CALLOGENESIS IN LEAF SEGMENTS OF *Mammea americana* L.

CALOGÊNESE EM EXPLANTES FOLIARES DE *Mammea americana* L.

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ABSTRACT

Apricot tree is a native species from West Indias belonging to the Clusiaceae family. The fruit is consumed *in natura* as juices, candies, liquors, etc. The *in vitro* propagation is presented as an alternative technique for the species propagation, contributing for the production of plants with uniform genetic characteristics, identical to the mother plant, diseases free, with the production of many of plants in a small space, independent of the time of the year. The objective of the present work was to establish a protocol for callogenesis induction in leaf segments of apricot tree. Leaf segments were used as explants, which were inoculated in MS, WPM and Knudson media, supplemented with 2,4-D (0.0; 2.0 and 4.0 mg L^{-1}) and BAP (0.0; 2.0 and 4.0 mg L^{-1}), sucrose (0.3%), agar (0.8%) and pH adjusted to 5.8. The Knudson medium pH was adjusted to 4.8. The calli formation was more efficient using the MS medium containing 2.0 mg L^{-1} 2,4-D + 2.0 mg L^{-1} BAP.

Index terms: *In vitro* propagation, Clusiaceae, cytokinin, auxin.

RESUMO

O Abricó-do-Pará é uma espécie nativa das Índias Ocidentais pertencente à família Clusiaceae. É consumida *in natura* e na forma de sucos, doces, licores, etc. A propagação *in vitro* apresenta-se como técnica alternativa para propagação da espécie, contribuindo para a obtenção de plantas com características genéticas uniformes, idênticas à planta mãe, livres de doenças, além da obtenção de um grande número de plantas em pequeno espaço, independente da época do ano. O presente trabalho tem como objetivo estabelecer um protocolo para indução da calogênese em segmentos foliares de abricó-do-pará. Foram empregados segmentos foliares como explantes, os quais foram inoculados em meios MS, WPM e Knudson, acrescidos de 2,4-D (0,0; 2,0 e 4,0 mg L^{-1}) e BAP (0,0; 2,0 e 4,0 mg L^{-1}), 3,0% de sacarose e geleificado com 0,8% de ágar e o pH foi ajustado para 5,8. O meio Knudson teve seu pH ajustado para 4,8. Os explantes foram mais reponsivos ao meio MS combinando 2,0 mg L^{-1} de 2,4-D e 2,0 mg L^{-1} de BAP.

Termos para indexação: Propagação *in vitro*, Clusiaceae, citocinina, auxina.

INTRODUCTION

Apricot tree is a native plant to the West Indies and northern South America that is grown throughout the entire Amazon region, especially in the state of Pará, hence the name abricó-of-Pará (CAVALCANTE, 1996). The fruits are used in salads, and in the forms of liquors, jellies and compotes, all which keep the taste and flavor for long periods of time. The bark latex and the seed powder of apricot are reported to have an insecticidal effect that efficiently control of ticks, other insects, and animal parasites. Its wood tannins are used in the leather industry (DONADIO, 2007).

The apricot seed germination process is slow, and the seedling emergence begins at 40 days, reaching the maximum after 260 days, with 90% germination (VILACHICA, 1996). The seeds are classified as recalcitrants, so can not be subjected to drying, and present low temperature sensitivity (below 15%) (CARVALHO et al., 2001).

The propagation of apricot tree using seeds for commercial orchards is not indicated, since this species is androdioic, which results in the appearance of approximately 50% male plants. Furthermore, plants obtained from seeds present a long juvenile stage, starting to produce fruits between 6 and 8 years after *in situ* planting. Thus the propagation by seeds presents a limited importance only to obtain rootstocks (NASCIMENTO et al., 2008) and grafted plants start production about four years after *in situ* planting (MÜLLER; CARVALHO, 2003).

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Tissue culture techniques should minimize the required time for the introduction of new cultivars into the commercial market by increasing the availability of plants with improved horticultural characteristics (RÊGO et al., 2009). Moreover, these techniques allow the mass production of genetically superior plants in short periods of time (SOARES et al., 2009).

Callus induction is one of the most used techniques in the rescue of entire populations of induced mutants, from somaclonal variation or transgenic production. The establishment of these populations results in the development of new cultivars. For this, studies of every aspect (kind of explant, medium composition, light and temperature of incubation) involved on the calli growth and plant regeneration are needed (SANTOS et al., 2005).

The regeneration can be defined as a process of vegetative multiplication that results in the obtention of an entire plant from only one cell (BELTRÃO et al., 2008). The knowledge over the cell division speed allows to infer about the physiological changes in the callus and consequently, aids to optimize the regeneration protocols (SERRA et al., 2000).

The plant growth and morphogenesis are regulated by the interaction and balance between growth regulators added to the medium, and by the endogenous levels produced in cells grown *in vitro*. In general, similar concentrations of auxin and cytokinin in the culture medium induce callus formation, however, the interaction response that classes of growth regulators may vary depending on the cultivation conditions, kind of explant and plant genotype (CORDEIRO et al., 2007).

The objective of this work was to establish a protocol for callogenesis induction in leaf segments of apricot tree.

MATERIAL AND METHODS

Explants were taken from plants of apricot tree, located at the fruit collection of Embrapa Rondônia, Porto Velho, Rondônia. Leaves were taken from apical part of the branch, which were placed in polystyrene box in order to prevent dehydration.

The explants were carried out at the Plant Tissue Culture Laboratory of Embrapa Rondônia, where the precleaning was performed, using a sponge, commercial detergent and distilled water. In a laminar flow cabinet, the leaves were segmented into smaller pieces with a scalpel and immersed in 70% alcohol (v/v) for 1 minute, followed by immersion in 0,125% NaOCl (active chlorine) for 30 minutes. After this time, the segments were rinsed three times in sterile distilled water, placed over a sterile filter paper and cut into fragments of about 1.0 cm^2. Then, they were inoculated in Petri dishes with the adaxial face in contact with different culture media: MS (MURASHIGE; SKOOG, 1962), WPM (LLOYD; McCOWN, 1980) and KNUDSON medium (KNUDSON, 1946). Each one was supplemented with different concentrations of 2,4-D and BAP (Table 1), 0.3% sucrose, 0,8% agar and pH adjusted to 5.8 (WPM and MS media) and 4.8 (Knudson medium).

TABLE 1 – Concentration of growth regulators (2,4-D and BAP) used for callus induction in leaf explants of *Mammea americana* for each culture media (MS, WPM or Knudson).

Treatments	2,4-D (mg L^{-1})	BAP (mg L^{-1})
1	0.0	0.0
2	2.0	0.0
3	4.0	0.0
4	0.0	2.0
5	2.0	2.0
6	4.0	2.0
7	0.0	4.0
8	2.0	4.0
9	4.0	4.0

The experimental design was entirely randomized as a 3x9 (three culture media, each one with nine combinations of 2,4-D and BAP) with twenty seven treatments in total. For each treatment, 10 replicates were employed, with five explants per dish. The cultures

were maintained in a growth room chamber at 24 ± 2 ºC under dark. The evaluation was performed after 40 days of culture, observing the percentage of calli induction (%CI) and the percentage of leaf area covered by callus (%LACC). The data were subjected to ANOVA and the averages compared by Tukey test at 5% probability.

RESULTS AND DISCUSSION

The evaluation of callus formation after 40 days treatment demonstrated that the control and treatment supplemented only with 4.0 mg L^{-1} BAP did not promote callogenesis (Table 2). The treatments supplemented with 2.0 or 4.0 mg L^{-1} 2,4-D or with 2.0 mg L^{-1} BAP showed percentages of calli induction below 60%. On the other hand, it was observed that 100% of the explants presented callus formation in the treatments supplemented with the following combinations 2.0 mg L^{-1} 2,4-D + 2.0 mg L^{-1} BAP; 4.0 mg L^{-1} 2,4-D + 2.0 mg L^{-1} BAP; 2.0 mg L^{-1} 2,4-D + 4.0 BAP mg L^{-1} and 4.0 mg L^{-1} 2,4-D + 4.0 mg L^{-1} BAP; moreover, the use of 2.0 mg L^{-1} 2,4-D combined with 2.0 mg L^{-1} BAP presented higher percentages of leaf area covered by callus (LACC%) (Table 2). SANTIAGO (2003) concluded that in leaf explants of *Piper hispidinervum* C. DC., the maximum calli production was achieved with the combination of 2,4-D and BAP. LIMA et al. (2008) studying the callus induction in leaf segments of *Croton urucurana* Baill. observed that 2,4-D without the presence of BAP was the most effective in promoting callus induction.

Callus induction in leaf explants cultivated in WPM medium was similar to the MS medium. The growth regulator free medium and the treatment where only the cytokinin BAP was used at 4.0 mg L^{-1} did not present callus induction. When the auxin 2,4-D was used at 2.0 or 4.0 mg L^{-1} or the use of BAP at 2.0 mg L^{-1}, a percentage of calli induction below 50% in the leaf explants was promoted. In the treatments where the auxin was combined with cytokinin, at the following concentrations (2.0 mg L^{-1} 2,4-D + 2.0 mg L^{-1} BAP); (4.0 mg L^{-1} 2,4-D + 2.0 mg L^{-1} BAP); (2.0 mg L^{-1} 2,4-D + 4.0 BAP mg L^{-1}) and (4 mg L^{-1} 2,4-D + 4 mg L^{-1} BAP), the percentage of callus induction reached 100%. As it was observed during callus induction on MS medium, the combination of 2,4-D and BAP, both at 2.0 mg L^{-1}, allowed higher percentages of leaf area covered by callus (Table 3). In this medium, it was also noted a friable callus. Similar results were found by SOARES et al. (2008) in leaf segments of *Hancornia speciosa* Gomes in which combination of 2.0 mg L^{-1} 2,4-D + 2.0 mg L-1 BAP was the most efficient for callus induction in leaf explants.

Table 2 – Percentage of calli induction in leaf explants of *Mammea americana* at 40 days culture in MS medium.

Treatments	Leaf area covered by callus (LACC)				
	0-25%	25-50%	50-75%	75-100%	% CI**
1	0	0	0	0	0 c
2	5 ab	5 c	0	0	28.57 b
3	10 a	10 c	0	0	57.14 b
4	10 a	8 c	0	0	51.43 b
5	2 b	20 b	10 a	3 a	100 a
6	0	30 a	5 a	0	100 a
7	0	0	0	0	0 c
8	0	30 a	5 a	0	100 a
9	0	30 a	5 a	0	100 a

**Percentage of calli induction (%CI). Different letters indicate significant differences among treatments, using Tukey test at 5% significance level.

At 40 days of cultivation, callus induction was observed in 4 treatments regarding the use of the Knudson medium. Treatment with 4.0 mg L^{-1} 2,4-D + 2.0 mg L^{-1} BAP presented 66.6% induction, with no significant difference from the following treatments: 2.0 mg L^{-1} 2,4-D + 2.0 mg L^{-1} BAP; 2.0 mg L^{-1} 2,4-D + 4.0 mg L^{-1} BAP; and 4.0 mg L^{-1} 2,4-D + 4.0 mg L^{-1} BAP, which showed the best results, all presenting 100% calli induction (Table 4).

The use of 2.0 mg L^{-1} 2,4-D combined with 4.0 mg L^{-1} BAP presented higher percentages of leaf area covered by callus, however, the calli were not friable and showed a small growth. There was no callus induction on the following treatments: control (growth regulator free medium); 2.0 or 4.0 mg L^{-1} 2,4-D with BAP absence and 2.0 or 4.0 mg L^{-1} BAP in the absence of 2,4-D (Tabela 4). SANTOS et al. (2003) studyied the effect of 2,4-D and BAP in the induction of callus leaf segments of *Coffea arabica* L.

TABLE 3 – Percentage of calli induction in leaf explants of *Mammea americana* at 40 days culture in WPM medium.

Treatments	Leaf area covered by callus (%LACC)				
	0-25%	25-50%	50-75%	75-100%	% CI**
1	0 b	0 c	0 b	0 a	0 b
2	9 a	0 c	0 b	0 a	30 b
3	10 a	0 c	0 b	0 a	40 b
4	5 ab	0 c	0 b	0 a	16.66 b
5	3 b	24 a	7 a	1 a	100 a
6	5 ab	15 b	5 a	0 a	100 a
7	0 b	0 c	0 b	0 a	0 b
8	0 b	30 a	5 a	0 a	100 a
9	0 b	28 a	7 a	0 a	100 a

**Percentage of calli induction (% CI). Different letters indicate significant differences among treatments, using Tukey test at 5% significance level.

Table 4 – Percentage of calli induction in leaf explants of *Mammea americana* at 40 days culture in Knudson medium.

Treatments	Leaf area covered by callus (%LACC)				
	0-25%	25-50%	50-75%	75-100%	% CI**
1	0 c	0 b	0 a	0 a	0 c
2	0 c	0 b	0 a	0 a	0 c
3	0 c	0 b	0 a	0 a	0 c
4	0 c	0 b	0 a	0 a	0 c
5	25 ab	5 a	0 a	0 a	100 a
6	20 b	0 b	0 a	0 a	66.6 b
7	0 c	0 b	0 a	0 a	0 c
8	30 a	7 a	3 a	0 a	100 a
9	32 a	3 ab	0 a	0 a	100 a

**Percentage of calli induction (%CI). Different letters indicate significant differences among treatments, using Tukey test at 5% significance level.

cv Rubi and observed that the explants did not present any callus induction when inoculated in the absence of growth regulators or only in the presence of BAP.

STEFANELLO et al. (2009) evaluated the effect of 2,4-D and BAP on the protocorm formation from meristematic areas of the root and leaf segments of *Miltonia flavescens* Lindl. and observed that the combination of 3 mg L^{-1} 2,4-D + 1 or 3 mg L^{-1} BAP provided the best results for protocorm formation.

In this work it was observed that, in all culture media tested in the percentage of leaf area covered by callus was higher when explants were cultured in the presence of 2,4-D and BAP, showing that the combination of auxin and cytokinin were essential for the callus formation (Table 2-4). SANTOS et al. (2010) evaluated the callus induction in *Bactris gasipaes* shoot tips and observed that higher callogenesis percentage was 60%, obtained by the use of 10.0 mg L^{-1} 2,4-D combined with 3.0 mg L^{-1} BAP.

Individually, the culture medium that most promoted callus induction was the MS medium, probably due to higher concentration of salts, as mentioned by KRIKORIAN (1991), justifying its extensive use in plant tissue culture. The explants inoculated in WPM medium also showed good results, attributing to its formulation developed especially for the *in vitro* culture of woody plants. The medium Knudson presented lower development of callus and higher oxidation, probably due to acidic pH and for being a medium particularly employed for orchid growing, presenting a reduced amount of salts in its composition. The results presented in this study are related with the composition of each medium and the combinations of auxin and cytokinin, allowing to distinguish the best concentrations for each one employed. The results achieved contribute to studies on the micropropagation of this species.

CONCLUSIONS

Individually, the MS medium was more efficient for callus induction of leaf segments of *Mammea americana*. The combination of BAP and 2,4-D provided the highest index cell development in the MS medium followed by WPM medium, while the development of explants inoculated in medium Knudson was slower. The treatments which had in their composition only auxin or cytokinin, showed no significant callus induction, confirming the need of combining auxin and cytokinin for callus induction in leaf explants of apricot tree.

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