4.422 |
| Media (M) | 3 | 2626.1 | 52.05 ** | 3.323 | 1199.3 | 13.42 ** | 4.422 |
| P x G | 6 | 171.6 | 3.40 ** | 5.755 | 638.3 | 7.14 ** | 7.659 |
| G x M | 9 | 426.7 | 8.45 ** | 6.646 | 540.2 | 6.04 ** | 8.844 |
| P x M | 6 | 728.0 | 14.43 ** | 5.755 | 205.4 | 2.29 * | 7.659 |
| P x G x M | 18 | 50.1 | 0.99 ns | 11.511 | 841.5 | 9.42 ** | 15.318 |
| Error | 96 | 50.4 | 1.00 | - | 89.3 | 1.00 | - |

** Significant at $P$=0.01; * Significant at $P$=0.05 level of probability; ns: not significant

pre-treatment has showed no significant differences for production of callus. Among all medium combinations and pre-treatments, CCRP 1 was performing significantly better than other genotypes for callus induction (Table 2). Among all genotype and pre-treatments, CIM 4 medium elicited a significantly greater frequency of callus production than other medium combinations (Fig. 3A).

For factorial interaction of pre-treatments and genotypes, significant and highest callus induction response was noticed in cold pre-treated CCRP 1 flower buds when compared to others (Table 2). For factorial interaction of genotype and medium combinations, CCRP 1, CCRP 5 and CCRP 3 in CIM 4 medium were on par, however, CCRP 1 and CCRP 5 produced highest percentage of calli than CCRP 3. For factorial interaction of pre-treatments and medium combinations, three pre-treatment conditions were on par but they all responded better in CIM 4 medium for callus induction than other medium combinations (Table 2). Combined interaction effect has not been observed between pre-treatments x genotypes x medium combinations (Table 3).

### Effect of 4 to 6 mm long flower bud on callus induction

The ANOVA on the calli producing anthers at 4 to 6 mm long flower bud with bi-nucleate microspores showed significant effects on formation of callus due to genotypes, pre-treatments and medium combinations alone; and factorial interactions such as pre-treatments x genotypes, genotypes x medium combinations, pre-treatments x medium combinations and pre-treatments x genotypes x medium combinations. Irrespective of genotypes and medium combinations, the flower bud treated with gamma irradiation and fresh were on par and revealed maximum callus induction frequency compared to cold treatment (28.8%). Irrespective of medium combinations and pre-treatments, CCRP 1and CCRP 2 showed highest percentage of callus production compared to other genotypes (Table 2). Irrespective of genotypes and pre-treatments, CIM 1 and CIM 4 medium were on par and displayed highest callus induction frequency than other media (Fig. 3B).

The genotypes CCRP 1 (71.4%) and CCRP 5 (62.2%) treated with gamma irradiation were on par with fresh CCRP 2 (66.4%) and CCRP 1 (61.1%) flower buds for best callus induction in all media combinations (Table 2). CCRP 1 showed the highest frequency of callus induction using CIM 1 medium among all pre-treatments. According to statistical interaction of pre-treatment conditions and medium combinations, callus formations were on par with untreated fresh and gamma irradiated flower buds than cold pre-treatment in both CIM 1 medium and CIM 4 medium.

The combined interaction effect of all factors with respect to 4 to 6 mm long flower buds, maximum callus induction frequency was noticed and were on par in fresh CCRP 2 (90.0%) and CCRP 1 (87.8%) genotypes in CIM 1; fresh CCRP 5 (90.0%) in CIM 4; gamma irradiated CCRP 1 in CIM 1 and CIM 4 (82.2%, 85.6%); gamma irradiated CCRP 5 (84.4%) in CIM 4 and cold pre-treated CCRP 2 (80.0%) in CIM 2 medium (Table 2).

### Comparison between 2 to 3 mm and 4 to 6 mm length flower buds

Certain similarities were observed across the flower bud sizes. Fresh flower buds of both 2 to 3 mm and 4 to 6 mm length were performed well. The CIM 4 medium is consistently superior to other media combinations. The effects of genotype, medium combinations and pre-treatments were not consistent across flower bud sizes.For instance, in 2 to 3 mm long flower buds possessed a significant cold pre-treatment effect on callus induction and 4 to 6 mm long flower bud had with gamma irradiation but not with cold pre-treatment. In both flower bud sizes, CCRP 1 was significantly better than the other genotypes for callus production. Examination of the data over the two flower bud sizes suggested 4 to 6 mm long flower buds anthers containing bi-nucleate microspore (50.1%) were inferior to 2 to 3 mm length flower buds anthers containing uni-nucleate microspore (67.8%) for callus induction (Table 2).

## Discussion

### Developmental stage of microspores correlates with length of flower buds

Choosing appropriate developmental stage of microspore is prerequisite for successful anther culture (Cimo *et al.*, 2016). Notably uni-nucleate to bi-nucleate microspore stages in papaya

(Rimberia *et al.*, 2006), late uni-nucleate or early bi-nucleate stages in *Brassica* had been documented as suitable candidates for successful anther culture (Fan *et al.*, 1988; Kott *et al.*, 1988; Pechan and Keller, 1988). Identifying visible and measurable traits like flower bud length linked with microspore developmental stage may fasten the process of choosing explant for anther culture (Srivastava and Chaturvedi, 2008). This report showed that 2 to 3 mm long flower buds (anthers containing uni-nucleate microspore stage) was found to be better than 4 to 6 mm long flower buds (anthers containing binucleate microspores stages) for callus induction.

**Effect of flower bud sizes in callus induction**

Overall, callus induction values suggested that 2 to 3 mm long flower bud was superior to 4 to 6 mm long flower buds. This result agrees with earlier report in neem *(Azadirachta indica)*, where 2 mm long flower bud (anthers containing early to late-uninucleate microspore stage) induced maximum callus/regeneration (Chaturvedi *et al.*, 2003).

**Effect of genotypes in callus induction**

With 2 to 3 mm long flower buds CCRP 1 and with 4 to 6 mm long flower buds, CCRP 1 and CCRP 2 genotypes were statistically similar and produced highest frequencies of callus production. CCRP 1, CCRP 2 and CCRP 5 genotypes performed well in CIM 4 medium with respect to 2 to 3 mm long flower bud with respect to pre-treatments. CCRP 1 produced more callus in CIM 1 medium with respect to 4 to 6 mm long flower buds. Accordingly, the results of this investigation confirm with previous reports which affirmed the existence of the effect of cocoa genotype on morphogenic response under *in vitro* conditions, physiological stage of plant materials, type of explant, the concentration and type of growth regulator used (Velasquez *et al.*, 2006).

**Influence of pre-treatment of floral buds in callus induction**

In order to induce pollen embryogenesis, temperature treatment is considered to be the most effective approach to compare with other pre-treatment methods. Pre-treatment of excised flower bud for certain period in cold conditions has been demonstrated to enhance the callus induction efficiency in anther culture (Mishra and Gowswami, 2014).

Our analysis revealed that, the effects of pre-treatments were not consistent across flower bud sizes. With 2 to 3 mm long flower buds, fresh and cold pre-treatment showed better results than gamma irradiation for callus induction. Culturing anthers at uni-nucleate microspores taken from 4°C pre-treated flower buds showed highest frequency of callus (Raghavan, 1986; Stoehr and Zsuffa,1990; Khatun *et al.*, 2012). The material exposed to low temperatures for long periods near to a week, had a positive effect on the cocoa morphogenic response (Teixeira *et al.*, 2002). The flower buds 4 to 6 mm long revealed maximum callus induction frequency when used either as fresh or treated with gamma irradiation, compared to cold pre-treatment. Another way of pre-treatment has been done by exposing flower buds to gamma irradiation and was reported to enhance microspore embryogenesis in some plants (Sangwan and Sangwan, 1986; Macdonald *et al.*, 1988; Pechan and Keller 1989). The use of irradiation on flower buds would be possible for obtaining haploid cocoa plantlets (Falque *et al.*, 1992), as observed in the present study. Pre-treatment conditions had significant effect on callus induction and it depended on factors like clones/genotypes (Powell, 1988; Osolnik *et al.*, 1993; Khiabani *et al.*, 2008) and flower bud size with appropriate microspore stage (Pedroso and Pais, 1994; Sopory and Munshi, 1996; Chaturvedi *et al.*, 2003).

The results of interaction effect between pre-treatments and genotypes revealed that anthers from cold pre-treated CCRP 1 buds significantly showed higher callus induction compared to others with 2 to 3 long flower buds. With respect to longer flower buds (4 to 6 mm) CCRP 1 and CCRP 5 treated with gamma irradiation and fresh buds from CCRP 1 and CCRP 2 were best for callus induction. This finding conveys that, each genotype requires specific pre-treatment conditions.

**Effect of media combinations**

The medium combination CIM 4 with 2 to 3 mm long flower buds and CIM 1, CIM 4 medium with 4 to 6 mm long flower buds respectively, induced significantly greater callus induction frequencies

from anthers than other medium combinations. In all the genotypes,three pre-treatment conditions were on par but they all responded better in particular at CIM 4 medium for callus induction than other media combinations when 2 to 3 mm long flower buds were used. The callus formations was on par with fresh and gamma irradiated flower buds than cold pre-treatment in both CIM 1 medium and CIM 4 medium with 4 to 6 mm long flower buds.

Selecting types of callus and time of transferring calli to new media is very important factor to get the embryos/plantlets. In this study, CIM 1, CIM 3 and CIM 4 were appropriate medium for producing friable callus. Supplementation of DKW medium with 2,4-D, TDZ and glutamine enhanced the callus formation from anthers that were isolated from 2 to 3 mm and 4 to 6 mm long flower buds. Treatment with DKW medium induced embryogenic callus growth and development of somatic embryos (Li *et al.*, 1998; Maximova *et al.*, 2002). The excess presence of $Ca^{2+}$, S and $Mg^{2+}$ions in DKW medium favours for *in vitro* propagation of woody perennial species than MS medium. Appropriate auxin to cytokinin ratio is necessary for successful *in vitro* culture (Ramawat *et al.*, 2014). The auxin 2,4-D is widely used in anther culture systems (Zheng and Konzak, 1999). Similarly the cytokinine TDZ is widely used in woody plant tissue culture and enables micro-propagation of the most recalcitrant species (Huetteman and Preece, 1993). The addition of other compounds like casein, biotin, coconut water and glutamine has improved callus induction during androgenesis of few woody plants (Drira and Benbadis, 1975; Custodio *et al.*, 2005).

### Regeneration

Embryogenic callus, somatic embryos and plants have been obtained in different cocoa genotypes and explants *viz.*, staminodes and petals (Ajijah *et al.*, 2016). Attempts were made to induce somatic embryo using various floral parts as explants, and successful report documented in staminode and stamen filament culture (Alemanno *et al.*, 1996). Santana *et al.* (2010) obtained rhizogenic callus from cocoa anthers of genotype Ocumare-61 and petals of genotype Chuao-2. The chromosomal analysis of roots derived from anther callus revealed haploid number of chromosome (2n=X=10), thus indicating the feasibility of haploid production through anther culture (Santana *et al.*, 2010). The genotypes under study showed recalcitrant behaviour to regeneration. The inability of callus to regenerate despite greening might be due to the very low regeneration ability of the genotypes under investigation. More cocoa genotypes have to be evaluated for identifying the contributing factors such as pre-treatment conditions and medium combinations for achieving better regeneration.

## Conclusion

Genotype specific optimization of culture media composition and pre-treatment of flower buds is necessary to succeed in anther culture in woody crops. Results in the present study indicate that, each genotype requires specific pre-treatment conditions and media combination for optimum androgenic callus response. The optimized protocol for induction and regeneration of callus from immature anthers in this report provides a step ahead in the development of *in vitro* cocoa haploid production.

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