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SHORT COMMUNICATION

Pro-embrionary Somatic Structure of Three Cacao Genotypes (*Theobroma Cacao* L.) Using Staminodes

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ABSTRACT

Staminodes of three cocoa genotypes (BT1, ICS-39 and CCN-51), were used to perform the characterization of somatic embryos induced and maintained by DKW culture medium enriched with 2% of sucrose, 10% of coconut water and 0.01% of cysteine, 2,5 mg/l 2,4 D, 5 µg/lTDZ respectively. The ANOVA and the comparison mean test, shown significant differences between globular-, heart-, torpedo- and cotyledonal-stages for each genotype. Additionally, the embryogenic stages and regenerants plants proportions were highest for Blanco Tarapotino 1 (BT1) genotype. A morphological comparison of somatic and zygotic embryos shows similarities in form and symmetry.

Keywords: Tissue culture, tropical crops, in vitro propagation, micro propagation

1. Introduction

Genera *Theobroma* its restricted and typical of neotropical regions, sheltering 22 species within its genus [1]. The most representative species is cacao (*Theobroma cacao* L.), due its importance in chocolate industry and content of polyphenols. Cocoa is also defined as a functional product because of its beneficial effects on cardiovascular diseases and high levels of antioxidants [2].

In Peru, cocoa production has been registered from the beginning of the XXI century, reaching global market visibility from 2012. Within the country, cacao cultivation is a main source of economic income. San Martin region is considered and recognized as a fine bean producing area in the country [3]. Productive areas from San Martin are being cultivated with commercial clones for more than 10 years. Nevertheless, the search for new genotypes with optimal characteristics for the region is carried constantly, therefore experimental station "Juan Bernito" from the Tropical Crops Institute-ICT in Peru, holds a collection of more than 900 cacao clones that are being selected, based in productivity and disease resistance. In our country, there is no clonal propagation protocol of promising cacao genotypes, much more for our region, with ideal genotypes in terms of disease resistance and good organoleptic characteristics, which can be competitive in national and international markets in terms of quality and quantity.

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Through the somatic embryogenesis technique, it is possible to obtain a large number of plants in a relatively short time, even more maintaining the genetic characteristics of the plants from which they come. For this reason, three cacao genotypes were evaluated in a basic culture medium supplemented with 2,4 D and TDZ, in order to induce the formation of embryogenic structures and to evaluate the potential to grow in vitro of each genotype, in order to cultivate them and later in the field.

Genetic characteristics of cacao are conserved via vegetative propagation, the most used technique is grafting, where productive clone is placed in a resistant genotype. Conservation of genetic material in experimental fields, generates high maintenance cost, while in vitro conservation by tissue culture has differentiated means of cost and space. Organogenesis and somatic embryogenesis are two techniques addressed in tissue culture [4]-[5], and particularly in cacao, evidence exists for organogenesis [6,7,8,9] and somatic embryogenesis from zygotic embryos [10]-[15]. Unlike zygotic embryos, the use of non-sexual somatic tissues such as nucella, leaf or floral tissues have major importance in somatic embryogenesis [4,5,8], developed a procedure to stimulate somatic embryogenesis and plant regeneration from floral cacao tissues (staminodes) using three stages of cultivation (callus induction, embryo development and plant regeneration), in combination with the use of 2,4-D (2,4-Dichlorophenoxyacetic acid) and TDZ (Tidiazuron). Based on previously mentioned somatic embryogenesis studies, we were interested in inducing this procedure in three cacao genotypes, aiming at the characterization and quantification of the pre-embryonic stages (globular, heart, torpedo and cotyledon) and the frequency of regenerants for each genotype. Complementarily, we compared two cotyledon stages, the somatic embryo and the zygote in one of the studied cacao genotypes.

2. Materials and Methods

The experiment was conducted at the Tissue Culture Laboratory of the Instituto de Cultivos Tropicales - ICT in Tarapoto, San Martín – Peru. For the experiment, floral buds and seeds of three cacao genotypes "BT1 (White Tarapotino 1)", "ICS-39 (Imperial Collage Selection)" and "CCN-51 (Colección Castro Naranjal)" (Table 1), were collected from the Germplasm bank of the "Juan Bernito" experimental station of the INstituto de Cultivos Tropicales - ICT in Tarapoto, San Martín – Peru.

Table 1: *Evaluated cacao clones characteristics*

Characteristics	Genotypes		
	BT1	**ICS-39**	**CCN-51**
Reaction to Frosty Pod	MR	S	MR
Compatibility	SI	SI	SC
Fruit color	Yellow	Yellow	Red-Orange
Apex fruit form	Obtuse	Acute	Obtuse
Seeds per Fruit	37	39	48
Seed shape	Ovoid	Ovoid	Elíptic
Seed color	White	purple	purple
Bean Index	1,0	2,2	1,5
Pod Index	30	13	15
Flower color	White	cream	Pink

MR=Moderately Resistant, S=Susceptible, SI=Self incompatible, SC=Self compatible

Floral buds (immature flowers) of three cacao genotypes were collected in flasks containing distilled water, to keep them hydrated and fresh and least 30 minutes after had taken them to the tissues culture laboratory for preparation, treatment, surface sterilization and establishment "in vitro". The floral buds were sterilized in a laminar flow camera with 1% sodium hypochlorite for 15 minutes, washed with sterile distilled water until residue from the disinfectant was removed. The flowers were dissected to obtain the staminodes, which were the explants inoculated into the culture medium for induction to somatic embryogenesis. The explants were maintained in different culture media for 22 days intervals. Somatic and zygotic embryos in the cotyledonary state were planted in glutaraldehyde for storage and evaluation.

The design used in the experiment, to induce somatic embryos, was the completely randomized design (CRD) with 5 replicates per treatment. The evaluated pre-embryonic stages (globular, heart, torpedo, cotyledonary) and regenerants were the variance (ANOVA) was

analyzed the medias compared with Tukey test (P <0.05).

Staminodes of immature floral cacao tissues were placed on modified PCG medium (grow of inmature calluses), containing DKW mineral salts [16], enriched with 2% sucrose + 10% coconut water, 10mg / l Cysteine + 2.5 mg / l 2.4 D, 5 Âµg / l TDZ and pH = 5.7. The incubation of the staminodes was done according to the protocol described by Guiltinan & Maximova [17], modified. Briefly, tissues were maintained in ED medium (embryo development: mineral salts and vitamins DKW + 3% sucrose + 0.1% glucose, hormone free) and PEC (Primary Growth Embryos: mineral salts and vitamins DKW + 300 mg / L potassium nitrate + amino acids + 1% sucrose + 2% glucose, hormone free), maintained at 22 day intervals for four months. After 88 days of culture, embryonic structures for each genotype were visualized and quantified by the Leica 2000 stereoscopic microscope.

3. Results and Discussion

The Analysis of Variance (ANOVA) shows for pre-embryonic and regenerating stages for the three cacao genotypes studies (Table 2). All phases presented significative differences between genotypes. Mean comparison by Tukey test ($p<0,05$) suggests that "Blanco Tarapotino 1-BT1" presented higher number of pre-embryonic stages (globular, heart, torpedo and cotyledonic) and regenerating frequencies as well (Table 3). In contrast, the ICS-39 genotype behaves differently due it`s lack of response. The results suggest that staminodes of the BT1 genotype are more responsive to the formation of embryonic structures than the other genotypes studied. The variation of the embryogenic response linked to the cocoa genotype, during the formation of embryonic and pre-embryonic structures, has been suggested by other authors [5,8,17]. Recent studies have shown that the transitional factor BABY BOOM (BBM) of cacao (TcBBM; Tc05_t019690), could probably, regulate the development of the embryonic stages of somatic and zygotic embryogenesis [19]. The cotyledonary stages obtained by somatic embryogenesis in BT1 and CCN-51 genotypes are similar to zygotic structures (Figure 1A-D), maintaining similar shape and symmetry (Figure 1).

Table 2: *Analysis of Variance (ANOVA)for cacao genotypes with respect to the four types of somatic and regenerating embryos.*

Mean Squares						
Source of variation	**Df**	**Somatic embryo type (%)**				**Regenerating (%)**
		Globular	**Heart**	**Torpedo**	**Cotyledonic**	
Genotypes	2	1621.7*	647.04*	1635.9*	558.88*	735.11**
Experimental Error	9	182.69	97.01	84.04	141.68	158.03
C.V.%		58.6	72.42	39.39	87.52	91.15
R^2 %		66	60	81	47	51

** Highly significant, * Significative

Table 3*: Percentage of four somatic and regenerating embryos*

Genotype	**Somatic embryo type (%)**				**Regenerating (%)**
	Globular	**Heart**	**Torpedo**	**Cotyledonic**	
BT1	36.4^{a*}	9.5^{a}	36.9^{a}	17.5^{a}	26.4^{a}
ICS-39	0.0^{c}	0.0^{c}	0.0^{c}	0.0^{c}	0.0^{c}
CCN-51	34.7^{b}	20.1^{b}	30.5^{b}	14.6^{b}	8.1^{b}

*Different letters in column represent significant differences by Tukey test ($p<0,05$)

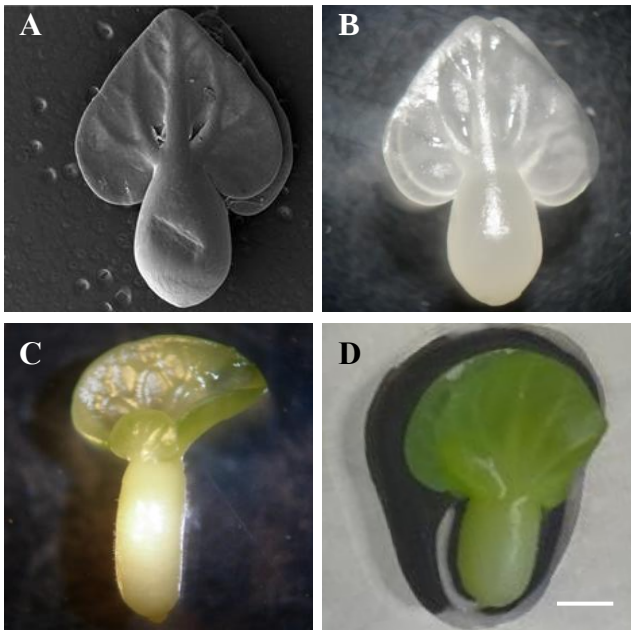

Figure 1*: Cotyledon stage of zigotic and somatic embryos. Zigotic embryo of BT1 genotype (A), and CCN-51 (B). Somatic cotyledon embryo of genotype BT1 (C), and CCN-51 (D). Line = 1 mm.*

4. Conclusions

The cotyledonary stages obtained by somatic embryogenesis in BT1 and CCN-51 genotypes are similar to zygotic structures and maintaining similar shape and symmetry. The knowledge of these characteristics will allow evaluating the embryogenic capacity of one or another elite cocoa, which allows us to continue the study. In addition to this, we can say that the staminodes are suitable for obtaining somatic embryos of cacao.

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