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CALLUS INDUCTION FROM FLORAL EXPLANTS OF CUPUASSU[1]

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ABSTRACT - There are few studies related to the *in vitro* cultivation of plants from the*Theobroma* genus and no effective micropropagation protocols for *T.grandiflorum*. The aim of this study was to evaluate the calli formation in cupuassu floral explants, targeting their organogenic or embryogenicdevelopment. Experiments were conducted in the Plant Tissue Culture Laboratory of EMBRAPA, Porto Velho, Rondônia, Brazil. Floral parts from unopened immature flower buds taken from seedless cupuassu trees were sterilized and employed as a source of explants. These explants were cultivated in Petri dishes in an induction medium consisting of MS salts and vitamins, supplemented with glycine(3 mg.L^{-1}), lysine (0,4 mg.L^{-1}), leucine (0,4 mg.L^{-1}), arginine (0,4 mg.L^{-1}), tryptophan (0,2 mg.L^{-1}), 2,4-D (1 mg.L^{-1}), kinetin (0,25 mg.L^{-1}), coconut water (50 ml.L^{-1}), sucrose (40 g.L^{-1}), Gelrite (2,2 g.L^{-1}) and pH adjusted to 5,8. Cultures were maintained in the dark for 3 weeks at 27°C and then subcultured for six weeks in medium without growth regulators supplemented with glycine (1 mg.L^{-1}), lysine (0,2 mg.L^{-1}), leucine (0,2 mg.L^{-1}), arginine (0,2 mg.L^{-1}), tryptophan (0,1 mg.L^{-1}), coconut water (100 ml.L^{-1}), sucrose (40 g.L^{-1}), Gelrite (2,2 g.L^{-1}) and pH 5,8. We used a completely randomized design with 10 replications of 5 explants per plate and four different explant sources: staminode, petal, ligule and ovary. As a result, we obtained a highercalli formation in theinduction medium when ovaries were used as source of explants.However, there was no development of somatic embryosor organogenic response in medium without growth regulators and further studies are being conducted.

Keywords: Micropropagation. Calogenesis. *Theobromagrandiflorum.*

INDUÇÃO DE CALOS DE EXPLANTES FLORAIS DE CUPUAÇU

RESUMO -Existem poucos estudos relacionados ao cultivo *in vitro* de plantas do gênero *Theobroma*, e nenhum protocolo eficiente de micropropagação de *T. grandiflorum.*Neste trabalho objetivou-se avaliar a calogêneseem explantes florais de cupuaçu, visando seudesenvolvimento embriogênicoou organogênico.Os experimentos foram conduzidos no Laboratório de Cultura de Tecidos Vegetais da Embrapa Rondônia, em Porto Velho, Rondônia. Partes florais de botões imaturos, não abertos, foram coletadas de árvores de cupuaçuzeiros sem sementes, desinfestadas e utilizadas como fonte de explantes. Osexplantes foram cultivados em placas de Petri em meio de indução, consistindo de meio MS e vitaminas, suplementado com glicina (3 mg.L^{-1}), lisina (0,4 mg.L^{-1}), leucina (0,4 mg.L^{-1}), arginina (0,4 mg.L^{-1}), triptofano (0,2 mg.L^{-1}), 2,4-D (1 mg.L^{-1}), cinetina (0,25 mg.L^{-1}), água de coco (50 mL.L^{-1}), sacarose (40 g.L^{-1}) eGelrite (2,2 g.L^{-1}), pH ajustado para 5,8. As culturas foram mantidas no escuro por três semanas a 27°C e, em seguida,subcultivadas por seis semanas em meio sem reguladores de crescimento, suplementado com glicina (1 mg.L^{-1}), lisina (0,2 mg.L^{-1}), leucina (0,2 mg.L^{-1}), arginina (0,2 mg.L^{-1}), triptofano (0,1 mg.L^{-1}), água de coco (100 mL.L^{-1}), sacarose (40 g.L^{-1}) e Gelrite (2,2 g.L^{-1}),pH 5,8.Empregou-se um delineamento inteiramente casualizado com 10 repetições de5 explantes por placa e quatro diferentes fontes de explantes: estaminódios, pétalas, lígulas e ovários.Como resultado, foi obtida uma alta formação de calos, no meio de indução, quandoovários foram utilizados como fonte de explantes. Porém, não houve desenvolvimento de embriões somáticos ou resposta organogênicano meio sem reguladores de crescimento e maiores estudos estão sendo conduzidos.

Palavras-chave: Micropropagação.Calogênese.Cupuaçu.

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INTRODUCTION

The cupuassu (*Theobromagrandiflorum*Schum.) is one of the most attractive amazonian fruit trees due to the flavor characteristics and aroma of its pulp, that is used in the commercial production of juices, ice creams, liquours, compotes, jellies, creams, sweets, etc (CALZAVARA et al; 1984; VENTURIERI et al; 1985). However, there are many agricultural problems related with cupuassu crop, such as the high susceptibility to witch broom (*Crinipellisperniciosa*) and the little period of fruit storage. Research institutions, in the Northern Region of Brazil, have implemented breeding programs emphasizing the selection of materials with high fruit production characteristics, high pulp yield and resistance to witch broom.The Codajás, Manacapuru, Belém and Coari clones of this species are the result of a selection work developed by Embrapa (Brazilian Agricultural Research Corporation) from a group of plants collected in northern Brazil in the 80's, due to their high productivity and tolerance to *C. perniciosa* (CRUZ; ALVES, 2002).

These problems can be minimized with the use of*in vitro* propagation, because this technique allows the generation of propagules without phytopathogens. Moreover, viamicropropagation, it is possible to obtain alarger number of plantules in a shorter period of time, when compared with traditional vegetative propagation.The micropropagation can make the breeding programs faster and therefore there is a great benefit in *in vitro* cloning of this material.

Callus induction is one of the most utilizedtechniques to rescue entire populations ofinduced mutants, from somaclonal variation ortransgenic production. The establishment of thesepopulations results on the development of newcultivars. For this, studies of diverse aspects such as kind ofexplant, medium composition, light and temperatureof incubation, involved on the calli growth and plantregeneration are needed (SANTOS et al., 2005;PINTO; LAMEIRA, 2001).The regeneration can be defined as a processof vegetative multiplication that results on thegeneration of an entire plant from only one cell (BELTRÃO et al., 2008). The determination of the calli cell division index can promote the understanding of these physiological changes and assisting in the protocols optimization for genetic transformation and regeneration(STEINet al., 2010).

Calli can be multiplied by successive subcultures and can be maintained *in vitro* for long periods of time, being important for morphogenetic studies *in vitro* and for obtainment of cell suspensions, secondary products including medicines, calli formation and multiplication presenting a great scientific and commercial interest (RODRIGUES; ALMEIDA, 2010).

Studies have been carried out onmicropropagation of cupuassu (RODRIGUES,2000; FERREIRA et al., 2001; FERREIRA et al., 2002; LEDO et al., 2002;VENTURIERI;VENTURIERI, 2004; FERREIRA et al., 2005; SILVA et al. (2006); CARDOSO et al. (2006); RAMOS et al., 2012a, b, c and d; RODRIGUES et al. 2012 a e b). However, an efficient protocol has not been achievedyet.Due to botanical proximity with cocoa, a protocol for the *T. grandiflorum*micropropagation has been adapted, based on researches done with *T. cacao*, testing floral parts as explants.The objective of this study was to evaluate the callus formation from flower explants of cupuassu, aiming to facilitate the development in embryos or organogenesis.

MATERIAL AND METHODS

Unopened immature flower buds with length of 1,0±0,5 cm were taken from seedless cupuassu trees on EmbrapaRondônia experimental field, Porto Velho, Rondônia, Brazil. The buds were conducted to Plant Tissue Culture Laboratory, where it was made a pre-cleaning, using a brush to remove the hairs, followed by washing with sponge, distilled water and some drops of commercial detergent and immersion in 70% alcohol (v / v) for 1 minute. In a horizontal laminar flow, the flower buds were removed from alcohol solution and immersed in a 0,125% (active chlorine) sodium hypochlorite solution for 20 minutes and then washed 3 times with sterile bidistilled water. Then, the explants were segmenteds in petal, staminode, ligule and ovary (Figure 1).

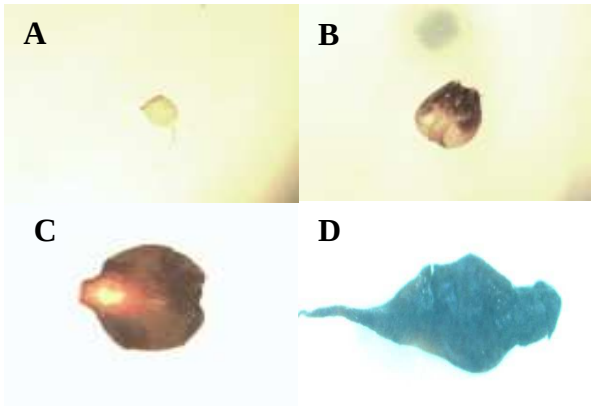

Figure 1.Segments of cupuassu floral explants.**A**. Ovary. **B**. Ligule. **C**. Petal. **D**. Staminode.

These segments were immersed in anantioxidant solution for 10 minutes, containing ascorbic acid (100 mg) and citric acid (150 mg), dissolved in water (1 L) and filter sterilized (FERREIRA et al., 2009). These explants were then cultivated in Petri dishes in an induction medium (IM), adapted from LOPEZ-BAEZ et al. (1993), consisting of MS salts (MURASHIGE; SKOOG,1962) and vitamins, sup-

plemented with glycine (3 mg.L-1), lysine (0,4 mg.L -1), leucine (0,4 mg.L-1), arginine (0,4 mg.L-1), tryptophan (0,2 mg.L-1), 2,4-D (1 mg.L-1), kinetin (0,25 mg.L-1), coconut water (50 ml.L-1), sucrose (40 g.L-1), Gelrite (2,2 g.L-1) and pH adjusted to 5,8. After autoclaving, was it was added cefotaxime (100 mg.L-1) to the culture medium. Cultures were maintained in dark for 3 weeks at 27°C, being evaluated the presence or absence of callus, and then transferred to a medium without growth regulators, consisting of MS salts ((MURASHIGE; SKOOG,1962) and vitamins, supplemented with glycine (1 mg.L-1), lysine (0,2 mg.L-1), leucine (0,2 mg.L-1), arginine (0,2 mg.L-1), tryptophan (0,1 mg.L-1), coconut water (100 ml.L-1), sucrose (40 g.L-1), Gelrite (2,2 g.L-1) and pH 5,8, for 6 weeks. At the end of this period, the explants were evaluated for the formation of somatic embryos or organogenic response.The experimental design used was entirely randomly, being employed 10 replicates for each explant, each one containing five explants per plate. The means were compared using the Tukey test at 1% of probability.

RESULTS AND DISCUSSION

The results evaluated after 21 days indicated higher callus formation using ovary and ligule as explants source in the induction medium, 82 and 34%, respectively (Figure 2). Only 12% of staminode tested were able to form calli and the petals were not responsive to the procedures for induction. The results differ from those obtained by SILVA et al. (2006) with species of the *Theobroma* genus, which used floral parts and observed higher calogenesis in staminodes, and from RAMOS et al. (2012[d]) and RODRIGUES et al. (2012[b]), employing staminodes of cupuassu. With respect to other explants, the staminodes showed the calli formation from the first three weeks and the petals just from the second cultive medium, and the results were insignificant.The calli originated from ovaries were big, compact, yellow and with friable aspect. The other explants showed callus formation smaller,with white and bright aspect and without friable aspect (Figure3). In general, embryogeniccalli are recognized by the colour. The white-translucid or yellow parts are considered friable and have potential for formation of somatic embryos (GUERRA et al., 1999).

In the present study there was no formation of somatic embryos or organogenic response after 42 days in medium without growth regulators.LEDO et al. (2002) evaluated the morphogenetic responses from different cupuassu tree explants under various conditions of *in vitro* culture and also did not observed the formation of somatic embryos. The authors affirm that the absence of embryogeniccalli observed in cultures may be related to several factors such as type and development stage of explants, medium culture composition and type and growth regulators concentration. VENTURIERI & VENTURIERI (2004) evaluated the calogenesis of three different tissues obtained from *T. grandiflorum* x *T. obovatum* hybrid seeds and found that the cotyledons were the explants that produced more calli, when the dose of TDZ was lower (5 µg/l), and the media containing NAA and KIN presented evidences of effectiveness in the somatic embryos formation.

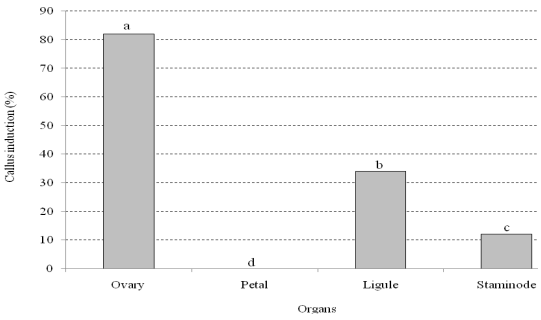

Figure 2.Percentages of calli induction in different types of *T. grandiflorum* floral explants after 21 days in induction medium.Means identified with the same letters do not differ according to the Tukey test at 1% of probability.

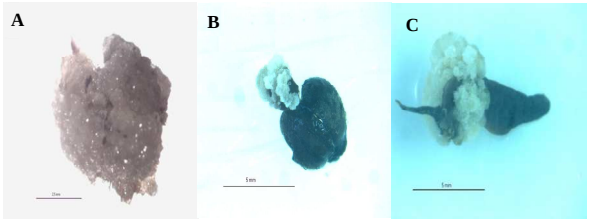

Figure3.Calogenesis in floral explants of cupuaçu after 21 days in induction medium (IM). **A**. Ovary. **B**. Ligule. **C**. Staminode.

Some studies have shown the ability of different cupuassu explants to form calli and then differentiate in embryogenic structures. RODRIGUES (2000) did not observed calli formation in cupuassu nodal segments in medium supplemented with different concentrations of 2,4-D.FERREIRA et al. (2001), evaluating the effectof auxin, observed that the combination of NAA and 2,4-D induced the rhizogenesis and calogenesisin hypocotyls segments of cupuassu. Also they observed that the presence of coconut water, in medium without growth regulators, promoted rhizogenesis and calogenesis. COSTA et al. (2008) studying the influenceof auxin upon different types ofexplantsin the induction of*Piper hispidinervum* callus, found that theaddition of NAAat concentrations of2.5and 5mgL-1 allowedthe highestpercentageof friable callus formation andthe type ofexplants andhad an strong influence onthis variable. STEIN et al. (2007) determined a protocol for the induction of callus in ovaries of *Inga vera* and observed that the MS medium supplemented with 4,5µM 2,4-D was the most indicated.

Studies related to the *Theobroma* genus are

still quite incipient and there are no conclusive results. Besides the literature about somatic embryogenesis in cupuassu is scarce and there is no report of a successful micropropagation protocol for cupuassu.Attempts with somatic embryogenesis just allowed obtainingembryogeniccalli that failed to produce viable seedlings (VELHO et al., 1990). FERREIRA et al. (2005) used cupuassu leaves for induction of somatic embryos and were able to detected using scanning electron microscopy structures with characteristics similar to somatic embryos in medium supplemented with TDZ. In the current study, higher calli frequency in ovaries suggests that these explants can be used to obtain an efficient propagation system, employing somatic embryogenesis.

CONCLUSION

The ovary was the most responsive explant for calli formation from cupuassu.

Future works should test different concentrations of 2,4-D as well as another source of auxin, using the ovary as explant.

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