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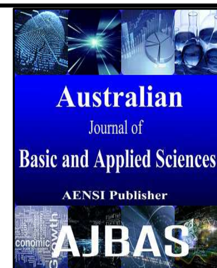

Callogenesis in leaf explants of *Annona glabra* L.

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ABSTRACT

Background: *Annona glabra* L. is a plant species native to South America used as rootstock to other Annonaceae. Its edible fruits offer great economic potential and are the reason of its cultivation in Brazil and Central America, even though the species has not yet been domesticated. **Objective:** The objective of this study was the disinfection and induction of callus in leaf explants of *A. glabra* using combinations of growth regulators. **Results:** Immersion of the explants in 10% calcium hypochlorite for 30 minutes was the most efficient treatment and resulted in 90% disinfection. The highest percentage of callus induction was 100% in the media supplemented with 18.17 and 36.33 µM TDZ, without 2,4-D.

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INTRODUCTION

The Annonaceae botanical family is composed of trees and shrubs with pantropical distribution and comprises approximately 130 genera and 2,300 identified species (Corrêa *et al*., 2007) including 26 genera and 260 species in Brazil (Maas *et al*., 2001). *Annona glabra* L. is a small tree with a wide geographical distribution in the tropics and subtropics and grows exclusively in swampy areas, periodically or permanently flooded (Zotz *et al*., 1997; Zotz *et al*., 1999), for it is a flood-tolerant species, with adaptations such as a swollen trunk base, many adventitious roots, aerenchyma in roots and lower trunk, and buoyant fruits and water-dispersed seeds (Zotz *et al*., 1997). In Brazil, it is known as “araticum-do-brejo” or “araticum-bravo” (Siebra *et al*., 2009) and occurs spontaneously from the Amazon in the North to the state of Santa Catarina in the South (Carvalho *et al*., 2001). Each plant has only one trunk, but the seeds in general germinate in groups, giving the appearance of shrubs with several trunks (Siebra, 2007).

A. glabra has great economic potential and has been cultivated in Brazil and Central America because of its edible fruits, which yield a yellow pulp, even though the species has not yet been domesticated (Villachica, 1996; Santos *et al*., 1998). Its leaves have been used in folk medicine as antihelminthic and antirheumatic and the roots as substitutes for cork and plastic materials in the manufacture of life-buoys, helmet-linings and shoe inner-soles (Santos *et al*., 1998). Also, this species can be used as rootstock for other economically important *Annona* species, as in the case of *A. muricata* L. with which it has an excellent level of compatibility (Pinto and Silva, 1994; Núñez-Elisea *et al*., 1999). *A. glabra* has dwarf genetic factors and its utilization as rootstock provides a small treetop for the grafted plant, which can facilitate the cultural procedures such as artificial pollination and control of pests (Carvalho *et al*., 2001).

The most used propagation method of this species is by seeds. However, this results in a large genetic variability, due to the genetic recombination inherent in the process. The outcome is the formation of orchards with uneven plants, with low productivity and poor quality of fruit. Another conventional form of propagation is made by cuttings. In general, this is the most adequate way to propagate fruit trees, but specifically in the case of *A. glabra*, it leads to the dissemination of diseases among plants (Braga, 2012).

In vitro culture is a viable method for propagation of several fruit tree species, and it is been used for decades for the propagation of high agronomic value carriers of rare genes and those at risk of extinction, for cleaning clonal plants and accelerating breeding programs by means of the development of new cultivars and expansion of genetic variability (Pasqual *et al*., 2012). The propagation by tissue culture can be direct or indirect, the latter by callus formation, which is considered a potential mass propagation method

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(Landa *et al*., 2000). Nevertheless, the *in vitro* multiplication of Annonaceae species has several limitations, as the endogenous contamination of explants, the high concentration of phenolic compounds inside the tissues and the premature leaf abscission (Santana *et al*., 2011). Considering the limitations of the *in vitro* propagation of *A. glabra* by tissue culture techniques, the objective of this study was the *in vitro* establishment of leaf explants and the induction of callus in these explants, aiming at the further regeneration of plants.

MATERIAL AND METHODS

Young leaves were excised from flowering, healthy, and disease free *A. glabra* stock plants from Embrapa (Brazilian Agricultural Research Corporation), in Porto Velho, RO, Brazil. Voucher specimens of these plants were identified and registered (number 6847) at the Dr. Ary Tupinamba Penna Pinheiro Herbarium, in Porto Velho. After washing with running tap water and a detergent agent for five minutes, the leaves were surface-sterilized in 70% ethanol for 1 minute and soaked in calcium hypochlorite at 5 and 10% (w/v) for 15 and 30 minutes under shaking, and then rinsed three times with sterile water. Explants were produced by cutting the leaves into 1 cm^2 pieces in sterile Petri dishes and individually transferred with the adaxial surface up, to test tubes (25 mm x 150 mm) containing 10 mL of an MS (Murashige and Skoog, 1962) basal culture medium supplemented with 3% (w/v) sucrose, 0.6% (w/v) agar and factorial combinations of 2,4-dichlorophenoxyacetic acid (2,4-D) (0, 9.05, 18.10, and 36.19 µM) and Thidiazuron (TDZ) (0, 9.08, 18.17, and 36.33 µM), totaling 16 treatments. The pH of the medium was adjusted to 5.8 before the addition of agar followed by autoclaving at 121°C for 20 minutes. After inoculation the cultures were kept in the dark, at 25±1°C, for four weeks. Treatments were arranged in a completely randomized design using five replicates of four explants. Contamination and oxidation of the explants were evaluated in the first seven days after inoculation. Callus formation was evaluated weekly until the 28th day, by assessing the number of callus induced per treatment. Variance analyses and polynomial regression were performed by using the Assistat 7.7 statistical program.

RESULTS AND DISCUSSION

The most efficient treatment for disinfection was the immersion in 10% calcium hypochlorite for 30 minutes, which resulted in 90% of the explants without contamination. There was no oxidation of the explants. Immersion in the same concentration of calcium hypochlorite for 15 minutes resulted in only 10% of the explants free of microorganisms, and 5% hypochlorite for 15 and 30 minutes led to the contamination of all the explants.

Different disinfection treatments and diverse results have been observed in the *in vitro* establishment of explants of *Annona* species. Castro *et al*. (1999) studied the effect of three disinfection treatments in the establishment of *A. cherimola* nodal explants: 3% sodium hypochlorite + Tween 20 for 15 minutes; 95% ethanol for 5 seconds + 0.25% sodium hypochlorite + Tween 20 for 10 minutes; and 35% H_2O_2 + Tween 20 for 10 minutes. The authors concluded that the type of disinfectant did not influence contamination rates, but rather the time and phenological state of the material under consideration. Garcia-Águila *et al*. (2012) evaluated the disinfection of nodal segments of *A. muricata* by using sodium hypochlorite at 0.5, 1.0, 1.5, and 2.0% for 15 minutes. The authors did not observe fungus occurrence. Contamination by bacteria only occurred in 3.8% of the explants treated with 0.5% sodium hypochlorite. Santana *et al*. (2003) studied the disinfection of leaf explants of the same species and found that sodium hypochlorite was inefficient (from 0.5 to 2.5% active chlorine). The authors then tested the incorporation of ampicillin at 0.0, 1.0, 2.0 and 4.0 mg L^{-1} and Benlate 500 at 0.0, 1.0, 2.0 and 4.0 g L^{-1} to the culture medium, and found that 1.0 g L^{-1} Benlate 500 was effective to promote 100% disinfection of the explants.

Regarding the occurrence of callogenesis, there was no callus induction on the medium without growth regulators. All the treatments where both regulators were supplemented resulted in callus induction. According to the variance analyses (Table 1), the effects of TDZ were highly significant on callus induction, but 2,4-D effects were not significant. Considering the lack of significance of 2,4-D treatments and the fact that TDZ treatments reached 100% callus induction only on the media where 2,4-D was not supplemented, it is possible to infer that 2,4-D has a negative effect on callogenesis and then the TDZ effect was evaluated in isolation, by polynomial regression (Figure 1).

Indirect propagation of *A. glabra* has not yet been achieved. In the present work abundant callus proliferation was promoted as the first phase for plant regeneration. Santana *et al*. (2011) tried to induce calluses in *A. glabra* by supplementing the medium with 2.22, 4.44, 8.88 and 17.76 µM BA in combination with 1.34, 2.68 and 5.37 µM NAA, without success in any of the combinations. Completion of direct propagation, without a callus phase, was described by Núñez-Elisea *et al*. (2000), who established a protocol for propagation of *A. glabra* by air-layering without the need of growth regulators to promote the rooting and by Oliveira *et al*. (2008), who regenerated plantlets from nodal stem segments.

Similarly to the observations of the present study, Nagori and Purohit (2004) noticed an efficient

effect of TDZ on callus induction in hypocotyl segments of *A. squamosa*. However, the authors achieved the regeneration of plantlets from shoot buds induced with BA at 22.20 µM and not from those treated with TDZ. Studying callogenesis in anthers of this species, Nair *et al*. (1983) achieved large frequency of calluses (80%) by supplementing the medium with 28.54 µM IAA and induced haploid plants from these calluses by using NAA in combination with BA. Studying the same species, Nair *et al*. (1984) observed the formation of calluses in leaf explants in medium supplemented with 2,4-D isolated, at the concentrations of 2.26 and 4.53 µM, but no callogenesis was observed with the concentration of 0.45 µM. Nair *et al*. (1986) induced callus proliferation on mature endosperm tissue of *A. squamosa* by using combinations of Kin (0.46 µM), BA (0.89 µM), NAA (2.69 and 5.37 µM), and GA_3 (1.44 and 2.89 µM). Bejoy and Hariharan (1992) tested several combinations of BA (2.2, 4.4, 8.9, and 13.3 µM) and NAA (0.27, 0.54, and 2.70 µM) in hypocotyl segments of *A muricata* and did not observe callus induction, but recorded the direct formation of shoot buds. Different results were found by Lemos and Baker (1998), who achieved callogenesis on internodal explants of the same species by supplementing the medium with 8.88 µM BA and 2.69 µM NAA.

Callus induction became apparent on the 14th day after inoculation, with the swelling of the leaf explants. On the 28th day, friable white calluses could be observed in 100% of the explants in the treatments with 18.17 µM and 36.33 µM of TDZ, but the latter concentration produced larger calluses, although they were not weighed or measured. The TDZ effect was represented by a quadratic regression (Figure 1), which confirmed that 36.33 µM was the most adequate concentration of TDZ to guarantee 100% of callus induction in the leaf explants of *A. glabra*.

Table 1: Variance analyses of the effect of 2,4-D and TDZ on callus induction in leaf explants of *A. glabra*, 28 days after inoculation.

Source	Degrees of Freedom	Mean Square	F
2,4-D	3	0.42	2.47 ns
TDZ	3	18.25	107.35**
CV (%)		6.48	

** - significant at 1% probability by F test; ns - not significant.

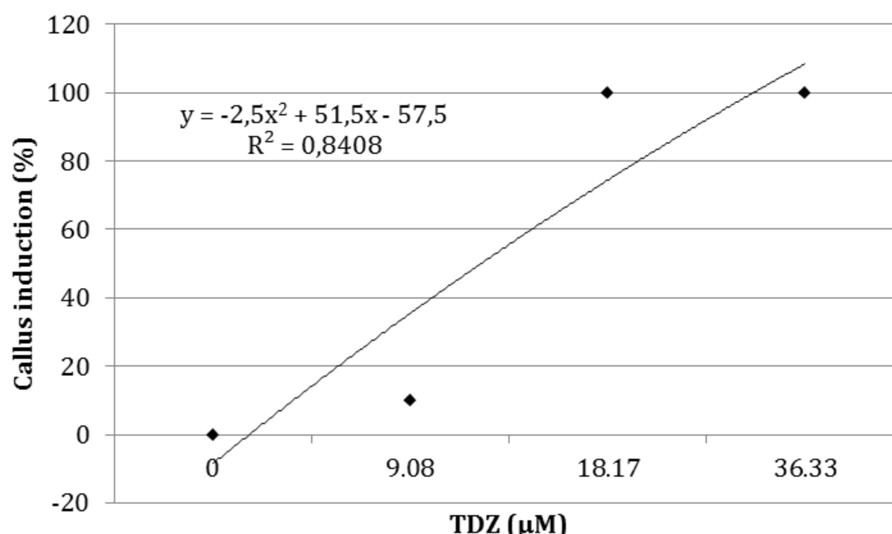

Fig. 1: Percentage of callus induction in leaf explants of *A. glabra* in MS medium supplemented with TDZ, 28 days after inoculation.

Conclusion:

Disinfection of *A. glabra* leaf explants can be achieved at a level of 90% with the immersion of the explants in 10% calcium hypochlorite for 30 minutes. Callus induction in 100% of leaf explants can be accomplished by supplementing the culture medium with TDZ at 36.33 µM.

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