

Research Article

Development of *in vitro* gynogenesis system in cocoa (*Theobroma cacao* L.)

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Abstract

In the present study, the effect of genotypes, flower bud sizes and pre-treatments on *in vitro* ovary culture of cocoa was studied. The first step, the association between floral bud size and the corresponding microspore developmental stages/ovary stages was established. Bud sizes of 4.0-6.0 mm long (representing the microspores at the bi-nucleate stage and mature ovary) provided the highest callogenesis levels. However, no significant difference in gynogenic response was found between flower bud sizes. Both cold pre-treatment at 4°C for four days alone and cold combined with gamma irradiation at 8 Gy for 12 seconds elicited a significant increase in callus formation as compared with fresh flower buds. The highest percentage of morphogenetic calli was obtained with 4.0-6.0 mm long flower buds of CCRP 5 on WPM medium supplemented with NAA (2.0 mg L⁻¹), BAP (1.0 mg L⁻¹), Coconut water (50.0 mL L⁻¹), Sucrose (70.0 g L⁻¹) with either cold alone (91.7%) or combined with gamma irradiation (90.0%) pre-treatment. Visible callus was observed about 30-40 days after inoculation of ovaries in callus induction medium. Both embryogenic friable and non-embryogenic compact calli were observed. Greening of the callus was observed and no regeneration from calli was noticed.

Keywords: Cocoa, flower bud size, explants stages, pre-treatments, ovary culture, callus induction, regeneration

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Introduction

Cocoa (*Theobroma cacao* L.) (Malvaceae family) is one of the world's most valuable crops mainly for production of chocolate. Cocoa, native to South America, is characterized by three main cultivar groups: *Criollo*, *Forastero* and *Trinitario* [1]. It is cultivated worldwide in an area of 10.43 million ha with total production of 4.45 million tonnes mainly in the (sub) humid tropics [2]. Cocoa was introduced in India during 1798 [3] and is cultivated predominantly in Kerala, Andhra Pradesh, Tamil Nadu and Karnataka, totally in an area of 78,000 ha with total production of 16,050 MT [4]. The availability of homozygous lines of cocoa would be of great use for its genetic improvement as the homozygous parents might improve the phenotypic homogeneity of hybrid progenies. Classical methods of inbreeding by successive self-pollinations are difficult and very long in the case of cocoa because of long reproductive cycles and frequent self-incompatibilities [5]. Spontaneous cocoa haploid plants have been obtained in some genotypes, particularly in polyembryonic seeds [6]. However, the frequency of spontaneous haploids is very low, mostly below 0.1%, whereas most genotypes giving near 0 per cent spontaneous haploids. In this regard, tissue culture can play an important role for genetic improvement of cocoa with artificial haploid induction. Embryogenic callus and somatic embryos have been obtained in different cocoa genotypes and explants such as the base of floral buds, petals, staminodes, stamens and ovaries [7] and [8], petal and staminodes [9-15] and [16]. Although cocoa somatic embryogenesis can be obtained using different culture conditions and explant types, best results have been shown on callus induction from staminode. Callus induction and regeneration of plants from ovary culture in cocoa genotypes is yet to be reported. Haploid plants can be achieved using several methods: *in vitro* androgenesis (anther-isolated microspore culture) and gynogenesis (ovule-ovary culture), *in situ* parthenogenesis (pollen irradiation or treatment with chemicals) [17] and [18]. The homozygous breeding population for genetic improvement can be easily obtained by doubling the chromosomes of the haploid progenies obtained through tissue culture methods.

Gynogenesis has been shown to be a possible alternative source for haploid production in plants, particularly in species where androgenesis is unsuccessful [19]. Successful gynogenesis is reported in many plant species, e.g., onion [20] and [21], sweet potato [22], maize [23], sugar beet [24], cucumber [25], and wheat [26]. There are several endogenous and exogenous factors which play important role for successful *in vitro* gynogenesis viz., donor genotype selection, developmental stage of the female/male gametophyte, pre-treatments, the nutrient medium composition, growth regulators, additives and culture conditions [19] and [27]. The present study was attempted to evaluate the effect of cocoa genotypes, flower bud size and pre-treatments to define an *in vitro* protocol for callus induction /somatic embryogenesis and plant regeneration from ovaries.

Materials and Methods

Plant material

The unopened flower buds of four cocoa genotypes viz., CCRP 1, CCRP 2, CCRP 3 and CCRP 5 were used for ovary culture study. The flower buds were collected from 5-6 year old field established plants in the coconut garden maintained by Department of Spices and Plantation crops, Horticultural College and Research Institute, TNAU, Coimbatore. The experiment was conducted in the Tissue Culture Laboratory at Department of Plant Biotechnology, Centre for Plant Molecular Biology and biotechnology, Tamil Nadu Agriculture University, Coimbatore.

Methods

The flower buds were collected in the morning hours. The first step, the association between floral bud size and the corresponding ovary stages/microspore developmental stages was established. For ovary culture studies, two different flower buds sizes were selected viz., flower buds at 2.0-3.0 mm long (Figure 2a) and 4.0-6.0 mm long (Figure 2e). The flower buds were used either fresh or subjected to pre-treatments (cold pre-treatment at 4°C for 4 days in dark or cold pre-treatment at 4 °C for 4 days followed by gamma irradiation at 8 Gy for 12 seconds).

The buds (either fresh or pre-treated) were surface sterilised in ethanol (70%) for 30 seconds, followed by immersion in sodium hypochlorite solution (1%) for 5 minutes and rinsed three times in sterile distilled water in a laminar flow chamber. Ovaries were isolated from 2.0-3.0 mm (Figure 2b) and 4.0-6.0 mm (Figure 2f) long flower buds by using scalpels and forceps, after removal of sepals and petals. The ovaries were inoculated on Woody Plant Medium (WPM) [28] supplemented with NAA (2.0 mg L⁻¹), BAP (1.0 mg L⁻¹), Coconut water (50.0 mL L⁻¹) and Sucrose (70.0 g L⁻¹) (Figure 4a and 4b). One petri dish constituted one replicate (containing 15 explants) and an average of 4 replicates was cultured for each treatment. The Cultures were incubated in incubator at 25 ± 2 °C under dark condition for 30 days for callus initiation. After 30 days, callus obtained from ovaries were sub-cultured to the same fresh medium composition for callus proliferation. The cultured plates were examined periodically to observe the progress with respect to callus formation and forty days after inoculation, percentage of callus induction was recorded. For regeneration, proliferated calli (90 days old) were transferred to MS medium [29] supplemented with Zeatin (1.0 mg L⁻¹), kinetin (3.0 mg L⁻¹), TIBA (1.0 mg L⁻¹), proline (100.0 mg L⁻¹), glutamine (200.0 mg L⁻¹), ascorbic acid (35.2 mg L⁻¹), FeEDDHA (200.0 mg L⁻¹), sucrose (30.0 g L⁻¹) and maintained at 25±2°C under 16h/8h light/dark period. Morphological observations (compact white/friable yellow) were made with the aid of a stereomicroscope, in explants with initial callus development and throughout the experiment, to confirm the callus development from ovary tissues only.

The callus induction frequency was calculated as:

$$\text{Callus induction frequency (\%)} = \frac{\text{Number of ovaries producing calli}}{\text{Number of ovaries plated}} \times 100$$

All data were analysed using SAS software v.9.3 [30] and Microsoft Excel. The experimental design was a 2 x 3 x 4 factorial. Two flower bud sizes (2.0-3.0 mm and 4.0-6.0 mm long), three pre-treatments (fresh flower buds, cold pre-treated at 4°C for 4 days and cold pre-treatment at 4°C for 4 days followed by gamma irradiated at 8 Gy for 12 seconds) and four genotypes (CCRP 1, CCRP 2, CCRP 3 and CCRP 5).

Histological analysis

Ovaries and calli samples were taken and fixed in 10 per cent neutral buffered formalin solution (50 ml of 37 % formaldehyde, 450 ml distilled water, 3.25 g sodium phosphate (Dibasic Na₂HPO₄), 2.0 g sodium phosphate (Monobasic-NaH₂PO₄) for 24 hour at room temperature. The material was passed through a tertiary butyl alcohol

series for dehydration, infiltrated with paraffin wax (Merck, Darmstadt, Germany), and embedded in pure paraffin wax. The paraffin blocks were sectioned at 10 μm thickness. The ribbons were placed on the slides smeared with Meyer's albumin and flooded with 4 per cent formalin. These slides were slightly warmed so as to stretch the sections. Sections were mounted and observed under light microscope (Leica).

Results and Discussions

Successful induction of callus; somatic embryos either directly or indirectly and subsequent plantlet regeneration depends upon the flower donor genotype selection, explant types, developmental stage of the female gametophyte, pre-treatments, the nutrient medium composition, growth regulators, additives and culture conditions [19]. Initially, experiments were carried out with 14 medium combinations (Three different medium *viz.*, MS medium [29], WPM medium [28] and DKW medium [31] with different growth regulator combinations) to establish the callus from ovary. In all three basal media, no callus induction was observed except WPM basal which showed 1-2 % callus induction frequency. Out of 14 medium combinations, the best combination WPM medium supplemented with NAA (2.0 mg L^{-1}), BAP (1.0 mg L^{-1}), coconut water (50.0 mL L^{-1}) and sucrose (70.0 g L^{-1}) showed more than 40 per cent callus induction frequency and proliferative callus morph types was shortlisted and used for further experiments (supplementary data).

The analysis of variance (ANOVA) revealed that the variation associated with pre-treatments, genotypes alone; and the factorial interactions such as pre-treatments x genotypes; and flower bud size x pre-treatments x genotypes were highly significant ($p=0.05$) on the capacity of gynogenesis (**Table 1**). The ovary culture response in the four cocoa genotypes ranged from 54.72% to 68.61%. Out of four elite genotypes selected for the study, CCRP 5 (68.61 %) and CCRP 2 (64.17 %) gave the highest and most consistent results in terms of callus formation followed by CCRP 1 and CCRP 3 (**Table 2**). Gynogenesis efficiency in plants is highly dependent on the variety used, the growth condition of the plants, and the quality of the donor material [27]. The genotype proved to be one of the most important factors affecting *in vitro* gynogenesis in squash as the percentage of gynogenic ovules ranged from 0% to 48.8%, depending on genotype [40]. The significant interaction effect between pre-treatment conditions x genotypes are presented in **Figure 1**. The genotypes CCRP 5 (90.00 %) and CCRP 2 (80.00 %) pre-treated with cold were on par with CCRP 5 (85.84 %) and CCRP 1 (79.17 %) pre-treated with cold followed by gamma irradiation and showed maximum number of callus, whereas fresh CCRP 1 (25.00%) produced lowest percentage of calli. Observations made in this study clearly suggest that, the degree of response varied with the genotype and the pre-treatment conditions used.

Table 1 Analysis of variance for effect of genotypes, flower bud size, pre-treatments and their interaction for induction of callus from unpollinated ovaries

Source of variation	df	MS	F value	SED	CD (0.05%)
Flower bud size (F)	1	253.59	3.08 ^{ns}	1.85	3.69
Pre-treatments (P)	2	6725.46	81.61 ^{**}	2.26	4.52
Genotypes (G)	3	443.12	5.38 ^{**}	2.62	5.22
F x P	2	131.10	1.59 ^{ns}	3.20	6.39
F x G	3	125.83	1.53 ^{ns}	3.70	7.38
P x G	6	701.64	8.51 ^{**}	4.53	9.04
F x P x G	6	183.21	2.22 [*]	6.41	12.79
Error	72	82.41			

** = significant at 0.01 level of probability; * = significant at 0.05 level of probability; ns= non-significant at 0.05 level of probability

Bud sizes of 2.0-3.0 mm long, corresponded to the immature ovary stage and uni-nucleate microspore stage (**Figure 2c** and **2d**); and 4.0-6.0 mm long flower buds showed mature ovary and bi-nucleate microspores stage (**Figure 2g** and **2h**). Ovaries collected from flower buds size 4.0-6.0 mm length (containing mature ovary/bi-nucleate microspore stage) responded better than 2.0-3.0 mm length (containing immature ovary/uni-nucleate microspore stage) (**Table 2**). However, no significant difference between flower bud sizes stages was found (**Table 1**). For successful ovary culture the stage of development of the embryo sac is very critical. Haploids of 21 angiosperm species have been obtained from *in vitro* unfertilized ovule/ovary culture since 1976, in most cases using explants at uni-nucleate to mature embryo sac stages [34]. The evaluation of the developmental stage of ovules at the moment of plating is difficult. An indirect method is based on the observation of microspores development that could be helpful in choosing appropriate flowers for *in vitro* cultures [35]. In onion, embryo sac is completely mature when most

microspores are in the uni- or bi-nucleate stage, flowers being just prior to anthesis [36] and the length of flower buds is 3.4-4.5 mm long [37]. These results are in agreement with the findings of [38]. In onion (*Allium cepa* L.), the high effectiveness of gynogenesis was seen in 3.5-4.5 mm long flower bud and it is related to the presence of mature embryo sacs, with anthers containing binucleate microspores [38], in *Allium porrum* small buds around 2 mm long were the most embryogenic [39].

Table 2 Effect of genotypes, flower bud size, pre-treatments and their interaction for induction of callus from cultured ovaries

Flower bud size	Pre-treatments	Genotypes				Total Mean
		CCRP 1	CCRP 2	CCRP 3	CCRP 5	
Means of callus induction frequency (%)						
2.0-3.0 mm long	PT1 [@]	20.00	38.33	41.66	23.33	30.83
	PT2 [@]	73.33	78.33	61.66	88.33	75.41
	PT3 [@]	81.67	56.67	66.67	81.67	71.67
Total mean		58.33	57.78	56.66	64.44	59.30 ^a
4.0-6.0 mm long	PT1	30.00	68.33	23.33	36.67	39.58
	PT2	68.33	81.67	46.66	91.67	72.08
	PT3	76.67	61.67	88.33	90.00	79.17
Total mean		58.33	70.56	52.77	72.78	63.61 ^a
Grant mean (genotypes mean)		58.33 ^b	64.17 ^a	54.72 ^c	68.61 ^a	

[@]PT1: Fresh flower buds; PT2: Cold pre-treatment at 4°C for 4 day; PT3: Cold pre-treatment at 4°C for 4 days followed by gamma irradiation at 8 Gy for 12 seconds. 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.

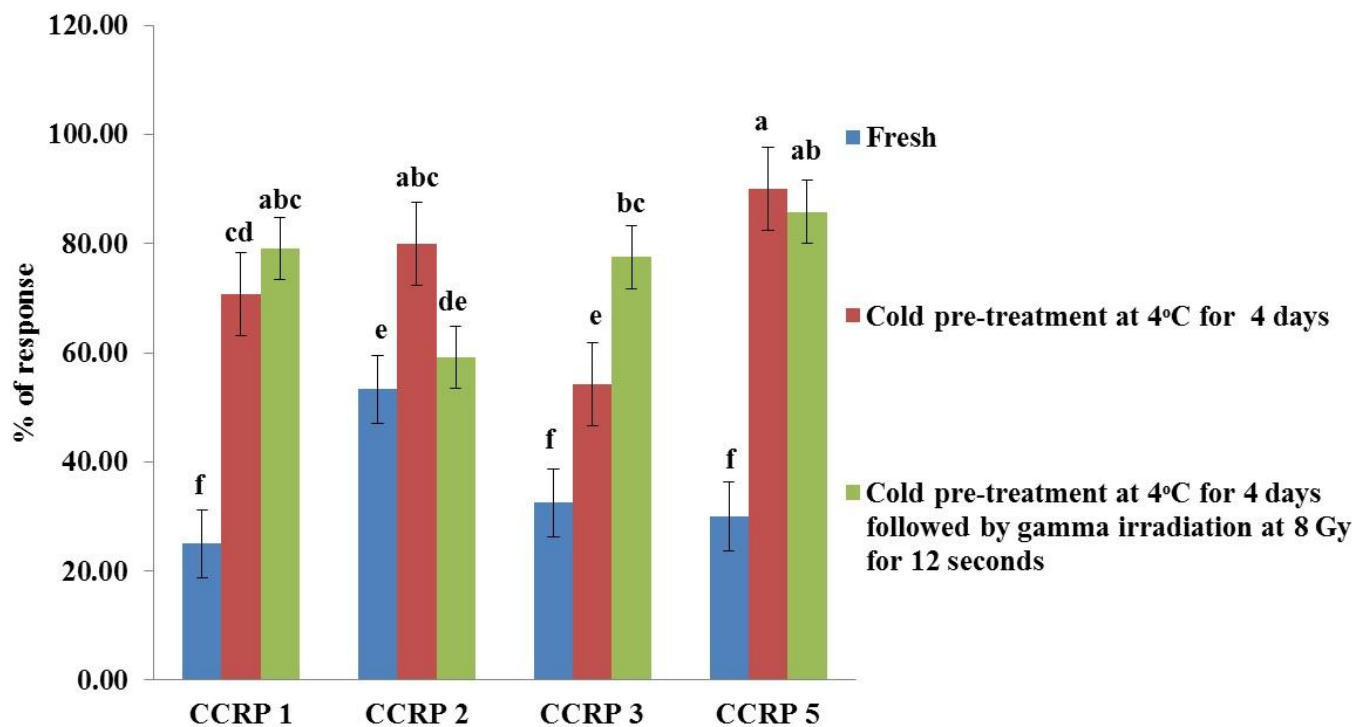


Figure 1 Pre-treatments x genotypes interaction effect for induction of callus from cultured ovaries. Analyses were performed on arcsine transformed data. Figure shows untransformed data. Means followed by the same letter are not significantly different at the 0.05 probability level using LSD test

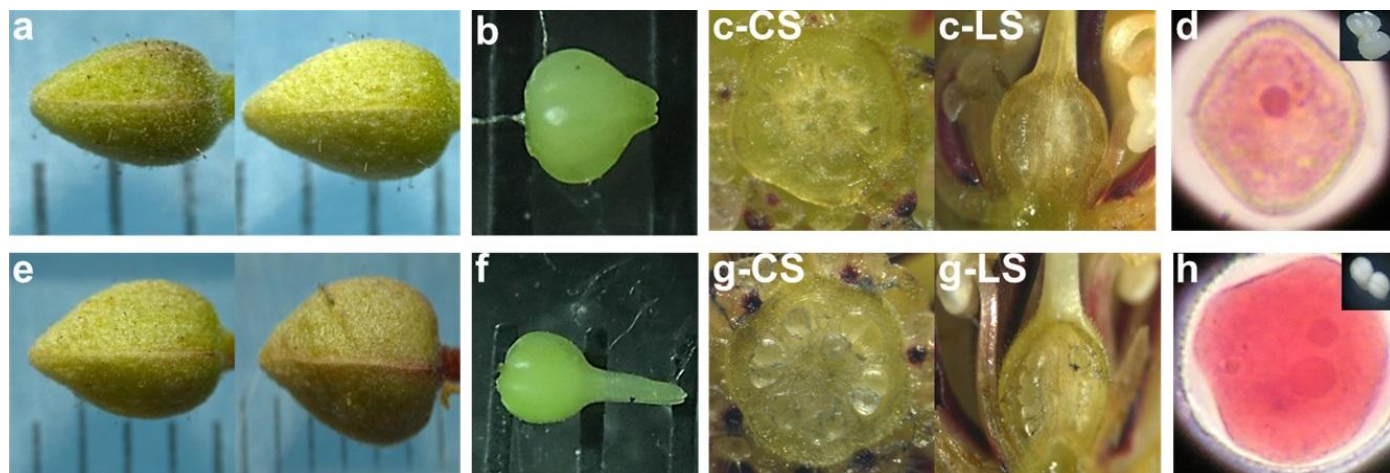


Figure 2 Association of floral bud sizes with stages of ovary development:

(a) 2.0-3.0 mm long flower bud; (b) Ovary isolated from 2.0-3.0 mm long flower bud; (c) Cross (c-CS) and longitudinal section (c-LS) of 2.0-3.0 mm long flower bud showing immature ovary and ovules. The anthers filament has begun to expanding and the style is not yet at the same length as the staminodes; (d) Anthers containing uninnucleate microspore development stage associated with 2.0-3.0 mm long flower buds; (e) 4.0-6.0 mm long flower bud; (f) Ovary isolated from 4.0-6.0 mm long flower bud; (g) Cross (g-CS) and longitudinal section (g-LS) of 4.0-6.0 mm long flower bud showing mature ovary and ovule just prior to flower bud opening; (h) Anthers containing binucleate microspore development stage associated with 4.0-6.0 mm long flower buds.

Among the pre-treatments tested, both flower buds treated with cold at 4°C for 4 days (73.75%) and cold pre-treatment followed by gamma irradiation at 8 Gy for 12 seconds (75.42%) were on par and performed better than fresh flower buds (**Figure 3**). Observations made in this study clearly suggest that the pre-treatments used critically influenced the *in vitro* response towards callus formation and nature of callus. Temperature shocks improve gynogenesis by diverting normal gametophytic development into a sporophytic pathway leading to the formation of haploid embryos [39]. The exact mechanisms that act on plantlet formation following temperature pretreatments are still unknown. The shock provided by low or high temperature probably establishes cellular conditions that are important for switching from the gametophytic to sporophytic developmental pathway. For different explants, the optimum types, levels, and durations of pre-treatment are different and the regeneration efficiencies also vary [27]. Dark incubation favors gynogenesis and minimizes somatic callusing [41] and [25]. Cold treatment was found to be beneficial in durum wheat [26], sugar beet [24] and niger [42]. Effect of gamma irradiation of ovaries and their subsequent response in gynogenesis has not been reported previously. In this study, cold followed gamma irradiated flower buds was found to be effective in gynogenic response of ovaries.

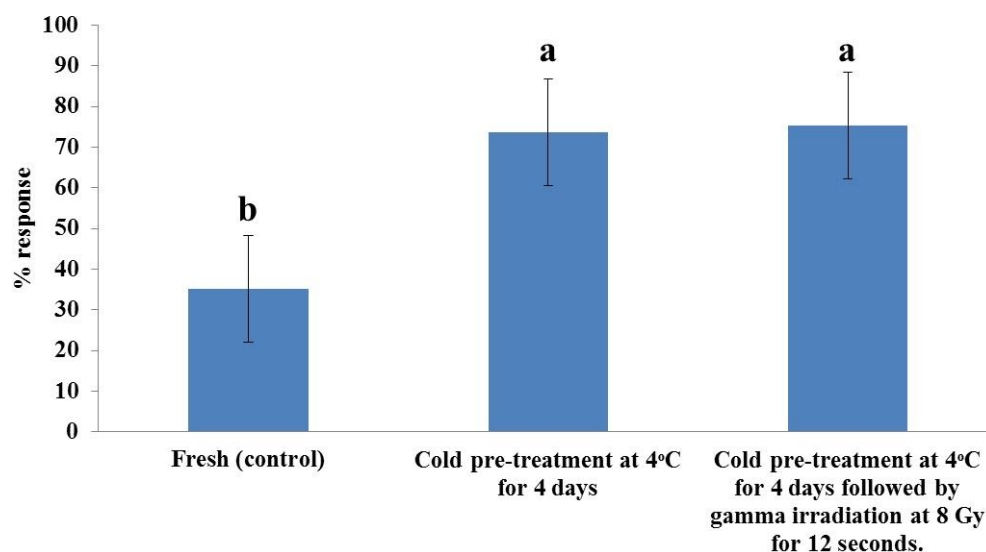


Figure 3 Effect of pre-treatments for the induction of callus from cultured ovaries. Analyses were performed on arcsine transformed data. Figure shows untransformed data. Means followed by the same letter are not significantly different at the 0.05 probability level using LSD test.

The significant interaction effect between flower bud size x pre-treatments x genotypes are represented in Table 2. The ovary culture response varied ranges from 20.00% to 91.67%. Ovaries from both cold pre-treated and cold followed by gamma irradiated flower buds (2.0-3.0 and 4.0-6.0 mm length) of CCRP 5; ovaries from cold followed by gamma irradiated flower buds (4.0-6.0 mm length) of CCRP 3; ovaries from cold followed by gamma irradiated flower buds (2.0-3.0 and 4.0-6.0 mm length) of CCRP 1 and ovaries from cold pre-treated flower buds (2.0-3.0 and 4.0-6.0 mm length) of CCRP 2 were on par and gave best callus induction frequencies on WPM medium supplemented with NAA (2.0 mg L^{-1}), BAP (1.0 mg L^{-1}), Coconut water (50.0 mL L^{-1}) and Sucrose (70.0 g L^{-1}).

Gynogenic callus induction was characterized by emergence of callus tissue from the ovules as well as from ovary cell wall (**Figure 4c**) and at later stage proliferative light brown friable and white compact callus was observed (Figure 4d and 4e). When proliferative calli transferred to regeneration medium, greening of callus was noticed without any further shooting under light conditions (Figure 4f). In one of experiment in cocoa base of floral buds, petals, staminodes, stamens and ovaries were used as explants for somatic embryogenesis but only staminode and stamen filament produced somatic embryos [7]. While culturing cocoa anthers and petals different types of non-embryogenic callus were noticed *viz.*, necrotic/browning callus, callus with globular structures in some cases similar to the shape of torpedoes, callus bifurcated elongated structures and Callus with clusters of elongated structures [32]. In this study also, same types of non-embryogenic calli were observed. Most of the calli observed in this study showed marked heterogeneity with non-embryogenic nature. There were globular, compact, watery, rhizogenic, necrotic and crystalline calli (**Figure 5a-f**).

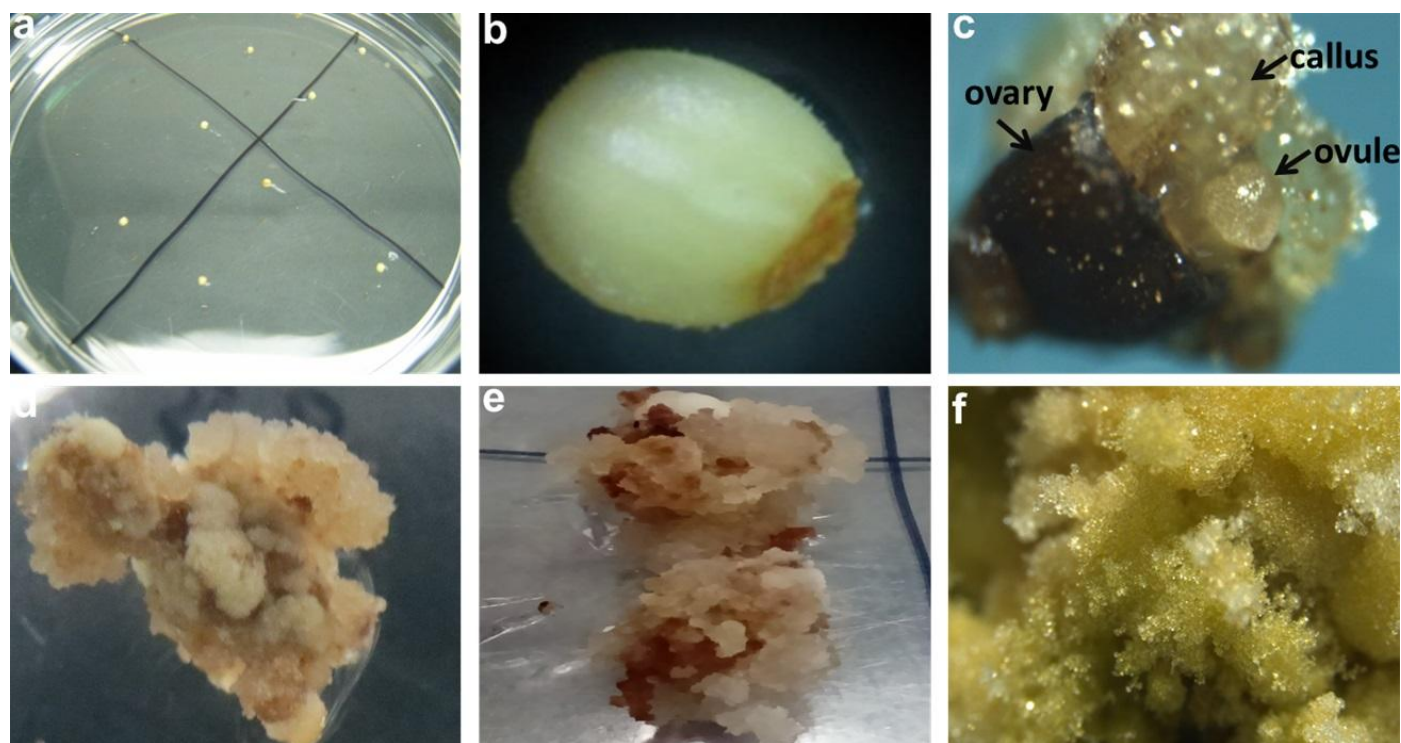


Figure 4 *In vitro* ovary cultures of cocoa. (a & b) Whole ovaries cultured on WPM medium supplemented with NAA (2.0 mg L^{-1}), BAP (1.0 mg L^{-1}), Coconut water (50.0 mL L^{-1}) and Sucrose (70.0 g L^{-1}); (c) Callus formation at 14 days after induction; (d) Gynogenic callus development; (e) Proliferated friable callus with areas of compact nature and; (f) Greening of gynogenic callus

Histological analysis of ovary callus was carried out to see the possibility of plant regeneration from organogenic masses. Prior to induction of callus/somatic embryogenesis, sectioning of ovary explants revealed five united carpels and inside the ovules are united with central axis through placenta (**Figure 6a** and **6b**). After 15 days of culture swelling of ovule and degeneration of the embryo sac was observed, along with callus formation not only from the explant but also from other surrounding tissues (Figure 6c and 6d). After 50 days friable calli (containing embryogenic cells) with areas of compact nature (Figure 6e and 6f) was noticed. In the white, compact non-embryogenic calli, internal cells were observed to be differentiated and accumulating polyphenolic compounds, whereas the outer cells were showing some active division forming closely packed chains of cells giving the white appearance (Figure 6g and 6h). Both embryogenic cells (rounded cell clusters thick cell wall and prominent nucleus) and non-embryogenic cells (packed chains of elongated cells with thin cell wall) were noticed in single callus.

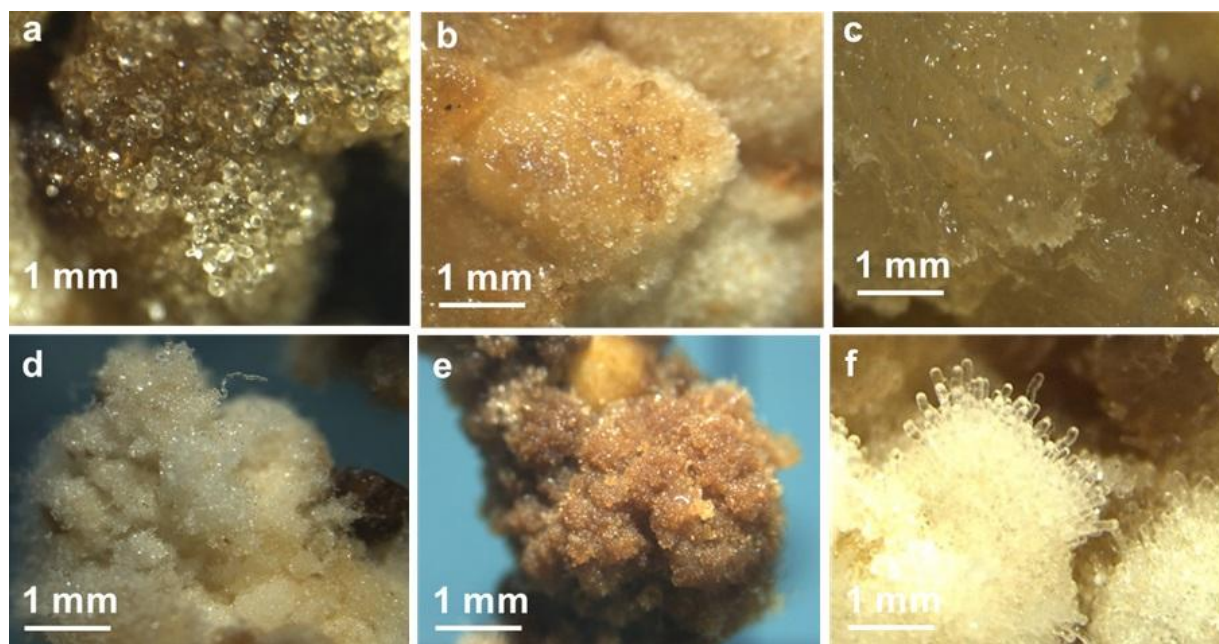


Figure 5 Morphological heterogeneity in non-embryogenic callus obtained from ovary culture. (a) Callus with globular structures; (b) White compact calli; (c) Watery callus; (d) Rhizogenic callus; (e) Dry brown callus; (f) Callus with clusters of elongated structures (like crystalline). Bars = 1mm (a-f)

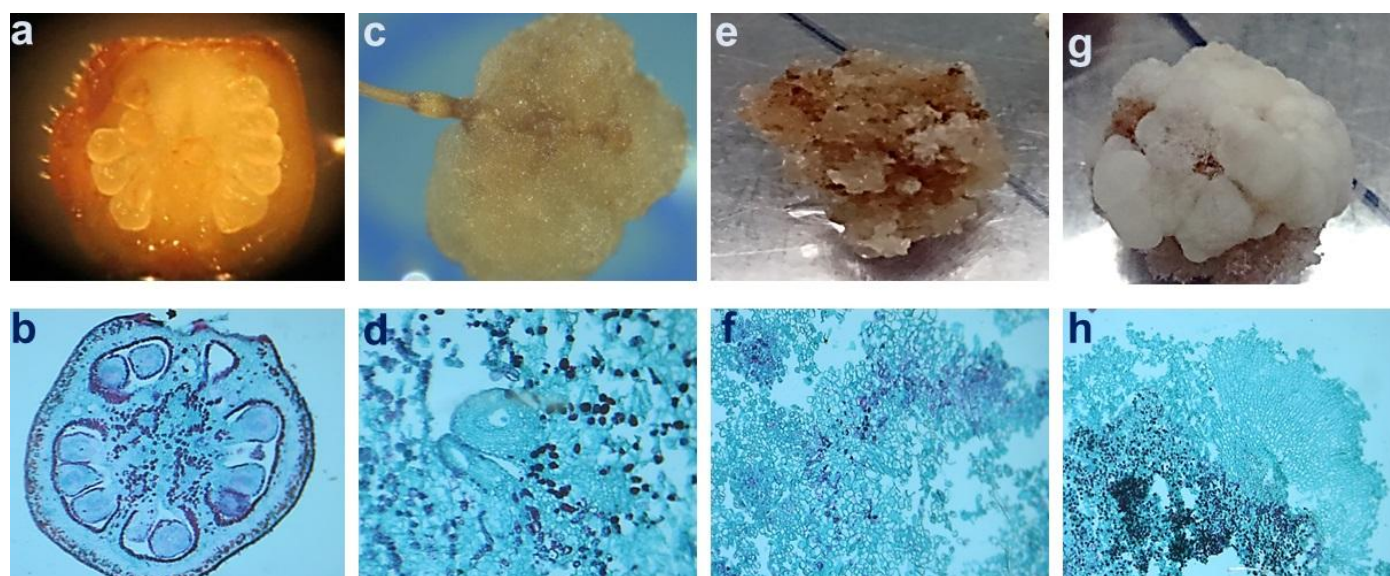


Figure 6 Histological representation of callus induction process from ovaries of 0, 15 and 50 days after inoculation. (a & b) Section of cocoa ovary showing ovules; (c & d) Section of a 'swollen' ovule, showing a degenerating embryo sac and callus formation at several locations after 15 days of inoculation; (e & f) Approximately 50 days old friable calli with areas of compact nature showing embryogenic cells; (g & h) Approximately 50 days old fully white compact calli showing both embryogenic cells (inside of the callus) and non-embryogenic cells (outside of the callus) with phenolic compounds

Histological observations in the developing callus revealed embryogenic cells but no developing structures for regeneration. In the non-regenerating variety of cocoa, embryogenic calli from staminodes/anthers were compact and such structures contained polyphenols randomly distributed across all tissues and in case of ovary polyphenols were present in all tissues except the epidermis [33]. Such phenomenon was also observed in the present study. 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. So, more genotypes have to be evaluated for the contributing factors such as media combinations and pre-treatment conditions for achieving regeneration.

Conclusion

Ovaries from either cold pre-treated (91.67%) or cold followed by gamma irradiated (90.00%) CCRP 5 flower buds at 4.0-6.0 mm length size responded well for gynogenesis on WPM medium supplemented with NAA (2.0 mg L⁻¹), BAP (1.0 mg L⁻¹), Coconut water (50.0 mL L⁻¹) and Sucrose (70.0 g L⁻¹). This observation indicates that, the ovaries collected from flower buds size 4.0-6.0 mm length (containing mature ovary/bi-nucleate microspore stage) responded better than 2.0-3.0 mm length (immature ovary/containing uni-nucleate microspore stage). Successful induction of callus and regeneration depends on the flower bud size with appropriate stage, genotypes and as well as pre-treatments. By studying these factors, we can improve the responsiveness of both genetically high and low responsive genotypes. Therefore, further investigations are needed to standardise the *in vitro* gynogenesis protocol with efficient regeneration to generate successful double haploid plants in cocoa for its genetic improvement.

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References

- [1] Afoakwa EO. Chocolate science and technology. John Wiley and Sons. 2016, PP 1-550
- [2] Food and Agriculture Organization. FAOSTAT (crop visualize data set). Retrieved 02 August 2017, from <http://www.fao.org/faostat/en/#data/QC/visualize>
- [3] Ratnam R. Introduction of Criollo cacao into Madras state. South Indian Horticulture, 1961, 9(4), 24-29.
- [4] Directorate of Cashewnut & Cocoa Development. Production scenario of Cocoa. Retrieved 02 August 2017, from <http://www.dccd.gov.in>
- [5] Dublin P. The haploids of *Theobroma cacao* L.: their possible utilization for improving and studying the genetics of the cacao tree. In Proc. First. Int. Symp. Guelph, 1974, (Vol.6, pp. 10-14).
- [6] Dublin P. Polyembryonie et haploïdie chez *Theobroma cacao*. Café Cacao Thé, 1972, 16(4), 295-310.
- [7] Alemanno L, Berthouly M and Michaux-Ferrière N. Histology of somatic embryogenesis from floral tissues cocoa. Plant Cell, Tissue and Organ Culture, 1996, 46(3), 187-194.
- [8] Santana M, Velasquez S and Mata J. Carbon source effect on cacao organogenesis and somatic embryogenesis. Cytogenetic analysis. Agronomía Tropical (Maracay), 2010, 60(2), 193-202.
- [9] Li Z, Traore A, Maximova S and Guiltinan, MJ. Somatic embryogenesis and plant regeneration from floral explants of cacao (*Theobroma cacao* L.) using thidiazuron. In Vitro Cellular & Developmental Biology-Plant, 1998, 34(4), 293-299.
- [10] Tan CL and Furtek DB. Development of an *in vitro* regeneration system for *Theobroma cacao* from mature tissues. Plant Science, 2003, 164(3), 407-412.
- [11] Traore A and Guiltinan MJ. Effects of carbon source and explant type on somatic embryogenesis of four cacao genotypes. HortScience, 2006, 41(3): 753-758.
- [12] Quainoo AK, Wetten AC and Allainguillaume J. The effectiveness of somatic embryogenesis in eliminating the cocoa swollen shoot virus from infected cocoa trees. Journal of virological methods, 2008, 149(1), 91-96.
- [13] Niemenak N, Saare-Surminski K, Rohsius C, Ndoumou DO and Lieberei R. Regeneration of somatic embryos in *Theobroma cacao* L. in temporary immersion bioreactor and analyses of free amino acids in different tissues. Plant cell reports, 2008, 27(4), 667-676.
- [14] Minyaka E, Niemenak N, Sangare A and Omokolo DN. Effect of MgSO₄ and K₂SO₄ on somatic embryo differentiation in *Theobroma cacao* L. Plant Cell, Tissue and Organ Culture, 2008, 94(2), 149-160.
- [15] Noah AM, Niemenak N, Sunderhaus S, Haase C, Omokolo DN, Winkelmann T and Braun HP. Comparative proteomic analysis of early somatic and zygotic embryogenesis in *Theobroma cacao* L. Journal of proteomics, 2013, 78, 123-133.
- [16] Ajijah N, Hartati RS, Rubiyo R, Sukma D and Sudarsono S. Effective cacao somatic embryo regeneration on kinetin supplemented DKW medium and soma clonal variation assessment using SSR markers. Agrivita, 2016, 38(1), 80-92.
- [17] Germana MA and Chiancone B. Gynogenetic haploids of Citrus after *in vitro* pollination with triploid pollen grains. Plant Cell, Tissue and Organ Culture, 2001, 66(1), 59-66.
- [18] Blasco M, Badenes ML and del Mar Naval M. Induced parthenogenesis by gamma- irradiated pollen in loquat for haploid production. Breeding science, 2016, 66(4), 606-612.
- [19] Mishra VK and Gowswami R. Haploid production in higher plant. International journal of chemical and biological sciences, 2014, 1, 26-45.

- [20] Bohanec B, Jakse M, Ihan A and Javornik B. Studies of gynogenesis in onion (*Allium cepa* L.): induction procedures and genetic analysis of regenerants. *Plant Science*, 1995, 104(2), 215-224.
- [21] Luthar Z and Bohanec B. Induction of direct somatic organogenesis in onion (*Allium cepa* L.) using a two-step flower or ovary culture. *Plant cell reports*, 1999, 18(10), 797-802.
- [22] Kobayashi RS, Sinden SL and Bouwkamp JC. Ovule culture of sweet potato (*Ipomoea batatas*) and closely related species. *Plant cell, tissue and organ culture*, 1993, 32(1), 77-82.
- [23] Tang F, Tao Y, Zhao T and Wang G. In vitro production of haploid and doubled haploid plants from pollinated ovaries of maize (*Zea mays*). *Plant cell, tissue and organ culture*, 2006, 84(2), 233-237.
- [24] Gurel S, Gurel E and Kaya Z. Doubled haploid plant production from unpollinated ovules of sugar beet (*Beta vulgaris* L.). *Plant Cell Reports*, 2000, 19(12), 1155-1159.
- [25] Gemes-Juhász A, Balogh P, Ferenczy A and Kristof Z. Effect of optimal stage of female gametophyte and heat treatment on in vitro gynogenesis induction in cucumber (*Cucumis sativus* L.). *Plant Cell Reports*, 2002, 21(2), 105-111.
- [26] Sibi ML, Kobaissi A and Shekafandeh A. Green haploid plants from unpollinated ovary culture in tetraploid wheat (*Triticum durum* Desf.). *Euphytica*, 2001, 122(2), 351-359.
- [27] Chen JF, Cui L, Malik AA and Mbira KG. In vitro haploid and dihaploid production via unfertilized ovule culture. *Plant Cell, Tissue and Organ Culture (PCTOC)*, 2011, 104(3), 311-319.
- [28] Lloyd G and McCown B. Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. 1980, 30, 421-427.
- [29] Murashige T and Skoog F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia plantarum*, 1962, 15(3), 473-497.
- [30] Base S. 9.3 Procedures Guide: Statistical Procedures SAS Institute Inc. Cary, NC, 2011.
- [31] Driver JA and Kuniyuki AH. In vitro propagation of Paradox walnut root stock. *Hortscience*, 1984, 19: 507-509.
- [32] Santana M, Velasquez S and Mata J. (2010). Carbon source effect on cacao organogenesis and somatic embryogenesis. Cytogenetic analysis. *Agronomía Tropical (Maracay)*, 2010, 60(2), 193-202.
- [33] Alemanno L, Ramos T, Gargadenec A, Andary C and Ferriere N. Localization and identification of phenolic compounds in *Theobroma cacao* L. somatic embryogenesis. *Annals of botany*, 2003, 92(4), 613-623.
- [34] Wu CH. In vitro culture and embryological studies on unfertilized ovules in Orchidaceae (Doctoral dissertation, Chinese academy of sciences), 2003.
- [35] Mukhambetzhonov SK. Culture of non-fertilized female gametophytes in vitro. *Plant Cell, Tissue and Organ Culture*, 1997, 48(2), 111-119.
- [36] Campion B and Alloni C. Induction of haploid plants in onion (*Allium cepa* L.) by in vitro culture of unpollinated ovules. *Plant cell, tissue and organ culture*, 1990, 20(1), 1-6.
- [37] Klein M and Korzonek D. The relationship between flower size and developmental stage of *Allium cepa* L. umbels. *Acta Biologica Cracoviensia Series Botanica*, 1999, 41, 185-192.
- [38] Michalik B, Adamus A and Nowak E. Gynogenesis in Polish onion cultivars. *Journal of plant physiology*, 2000, 156(2), 211-216.
- [39] Smith BM, Godwin RM, Harvey E and Werner CP. Gynogenesis from whole flower buds in bulb onions (*Allium cepa* L.) and leeks (*Allium porrum* L.). *Journal of Genetics and Breeding*, 1991, 45(4), 353-357.
- [40] Shalaby TA. Factors affecting haploid induction through in vitro gynogenesis in summer squash (*Cucurbita pepo* L.). *Scientia horticultrae*, 2007, 115(1), 1-6.
- [41] Bhagyalakshmi, N. Factors influencing direct shoot regeneration from ovary explants of saffron. *Plant cell, tissue and organ culture*, 1999, 58(3), 205-211.
- [42] Bhat JG and Murthy HN. (2007). Factors affecting in-vitro gynogenic haploid production in niger (*Guizotia abyssinica* (L. f.) Cass.). *Plant growth regulation*, 2007, 52(3), 241-248.

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