Organogenesis in three species of *Annona* (Annonaceae)

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Abstract – This study aimed to evaluate, under *in vitro* conditions, the regenerative potential of leaves of three species of *Annona* (Annonaceae), *A. squamosa*, *A. bahiensis* and A. *glabra*, and hypocotyl and epicotyl segments of *A. squamosa*. In the first study, organogenesis was induced by inoculating the first leaves of three species of *Annona* in WPM medium supplemented with all combinations of concentrations of BAP (2.22, 4.44, 8.88 and 17.76 µM) and NAA (0.00, 1.34, 2.68 and 5.37 µM). The cotyledons and first leaves of *A. squamosa* were also cultivated in the same culture media. In the second study, hypocotyl and epicotyl segments of *A. squamosa* were inoculated in a medium supplemented with different concentrations of BAP (0.00, 2.22, 8.87, 17.74 and 35.48 µM). The results showed that organogenesis in *Annona* leaves is dependent on the type of explant, genotype and auxin-cytokinin balance used. In *A. squamosa*, the cotyledons had higher organogenic potential for micropropagation. Multiple shoots were regenerated from *A. squamosa* hypocotyls and epicotyls, but the regenerative potential of the hypocotyl was higher than that of the epicotyl.
Additional key words: cotyledons, epicotyl, explant, hypocotyl.

Resumo (Organogênese em três espécies de *Annona* (Annonaceae) **–** Este trabalho teve como objetivos avaliar, sob condições *in vitro*, o potencial regenerativo de folhas de três espécies de *Annona* (Annonaceae), *A. squamosa*, *A. bahiensis* e *A. glabra* (Annonaceae), e de segmentos de hipocótilo e epicótilo de *A. squamosa*. No primeiro estudo, a organogênese foi induzida através da inoculação de primeiras folhas definitivas das três espécies de *Annona* em meio WPM suplementado com todas as combinações entre as concentrações de BAP (2,22; 4,44; 8,88 e 17,76 µM) e ANA (0,00; 1,34; 2,68 e 5,37 µM). Nesses mesmos meios de cultura, também foram inoculados os cotilédones e as primeiras folhas definitivas de *A. squamosa.* No segundo estudo, segmentos de hipocótilo e epicótilo de *A. squamosa* foram inoculados em meio WPM suplementado com diferentes concentrações de BAP (0,00; 2,22; 8,87; 17,74 e 35,48 µM). Os resultados mostraram que a organogênese em folhas de *Annona* é dependente do tipo de explante, do genótipo e do balanço auxina-citocinina utilizado. Em *A. squamosa*, os cotilédones apresentaram maior potencial organogênico para a micropropagação. Múltiplas brotações adventícias foram obtidas em hipocótilos e epicótilos de *A. squamosa*, porém o potencial regenerativo do hipocótilo foi maior do que o do epicótilo.
Palavras-chave adicionais: cotilédones, epicótilo, explante, hipocótilo.

The family Annonaceae consists of 128 genera and 2,300 species, represented by trees and shrubs with fibrous bark, whose cells contain oils rich in aromatic terpenoids, alkaloids and often tannins. It is widely distributed in tropical and subtropical regions, being found mainly in humid forests at low altitudes. *Annona* L. stands out among the various genera of this family. It possesses widely cultivated species, such as *A. muricata* L. and *A. squamosa* L., and many native species with great economic potential, which produce flavorsome fruits and contain substances with medicinal properties and insecticides in their leaves and fruits, an important source of active compounds with fungicidal, insecticidal, and cytotoxic properties (Oliveira et al. 2008; Santana et al. 2008; Judd et al. 2009). Some native species, such as *A. glabra* L., have been extensively researched as a grafting rootstock due to the root system's high tolerance to conditions of excess moisture in the soil and the induction of dwarfing in the scion (Oliveira et al. 2007). However, the commercial use of these species has been limited by high levels of segregation and genetic recombination, due to their cross-fertilization and the difficulty in propagation by seed, with slow, uneven, and not very significant germination (Oliveira et al. 2008).

In this context, tissue culture techniques have been widely used, enabling the rapid propagation of selected plants, with high levels of uniformity and a high degree of sanity. However, in the Annonaceae, micropropagation has faced great obstacles, such as high endogenous contamination of the explants (Santana et al. 2003), a high rate of leaf abscission in shoots (Oliveira et al. 2007) and the difficulty in obtaining multiple shoots in nodal explants, which has limited the rate of propagation (Oliveira et al. 2008).

The success of *in vitro* plant organogenesis is dependent on characteristics related to the graft, the

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physical conditions of cultivation and the interaction between endogenous hormones and growth regulators added to the medium (Matt & Jehle 2005; Ntui et al. 2009; Rathore & Shekhawat 2009; Sun et al. 2009; Tavano et al 2009). Explants of new tissues, still undergoing cell division are, in general, more responsive to growth regulators and less recalcitrant to culture conditions *in vitro*, and therefore frequently used as the explant source, with great regenerative potential (George 1993). Tissues with high meristematic capacity can be obtained from a range of differentiated structures, such as shoot tips, axillary buds, leaves, nodal segments, seedlings, cotyledons and embryos (Siddique & Anis 2009). Several studies using meristems, buds or nodal segments have been conducted aiming at the spread of fruit trees (Jordan 1988; Matt & Jehle 2005; Telles & Biasi 2005; Costa & Aloufa 2006). In the Annonaceae, Rasai et al. (1994) obtained multiple shoots induced from excised hypocotyls of atemoya (*Annona cherimola* × *squamosa*) cv. 'African Pride' seedlings. Lemos & Blake (1996) succeeded in the micropropagation of *Annona muricata* using nodal segments and hypocotyls of seedlings obtained from seeds germinated *in vitro*. However, studies seeking to induce the regeneration of shoots on the leaves of woody species are still rare (Nair et al. 1984; Rasai et al. 1994). Nonetheless, plant regeneration from leaf segments as explants has been common in ornamental species, such as the work of Schiavinato et al. (2008), where a successful regeneration of *Anthurium plowmannii* Croat (Araceae) through the use of leaf explants and root tips was achieved.

The addition of 6-benzylaminopurine (BAP) and naphthaleneacetic acid (NAA) has been essential in the induction of organogenesis in many woody species (Ntui et al. 2009; Sun et al. 2009). In the Annonaceae, Jordan (1988), working with leaf explants of *Annona cherimoia* cv. 'Concha Lisa', obtained 100% of shoots in Murashige and Skoog (1962) medium supplemented with 8.87 μM BAP, 2.68 μM NAA and 200 mg l^{-1} hydrolyzed casein. However, there were fewer shoots per explant. On the other hand, Oliveira et al. (2008) found that the formation of multiple shoots in nodal explants of *A. glabra* using zeatin was possible, although this natural source of cytokinins has not been widely used in micropropagation protocols due to its high cost, making it necessary to seek new explant sources that are more responsive to the regulators routinely used in plant micropropagation.

In view of the foregoing, and to expand the study of organogenesis in the Annonaceae, this study was undertaken to evaluate, under *in vitro* conditions, the regeneration potential of leaves of species *Annona squamosa*, *A. bahiensis* and *A. glabra*, and hypocotyl and epicotyl segments of *A. squamosa*.

Material and Methods

Experiment I – Direct organogenesis in leaves

Collection of explants. The seeds of *A. squamosa* were washed in running water for 10 min followed by immersion in 70% alcohol for 2 min and then in a solution of sodium hypochlorite (commercial bleach with 2.5% available chlorine) for 15 min. After each treatment, three successive washes in sterile distilled water were carried out. The seeds were inoculated in "Woody Plant Medium" (Lloyd & McCown 1980), supplemented with 10 g l^{-1} sucrose. After inoculation the tubes were kept in a growth chamber at 25 ± 3°C under photosynthetically active radiation of 5 μmol m^{-2} s^{-1} for a period of 15 days. After this period, the cotyledonary nodes and the first leaves were excised and then used as the source of the explants.

For *Annona bahiensis* and *A. glabra* the leaves obtained from the shoots established *in vitro* from nodal segments were kept in test tubes completely sealed for five weeks in culture medium supplemented with 30 g l^{-1} sucrose and 8.88 μM of 6-benzylaminopurine (BAP).

Basic medium and experimental design. The explants were inoculated in test tubes containing 10 ml of culture medium enriched with 30 g l^{-1} sucrose, solidified with 7 g l^{-1} agar and pH adjusted to 5.7 before autoclaving. The effects of different combinations and concentrations of BAP (2.22, 4.44, 8.88 and 17.76 μM) and NAA (naphthalene acetic acid) (0.00, 1.34, 2.68 and 5.37 μM) were analyzed. The experimental design was completely randomized, arranged in a factorial design 4 × 4 × 3 (BAP concentrations × NAA concentrations × species), with eight replications, each consisting of a tube with a single explant.

For the species *A. squamosa* the effect of type of explant (cotyledonal and first leaves with petiole) was also evaluated. For this, a completely randomized design in a 4 × 4 × 2 (BAP concentrations × NAA concentrations × types of explant) with eight replicates was used, each containing a tube with a single explant.

In both experiments, the percentage of callus formation 30 days after inoculation and percentage of induced shoots 30 and 60 days after inoculation were evaluated.

Experiment II - Induction of adventitious shoots on hypocotyl and epicotyl

Collection of explants. The hypocotyl and epicotyl segments were obtained from seeds of *A. squamosa* germinated *in vitro* under the conditions listed above. The hypocotyl was cut below the cotyledons and above the radicle and divided into segments 15–20 mm long. The epicotyl was excised above the cotyledons and below the first pair of first leaves and divided into segments 10–15 mm long.

Basic medium and experimental design. The explants were inoculated in test tubes containing 10 ml of culture medium enriched with 30 g l^{-1} sucrose, solidified with 7 g l^{-1} agar and pH adjusted to 5.7 before autoclaving. The effect of different concentrations of BAP (0.00, 2.22, 8.87, 17.74

and 35.48 μM) was evaluated. The experimental design was completely randomized in a factorial design 2 × 5 (explant types × BAP) with five replicates, each replicate consisting of tubes with four plants.

The percentage of callus formation, the length and number of internodes of the largest shoot and the average number of shoots at 30, 45 and 60 days after inoculation were evaluated.

Growing conditions and statistical analysis. After inoculation of the explants, the cultures were maintained under photosynthetically active radiation of 45–56 μmol m^{-2} s^{-1}, 16 h photoperiod and temperature of 25 ± 3ºC. The data were subjected to analysis of variance with application of the F test at 5% or 1% probability of error and means were compared by use of the Tukey test at 5% probability error, using the software Sisvar (Ferreira 2003). Data in percentages were transformed into arcsine $\sqrt{\%}$ and number count into $\sqrt{x+1}$.

Results and Discussion

Experiment I – Direct organogenesis in leaves

Of the three species studied, *Annona squamosa* was the only one that produced both calogenesis and regeneration at the base of the explants in some combinations of BAP and NAA concentrations. Direct organogenesis without callus formation was obtained in explants of *A. bahiensis* and *A. squamosa* (Table 1). In the cultivation of *Prunus avium* (L.) L. (Rosaceae), similar responses in relation to callus formation from leaf segments were found, obtaining higher results in the basal than apical region, albeit in an indirect way (Matt & Jehle 2005). By contrast, Khan et al. (2009), in studies of *Citrus sinensis* (L.) Osbeck (Rutaceae), found sprout formation without callus induction, although cuts in the leaves were made, which possibly lead to callus formation.

It can be seen that genotype was the factor that had the highest contribution in the induction of buds amongst the combinations of growth regulators. The regeneration of shoots in *A. squamosa* was observed only when the lowest concentration of BAP (2.22 μM) was used, alone or in combination with concentrations of NAA. However, the maximum regeneration (100%) was obtained when combined with the highest concentration of NAA (5.37 μM) sixty days after explant inoculation (Table 1).

For *Annona bahiensis*, there was no response to the regeneration of the explants with the use of BAP alone. The highest percentage of shoots (100%) occurred in the culture medium when 8.88 μM BAP + 2.68 μM NAA and 17.76 μM BAP combined with 2.68 and 5.37 μM NAA were added. As for *A. glabra*, no combination of BAP and NAA was able to induce shoots from first leaves (Table 1). Azad et al. (2005) achieved 85% explant regeneration in cultures of *Phellodendron amurense* Rupr. (Rutaceae) in the presence of 4.44 μM BAP and 1.0 μM NAA. In cultures of *Pistacia vera* L. (Anacardiaceae), Tilkat et al. (2009) obtained 35% of direct regeneration of shoots from leaves with concentrations of 8.8 μM of BAP together with 5.71 μM of IAA. Comparing these results with those found for the species of *Annona* studied here, it appears that the balance between the concentrations of auxins and cytokinins is crucial for the direct regeneration of shoots from leaves. Furthermore, the genotype influences the induction of adventitious shoots, with different responses. So, it can be seen that for each species/genotype, the type and concentration of plant growth regulators affect the induction and regeneration of shoots differently, as does the type of explant.

The results reported in this paper in relation to the organogenic potential of *Annona* genotypes for the induction and regeneration of shoots from leaf explants corroborate those found by Zheng et al. (2009) in the evaluation of the regenerative behavior of three species of *Lysimachia* L. (Primulaeae). These authors found a variation in responses between the species of *Lysimachia* grown on the same medium supplemented with combinations of plant growth regulators, but the highest percentage of direct shoot regeneration from leaves were obtained with *L. christiniae* Hance in the presence of 13.32 μM BAP and 0.54 μM NAA and *L. nummularia* L. with 4.44 μMBAP and 0.54 μM NAA. As for *Lysimachia rubinervis* F.H.Chen & C.M.Hu, no regeneration of shoots on the leaves was observed. However, Khan et al. (2009), when assessing the percentage of shoot regeneration for two cultivars of *Citrus sinensis*, found no significant differences in the responses.

As to the results obtained with the species *A. glabra*, it was observed that the first leaves were completely yellowish thirty days after inoculation. It is possible that this response occurred due to the physiological state of the explants, which were derived from shoots kept in tightly sealed tubes after five weeks of cultivation. Under these conditions the senescence process was probably already initiated and the cells were no longer competent. In the *in vitro* cultivation of *Rubus* cv. 'Black Satin' (Rosaceae), it was observed that leaf explants from plants grown in culture for three months showed higher regenerative response (91.7%) compared to the explants at five months (33.3%) in the culture medium (Gupta & Mahalaxmi 2009). According to Khan et al. (2009), the physiological age of explants is a determining factor in plant regeneration response, since, in the evaluation of the induction of shoots and age of explants, it was found that the stage of development influences the regeneration response.

It is also possible to infer that the type of growth regulator and the concentrations used were not efficient in stimulating organogenesis in *A. glabra*. According to George et al. (2008) cells can respond to an extracellular stimulus provided it is appropriate. In this case, the use of

Table 1. Interaction effect between naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP) on the percentage of leaf explants with calluses at 30 days (C), percentage of explants with shoots at 30 days (S1), and 60 days (S2), after inoculation of first leaves of *Annona bahiensis*, *A. glabra* and *A. squamosa*.

NAA	BAP (µM)											
(µM)	2.22			4.44			8.88			17.76		
	C	S1	S2	C	S1	S2	C	S1	S2	C	S1	S2
A. bahiensis [y]												
0.00	-	-	-	-	-	-	-	-	-	-	-	-
1.34	-	-	50	-	-	50	-	-	50	-	-	50
2.68	-	-	-	-	-	-	-	-	100	-	-	100
5.37	-	-	50	-	-	50	-	-	50	-	-	100
A. glabra [y]												
0.00	-	-	-	-	-	-	-	-	-	-	-	-
1.34	-	-	-	-	-	-	-	-	-	-	-	-
2.68	-	-	-	-	-	-	-	-	-	-	-	-
5.37	-	-	-	-	-	-	-	-	-	-	-	-
A. squamosa [z]												
0.00	100	50	50	50	-	-	50	-	-	100	-	-
1.34	50	50	50	87	-	-	50	-	-	100	-	-
2.68	50	50	50	50	-	-	50	-	-	100	-	-
5.37	100	62	100	62	-	-	100	-	-	100	-	-

[Y] explants from shoot explants *in vitro* nodal segments, [z] explants from plants germinated *in vitro*.

TDZ (thidiazuron) in the culture medium could be an option, since this control is one of the most active sources of cytokinin, especially for recalcitrant woody species (Oliveira et al. 2008).

In *Annona squamosa*, it was found that the cotyledons had the highest percentage of shoots compared to first leaves in most of the combinations and concentrations of BAP and NAA, except in the culture medium in which it was added to the lowest concentration of BAP (2.22 µM) with the highest concentration of NAA (5.37 µM), in which final leaves and cotyledon showed 100% of shoots (Table 2). However, in this same concentration of BAP (2.22 µM) the cotyledons also attained the highest shoot rates (100%), irrespective of the concentration of NAA.

Thus, it appears that the type of explant used is of paramount importance in the induction of *Annona squamosa* shoots, where the cotyledons showed higher organogenic potential and can be considered, therefore, an excellent source of explants for micropropagation of this species. These results are relevant, considering that Annonaceae still has great difficulty in regenerating *in vitro*. Using the regeneration process described here, depending on the balance between BAP and NAA, the production of shoots on the *A. squamosa* leaves reached 100%. These results corroborate those reported by Nair et al. (1984) when working with the same species. According to these authors, the portion of leaf used as explant is a very important factor in the formation of shoots, the base of the petiole being the most responsive region for the induction of shoots. However, Hervé et al. (2001) report that only 10% of the leaves of an elite clone of *Eucalyptus gunnii* Hook. f. (Myrtaceae) produced adventitious buds.

During the process of organogenesis observed in *Annona squamosa* and *A. bahiensis*, a bulge near the petiole of the leaf was formed, on the surface of which bud initiation occurred. Similar structures have been described by Tilkat & Onay (2009) in the *in vitro* culture of *Pistacia vera*, and which only appeared in the marginal region and midrib of the leaves. Khan et al. (2009) also verified the presence of such structures in the final portion of the leaf base, equally in the marginal/border region as in the venation in *Citrus sinensis*. The appearance of adventitious buds is explained by van Arnold & Hawes (1989) as being a result of meristems generated by the differentiation of epidermal and sub-epidermal tissues, caused by the action of cytokinins.

Experiment II – Induction of adventitious shoots on hypocotyl and epicotyl

All explants produced a callus mass on media containing BAP (Table 3). Initial callus induction occurred directly on the cut surface of explants, but the calluses were totally unable to produce adventitious shoot via indirect organogenesis. In addition, a direct correlation was observed between the concentration of BAP and the size of callus formation (data not shown): an increase in the size of the callus with an increase in the regulator concentration. In the *in vitro* cultivation of *Citrus volkameriana* Ten. & Pasq. and *C. aurantium* L. (Tavano et al. 2009), the use of BAP also promoted callus induction and is essential for the development of adventitious buds, since organogenesis resulted from callus formation in both the epicotyl and the hypocotyl.

The adventitious shoots formed directly over the entire length of the hypocotyl, while occurring only on the apical end of the epicotyl explant (Table 3). Although the formation of calluses in the absence of BAP was not observed, this directly affected the size and number of internodes of the shoots. The length of shoots in hypocotyl and epicotyl segments was inversely proportional to the concentration of BAP used. The largest hypocotyl (28.4 mm long) and epicotyl (14.0 mm) explants were obtained with the lowest level of BAP (2.22 µM) (Table 3). Similar results were reported in cultured *Psoralea corylifolia* L. (Leguminosae) (Baskaran & Jayabalan 2009); when the medium was supplemented with a low concentration (1.0 µm) of TDZ greater shoot length was obtained, although the number of shoots per explant was reduced.

In Table 4, it can be observed that for regeneration from the hypocotyl, the concentration of 8.87 µM BAP induced the highest number of shoots (4.47) and this differed significantly from concentrations 0.00 µM (control) and 2.22 µM BAP. However, there was a trend toward a reduction in the number of shoots per explant, when the BAP concentration was higher than 8.87 µM. As for the number of shoots formed in the epicotyl, it was found to be greater with increasing concentrations of BAP, although no significant difference was detected. Shan et al. (2009), working with some cultivars of *Arachis hypogaea* L. (Leguminosae), found similar results for the epicotyls subjected to high concentrations of BAP. These authors claim that the cells have the capacity to dedifferentiate and recover their meristematic ability, resulting in the formation of adventitious buds after being stimulated with cytokines. Similar results were also found by Tavano et al. (2009) in which an increase in the number of shoots when comparing hypocotyl and epicotyl in relation to the species *Citrus volkameriana* in culture medium supplemented with 4.44 µM of BAP was observed. These authors attributed this response to the use of hypocotyl with cotyledon fragments,

Table 2. Interaction effect between 6-benzylaminopurine (BAP) and naphthaleneacetic acid (NAA) on the percentage of shoots in explants consisting of first leaves and cotyledons of *A. squamosa* after 60 days of inoculation in WPM medium.

	BAP (µM)			
Type of explant	**2.22**	**4.44**	**8.88**	**17.76**
			0.00 µM ANA	
First leaves	37.5	0.0	0.0	0.0
Cotyledons	100.0	87.5	0.0	0.0
			1.34 µM ANA	
First leaves	50.0	0.0	0.0	0.0
Cotyledons	100.0	100.0	100.0	0.0
			2.68 µM ANA	
First leaves	37.5	50.0	0.0	0.0
Cotyledons	100.0	100.0	100.0	87.5
			5.37 µM ANA	
First leaves	100.0	0.0	0.0	0.0
Cotyledons	100.0	100.0	100.0	100.0

noting that such explants can be used to improve the rate of shoots in recalcitrant genotypes.

In the Annonaceae, Rasai et al. (1994) found similar results when using atemoya (*A. cherimola* × *squamosa*) hypocotyls of cv. 'African Pride'. The maximum number of buds found in this cultivar was 12.6 per explant at the distal part of the hypocotyl inoculated in MS medium supplemented with 8.87 µM BAP and 0.46 µM KIN. However, increasing BAP levels above 8.87 µM provoked a reduction in the number of buds, the shoot length and fresh weight of shoots. Jordan (1988) also obtained the formation of multiple adventitious shoots from hypocotyl segments of *Annona cherimola* L. without going through the callus phase, and the highest percentage of shoots were obtained in MS medium supplemented with regulators BAP and NAA (8.87 and 2.68 µM, respectively).

Conclusions

The organogenic responses in *Annona* are dependent on the species. Explants from cotyledonary and first leaves are promising explants for the proliferation of shoots of species *A. squamosa*, whereas in *A. bahiensis* only the first leaves present organogenic potential. Multiple adventitious shoots can be obtained from hypocotyl and epicotyl of *A. squamosa* with the use of BAP.

Table 3. Percentage of callus, shoot length and number of internodes on the longest shoot obtained in hypocotyl (H) and epicotyl (E) of *Annona squamosa* for different concentrations of 6-benzylaminopurine (BAP).

BAP (µM)	Callus at the base of the explant (%)		Shoot length of the longest shoot(mm)		Number of internodes of the longest shoot	
	H	E	H	E	H	E
0.00	0.0	0.0	8.0	2.4	1.8	0.8
2.22	100.0	100.0	28.4	14,0	4.6	3.0
8.87	100.0	100.0	25.0	13.4	3.6	3.0
17.74	100.0	100.0	21.4	9.8	3.2	2.4
35.48	100.0	100.0	8.0	8.6	2.0	2.0

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