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Assessment of the effects of physiological development of cocoa (*Theobroma cacao l.*) explant on somatic embryogenesis

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Abstract

Cocoa trees have shown a high degree of segregation for many traits when propagated by seeds. Somatic embryogenesis is an efficient *in vitro* propagation method which allows the production of several embryos capable of generating plants similar to the initial one from somatic tissues. The use of cocoa floral parts has been reported for regeneration of elite cocoa genotypes. This research is targeted in evaluating the effect of physiological development of the explants (staminode) and its response to two different cytokinase (kinetin and BAP) on embryogenesis. The experiment was laid in factorials with three replications in CRD. Three different physiological ages of the explant were examined: 1 week old (unopened, about 3-4 mm in length),2 weeks old (unopened, about 5-/6 mm in length) and 3 weeks old (unopened; matured flowers). Staminode was studied and explants were initiated for callus induction on Primary Callus Growth medium. The following data were scored for: Explants Induction Percentage, Percentage of callus induced and Percentage of Embryogenic callus. Results showed the interactions among the genotypes, hormones and the physiological age of the explants were significantly different at 0.05% probability level. Explants at 3 and 2 weeks respectively had higher efficiency for embryogenesis while the average performance was observed for explants at a week. Also, BAP recorded higher frequency 80% for embryogenesis compared to kinetin 70% under the present study. Physiological age of explants and the choice of callus development hormone have been found to play significant role in the embryogenesis of cocoa genotypes examined.

Keywords: *Theobroma cacao*; Somatic embryogenesis; Explants; Physiological; cytokinase; staminodes and segregation

1. Introduction

Theobroma cacao L. (chocolate tree) is grown in the humid tropics and constitutes an important source of incomes for many countries of the West and Central Africa regions. *Cacao* trees are predominantly propagated by seedlings, from seeds selected by farmers from their own materials (Chaidamsari*et al*., 2005). The seeds used for propagation are selected to be uniform of good quality and from seed derived clones. Propagation by seeds results in a high level of heterogeneity of the crop, and genetic variation of low yielding trees. Vegetative propagation is essential to produce true to type trees. Budding and the use of rooted cuttings are common practice throughout the cocoa growing regions (Eskes B., 2001). Propagation of *cacao* trees by rooted cuttings involves the use of orthotropic and plagiotropic shoots. Orthotropic (chuppons) shoots produce cocoa clones with the same morphology as seed derived trees, but only small quantity can be sourced.

Cacao breeding usually takes a long time and this is because of its long-life cycle and the narrow genetic background (Brown *et al*., 2007). Utilizing plant tissue culture technology is expected to accelerate constrain in achieving *cacao*

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improvement programs. Plant tissue culture relies on the fact that many plant cells have the ability to regenerate to a whole plant (totipotency).The flowers are hermaphroditic, with small sizes (diameters ranging from 0.5 to 1cm), regular and composed of 5 sepals, 5 petals, 5 staminodes, a pistil and an ovary. Pollination is predominantly entomophilous although it can be done manually in the experimental fields (Rodrigue *et al*., 2016). Flowering in cocoa is manifested by the production of a minimum of 50 000 flowers during the term with less than 5% of production pods (Lass, 1999).

Somatic embryogenesis is an efficient *in vitro* method for regenerating plantlets because it has a high multiplication rate. *In vitro* plantlet regeneration via somatic embryos has been developed in many plants' species, such as coffee (Ibrahim *et al.,* 2012; Ibrahim *et al.,* 2013a; Ibrahim *et al.*, 2013b), soybean (Widoretno*et al.*, 2003a) and one of the rare Indonesian medicinal plants (purwoceng - *Pimpinellapruatjan*Molk.) (Ajijah*et al.*, 2010). The present study was to examine the effect of physiological development of explants and the effect of hormone (for callus development) on the cocoa embryogenesis.

2. Materials and Methods

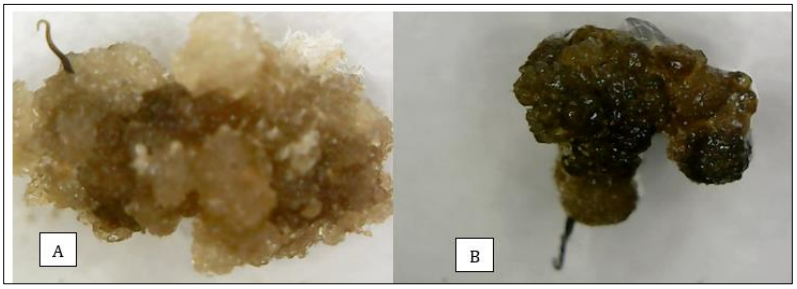
Five *cacao* genotypes (TC-1 (G5), TC-5 (G9),TC-8 (G12), Spec 54-1 (G2) and PA150 (G3)) were used. The *cacao* genotypes were tagged at the point of flower initiation, harvested for in-vitro culture initiation at: 1week, 2weeks and 3weeks respectively. A floral part (staminode) was studied. The experiment was laid in factorials with three replications in CRD. Three different physiological ages of the explants were examined: 1 week old (unopened, about 3-4 mm in length), 2 weeks old (unopened, about 5-/6 mm in length) and 3 weeks old (unopened; matured flowers).The explants were initiated (for callus induction) on PCG medium for all the ages of the flower for the period of fourteen days; respectively for the five genotypes under study. The callus produced were then transferred into two different Secondary Callus Growth medium (SCG: containing 1mg/ml kinetin and 1mg/ml BAP respectively) for fourteen days and Embryo Development (ED) medium for four weeks with daily maintenance on the media. SCG medium containing kinetin was prepared according to the recipes in the protocol manual (Penn.,2010), with filter-sterilized kinetin into an autoclaved medium after being cooled to a temperature of about 60^0C in a laminar-flow hood. The 0.2um filter was used. This was done to prevent the denaturing of kinetin during autoclaving.

2.1 Data collection

- Explants Induction Percentage was scored at twenty-four hours after explants initiation on induction medium, PCG
- Percentage of callus induced was scored from three to fourteen days after explants initiation on both the PCG and SCG (kinetin and BAP) medium respectively
- Percentage of Embryogenic callus was evaluated at 2 weeks to 4 weeks after initiation on ED medium

2.2 Data Analysis

Data were analysed using means and standard errors (S.E.M.) and statistical significance among values were assessed using ANOVA at a probability of B/0.05.



A = embryogenic callus developed on SCG (with BAP hormone); B = embryogenic callus developed on SCG (with Kinetin hormone)

Figure 1 Embryogenic callus of the *cacao* staminode tissue

3. Results

Table 1 showed the mean squares of inductions and callus formation of the five *cacao* genotypes (in staminode tissues) in three different physiological ages. Staminode tissues at 2 weeks of age showed significant differences among the five *cacao* genotypes tested for the explants induction at three days after initiation. Also, callus formation was significantly

different among the *cacao* genotypes at 3AI and 7AI for the physiological age at week1 and 2 respectively. In Table 2, there were significant differences among the genotypes, hormones, physiological age, and their interactions for the embryogenic callus formation for the five *cacao* genotypes tested. Table 3 revealed means of the embryogenic callus of the *cacao* genotypes in different hormones at different physiological age. The highest mean for embryogenic callus was observed in G3 at two weeks (81.39%) and four weeks (83.88%) while G12 had the least mean at two weeks (66.67%) and four weeks (67.22%) respectively. Also, BAP had the highest mean for embryogenic callus (83%, 83.89%) while the least mean was recorded for kinetin (71.22%, 73.22%) at two and four weeks respectively. For the physiological age of the floral explant (staminode), highest mean was recorded at week 3(99.50%, 100%), followed by week 2 (79.33%, 81.67%) while week 1 had the least mean (52.50%, 54%) at two and four weeks respectively. The interactions among the genotypes, hormones and the physiological age of the staminodes were significantly different at 0.05% probability level.

Table 1 Mean squares of induction and callus formation of the five *cacao* genotypes (in staminode tissues) in three different physiological ages

Treatment		**Induction**	**Callus formation**		
Source	**DF**	**3AI**	**C3AI**	**C7AI**	**C8AI**
1 week Genotype	4	40.42^{ns}	186.67**	563.55^{ns}	36.67^{ns}
Error	25	22.33	59.50	332.39	18.00
2 weeks					
Genotype	4	115.42**	123.75^{ns}	42.92**	11.25^{ns}
Error	25	18.33	111.67	12.33	7.67
3 weeks					
Genotype	4	40.42^{ns}	142.92^{ns}	5.42^{ns}	2.08^{ns}
Error	25	26.33	123.00	23.83	13.17

** Significant at P <_ 0.05; Ns = Non significant; AI = After initiation; C = Callus

Table 2 Mean squares of the embryogenic callus of the cacao genotypes and its response in two different hormones at different physiological age

Treatment		**Embryogenic callus**	
Source	**DF**	**2 Weeks**	**4Weeks**
Genotype(G)	4	633.06**	759.31**
Hormone(H)	1	3121.11**	2560.00**
PHYAGE	2	16678.61**	16087.78**
G*H	4	287.78**	317.64**
G*PHYAGE	8	458.47**	513.47**
H*PHYAGE	2	846.94**	693.33**
G*H*PHYAGE	8	478.19**	528.06**
Error	58	104.47	111.48

** Significant at P <_ 0.05; PHYAGE = Physiological age; 2 weeks = Embryogenic callus at 2 weeks; 4 weeks = Embryogenic callus at 4 weeks

Table 3 Means of the embryogenic callus of the cacao genotypes in different hormones at different physiological age

Treatment	**Embryogenic callus**	
Genotype(G)	**2 Weeks**	**4 Weeks**
G2	78.61a	79.44a
G3	81.39a	83.33a
G5	79.72a	81.94a
G9	79.17a	80.83a
G12	66.67b	67.22b
LSD	6.82	7.05
Hormones(H)		
BAP	83.00a	83.89a
Kinetin	71.22b	73.22b
LSD	4.31	4.46
PHYAGE		
1 week	52.50c	54.00c
2 weeks	79.33b	81.67b
3 weeks	99.50a	100.00a
LSD	5.28	5.46
G*H	**	**
G*PHYAGE	**	**
H*PHYAGE	**	**
G*H*PHYAGE	**	**
CV%	13.26	13.44

** Significant at P <_ 0.05; PHYAGE = Physiological age; BAP = Benzyl Amino Purine; Means with the same letter along the column are not significantly different at 5% level of probability. LSD = Least Significant Difference, CV% = Percentage of Coefficient of Variation

4. Discussion

Inspite of differences in the physiological age of the explants across the genotypes tested, over 90% induction within three days of culture initiation. Also, the development of the callus was not different from one another. Hence, explants at different physiological developments had a great potential for explants induction and callus development for the genotypes under study. Differentiation of the developed calli into embryogenic callus was different among the physiological development of the explants examined. Explant at 3 weeks gave the best frequency of embryogenesis (100%), followed by explants at 2 weeks (over 80%) while the explants at a week gave the least frequency (over 50%). Thus, explants at 3 and 2 weeks respectively had a higher efficiency for embryogenesis while the average performance was observed for explants at a week; for the genotypes examined. This was in line with the study of (Maximova *et al*.,2002 and Tan *et al*., 2002).

The assessment of the response of two different cytokinins (Benzyl Amino Purine (BAP) and Kinetin) on callus development revealed the better performance of BAP over kinetin across the three physiological developments of the explants examined. Though; the two hormones had a great potential in the callus development of the *cacao*; but BAP recorded higher frequency (over 80%) for embryogenesis compared to kinetin (over 70%). Among the five genotypes examined under this study, G12 had the least response for embryogenesis (over 60%) while the remaining genotypes were not significantly different from one another in embryogenic response (over 80%).

5. Conclusion

Physiological age of explant and the choice of callus development hormone have been found to play a significant role in the embryogenesis of cocoa genotypes examined. This could help in an effort to advance the improvement of *T. cacao* for further breeding research program.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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