Influence of explant polarity on morphogenesis responses of *Annona squamosa* L. cultivated *in vitro*

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(Influence of explants polarity on morphogenesis responses of *Annona squamosa* L. cultivated *in vitro*) – The influences of explant orientation on *in vitro* responses of *Annona squamosa* L. were evaluated. Hypocotyl and epicotyl segments were inoculated into test tubes with WPM medium supplemented with 8.87 µM BAP in the following orientations: horizontally on the medium surface, vertically upright retaining their natural polarity and vertically upright but inverted from their natural polarity (hypocotyl segments only). The test tubes were sealed with normal transparent plastic caps (either covered or not covered with PVC film) or with cotton plugs. After inoculation the tubes with the explants were maintained in a growth room under photosynthetically active radiation levels of 45-56 ìmol.m^{-2}.s^{-1} and temperatures of 25 ± 3ºC. The results showed that explant polarity affected organogenesis, and that the largest numbers of shoots (8.3 per explant) were found on hypocotyl explants placed vertically and retaining their natural polarity. No influences of the different test tube caps on morphogenetic responses of the explants were observed. The incubation of hypocotyl and epicotyl segments in a vertical position constitutes an efficient system for generating *A. squamosa* plants.

Key words: Annonaceae, "pinha", micropropagation.

(Influência da polaridade do explante na resposta morfogênica de *Annona squamosa* L. cultivada *in vitro*) – O trabalho teve como objetivo avaliar a influência do tipo e da polaridade do explante na resposta morfogênica *in vitro*, utilizando três tipos de vedação. Segmentos de hipocótilo e epicótilo de *Annona squamosa* L. foram inoculados em tubos de ensaio contendo meio de cultura WPM acrescido de 8,87 ìM de BAP nas orientações horizontal, vertical ou vertical invertida (somente hipocótilo). Os tubos foram fechados com tampa plástica (vedada ou não com película de PVC) ou tampão de algodão e mantidos em sala de crescimento sob radiação fotossintética ativa de 45-56 ìmol.m^{-2}.s^{-1} à 25 ± 3ºC. Os resultados mostram que a polaridade do explante afeta a organogênese e que o maior número de brotações (8,3 por explante) foi obtido quando os segmentos de hipocótilo foram inoculados na orientação vertical mantendo sua polaridade natural. O tipo de tampa usada para fechar os tubos de ensaio não afetou a capacidade organogênica dos explantes. A utilização de segmentos de hipocótilo e epicótilo, inoculados verticalmente, constitui o método mais eficiente para a obtenção de brotações em *A. squamosa*.

Palavras-chave: Annonaceae, pinha, micropropagação.

Introduction

Annona squamosa L. is an exotic species that is well adapted to the climate found in northeastern Brazil (Sousa *et al*., 2006). It is commercially valued as a fresh fruit in different parts of the country and is also used in folk medicine (Araújo, 1991; Erig *et al*., 2001).

Attempts to commercial use of *A. squamosa* have encountered problems related to their sexual propagation due the occurrence of plants with undesirable agronomic characteristics (Rasai *et al*., 1995). However, conventional vegetative propagation has not proved to be very viable alternative for reproducing this species (Santana *et al*., 2006), due to difficulties in finding compatible root-graft stock and increasing the dispersion of plants infected with virus.

According to Santana *et al*. (2006), micropropagation has been used in reproducing numerous species of *Annona*. This technique allows growers to obtain large numbers of plants from a single individual, and it has been useful during large scale production of commercially plants. The identification of the factors involved in the control of *in vitro* morphogenesis in *A. squamosa* is fundamental to the establishment of commercial protocols for plant production and clarify if they are stimulated by environmental parameters or intrinsic traits from the explants.

In vitro explants normally demonstrate accentuated polarity in terms of cell growth and morphogenesis. Hartmann *et al*. (1997) suggested that the orientation of plant organs or tissue segments used as explants affects the redistribution of certain plant substances such as auxins, which would explain the different growth responses observed. This was verified in apple cv. "Delicious", where the largest production of axillary shoots was observed when the explants were placed upside down into the culture medium, as compared as to the normal orientation

(ZIMMERMAN & FORDHAN, 1985) and the horizontal orientation of the explant in the culture medium also significantly increased the total number of shoots for the same species (BYEONG *et al*., 1987).

The effects of explant polarity during *in vitro* culture vary with the species genotype and with the type of explant used, and can often be influenced by treatment with growth regulators (GEORGE, 1993). As was observed in *Zamioculcas zamiifolia* (Lodd.) Engl., where the horizontal placing of explants on medium surface reduced its response compared to vertical orientation, probably because of better absorption of plant growth regulators and nutrients through the wounded ends and the polarity (PAPAFOTIOU & MARTINI, 2008).

In addition to explant polarity, the microenvironment within the culture vessel itself can have a significant influence on *in vitro* morphogenesis (CHEN & CHEN, 2002). An increase in the rate of gas exchange within vessels using permeable caps can result in a reduction of the internal relative humidity (SEELYE *et al*., 2003) and changes in explant differentiation, growth, rooting, and acclimatization have been reported by numerous authors (ZOBAYED *et al*., 2000; ZOBAYED *et al*., 2001; TREVISAN & MENDES, 2005; BELLINTANI, 2006) as consequence of this reduction.

The aim of this work was the evaluation of explant polarity orientations and different types of test tube seals on the morphogenesis response of *A. squamosa* during *in vitro* culture.

MATERIALS AND METHODS

The hypocotyls and epicotyls of *A. squamosa* were removed from seedlings that had been previously established under *in vitro* conditions and maintained in growth rooms under low photosynthetically active radiation (2-5 $\mu mol.m^{-2}.s^{-1}$). The hypocotyls were excised below the cotyledons and above the radicule, and were then subdivided into 15 to 20 mm long segments. The epicotyls were cut above the cotyledons and below the first pair of leaves, and then subdivided in two segments about 10 to 15 mm length.

The resulting explants were inoculated into test tubes (25 x 150 mm) containing WPM culture medium (LLOYD & MCCOWN, 1980) supplemented with 30 $g.L^{-1}$ of sucrose, 8.87 µM BAP (6-benzilaminopurine), and 7 $g.L^{-1}$ of agar (SANTANA, 2003). The pH of the culture medium was adjusted to 5.7 before autoclaving. After explant inoculation, the cultures were maintained under 45-55$\mu mol.m^{-2}.s^{-1}$ of photosynthetically active radiation, with a photoperiod of 16 hours at 25 ± 3ºC.

Experiment I – Polarity

In order to evaluate the effects of polarity, hypocotyl segments were inoculated into the culture medium with three different orientations: horizontal and vertical retaining their natural polarity, and vertical reversing their natural polarity. Segments cultured in either of the two vertical orientations had one of their extremities inserted into the culture medium to a depth of from 2 to 3 mm. Three types of seals were used to close the test tubes: simple plastic caps, plastic caps wrapped with PVC film and cotton plugs. The experiment was conducted in a completely randomized design in 3x3 factorial scheme (orientation x seal) with four replicates of four tubes each (one explant/ tube) per treatment.

Experiment II – Explant type

In order to evaluate the occurrence of interactions between the types of explant used and their polarity, hypocotyl and epicotyl segments were placed into the culture medium in horizontal and vertical orientations. Three different types of caps were used to close the test tubes: simple plastic caps, plastic caps wrapped with PVC film, and cotton plugs. The experimental design was conducted in a completely randomized design in 2x2x3 factorial scheme (explant x orientation x seal) with four replicates composed of four tubes each (one explant/ tube).

Variables evaluated and statistical analysis

The explants were evaluated after 45 days to determine the numbers of shoots per explant and the percentages of responsive explants. The data was submitted to variance analysis, F tests at 5% or 1% were applied, and the averages were compared using the Tukey test at a 5% de probability level; all calculations were performed using the Sisvar software package (FERREIRA, 2003). Data in percentages were arc-sine $\sqrt{\%}$ transformed, and the numbers of counts transformed by $\sqrt{x+1}$.

RESULTS AND DISCUSSION

Our results indicated that the type of explant and explant orientation can affect shootding responses during *in vitro* culture of *A. squamosa*.

Experiment I – Polarity

Statistical analyses indicated that explant orientation greatly affects the number of shoots formed (*P*d" 0.01) as well as the percentages of responsive explants (*P*d"0.01). Interactions between explant orientation and the type of test tube cap were significant only for the percentage of responsive explants (*P*d"0.05) (Table 1). Hypocotyl segments placed vertically retaining their natural polarity produced the largest numbers of adventitious shoots (8.30 per explant) and the greatest percentage of responsive explants (72.25%) - differing significantly from segments inoculated horizontally (Fig. 1). All explants formed shoots along the entire length of each segment, independent of their orientation. This same phenomenon was not observed to *in vitro* cultures of *Vaccinium vitis-idaea*, for the apical region of the hypocotyl segments in this species formed

Table 1. Analysis of variance summary for the average numbers of shoots per explant and of the percentages of responsive explants of hypocotyl segments of *Annona squamosa* L. inoculated in different orientations and in test tubes with different cap types.

Source	DF	Mean Square	
		Number of shoots[y]	Responsive explants[z]
Explant orientation (A)	2	1.5104**	14.3114**
Type of cap (B)	2	0.1479 ns	0.9852[ns]
A x B	4	0.0954 ns	5.3171*
Error	27	0.1452	1.2988
C.V. (%)		14.31	14.86

(**) or (*): Significant by the F Test to *P*d"- 0.01 or *P*d"- 0.05, respectively. ns: not significant.

[y], [z]: Original data transformed by or by arc-sine $\sqrt{\%}$, respectively.

larger numbers of shoots than the central or basal region (DEBENATH, 2003). In *Bixa orellana* L., the sub-basal region exhibited a greater regeneration frequency than the apical region, although all of the hypocotyl regions of this species demonstrated regeneration percentages above 60% (CARVALHO *et al*., 2005). According to NAGESH *et al*. (2009), in *in vitro* culture of *Curculigo orchioides* Gaertn., a herbaceous plant, showed that the capacity for *de novo* shoot shoot formation gradually decreased in the explants from the proximal end to distal end as reflected by the formation of lesser number of shoot shoots in explants from distal end.

Segments inoculated vertically, but with inverted polarity, demonstrated non-friable callus formation at the extremity in contact with the culture medium (data not shown). Visually, this treatment seemed to show the smallest number of shoots – although their number did not, in fact, differ statistically from the number of shoots seen on segments inoculated vertically and maintaining their natural polarity. These results differ from those reported by ZIMMERMAN & FORDHAN (1985) for the "Delicious" apple variety, which demonstrated a greater production of shoots with explants inverted in terms of their natural polarity. While TIWARI & TULI (2008) observed reduction in the percentage of induction of shoots of the species *Arachis hypogaea* L. when it reversed the orientation of the explant (20%) compared to the natural orientation (> 80%). The same occurred in the *in vitro* cultivation of the herb, *Spilanthes acmella* (L.) Murray, when the explants were inoculated in the inverted orientation, showing 45% of responsive explants for 97% when placed in the normal orientation (SINGH *et al*., 2009).

The smallest number of shoots (4.49 shoots per explant) and the smallest percentage of responsive explants (44.44%) were observed among horizontally inoculated explants of *A. squamosa* (Fig. 1).

These results differ from those reported for other species, where a horizontal orientation normally favors shoot induction; this may be due to the fact that the horizontal position breaks apical dominance by interrupting auxin flow and/or results in greater contact between the explant and the culture medium. In *Annona muricata* L., a horizontal position significantly increased explant shoot

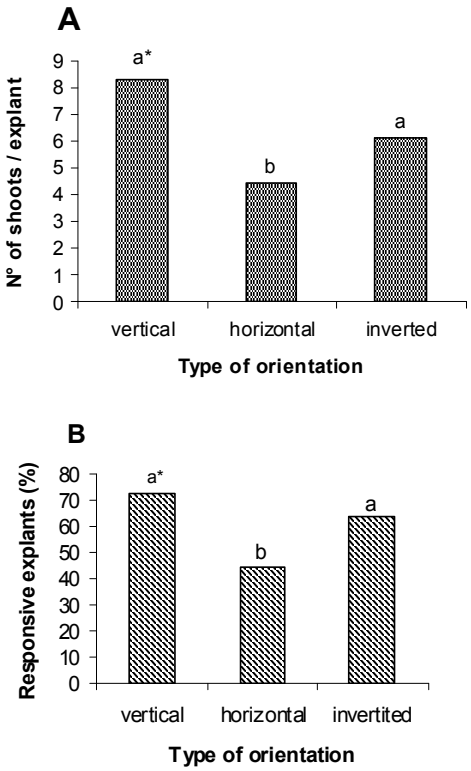

Fig. 1. Average number of shoots per explant (**A**) and percentage of responsive explants (**B**) in hypocotyls of *Annona squamosa* L. inoculated with different orientations. *Averages followed by the same letter do not differ by the Tukey test (Pd" 0.05).

production (BYEONG *et al*., 1987). Similar results were reported by ERIG & SCHUCH (2002) who obtained 5.81 shoots per explant in the horizontal position but only 3.78 in the vertical position, using the Marubakaido apple variety. The same was observed for *Uncaria guianensis* (Aubl.)Gmel. and *Albizia odoratissima* (L. f.) Benth. (PEREIRA *et al*., 2006; RAJESWARI & PALIWAL, 2008), which also reported a larger number of shoots among explants inoculated horizontally.

No significant differences were detected between the three types of caps used to seal the test tubes and the numbers of shoot or the percentages of responsive explants (Fig. 2).

These results were similar to those reported by SOUZA *et al*. (1999) who observed that the type of cap used

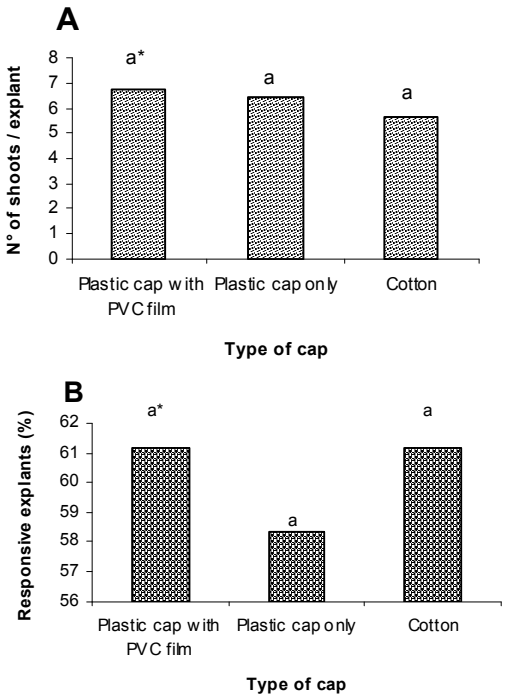

Fig. 2. Average number of shoots per explant (**A**) and percentage of responsive explants (**B**) in hypocotyls of *Annona squamosa* L. inoculated in different cap types. *Averages followed by the same letter do not differ by the Tukey test (Pd" 0.05).

did not influence shoot generation in *Brassica oleraceae* L (cabbage). However, shoots in test tubes sealed with cotton plugs showed less overall growth than those raised in tubes with plastic caps (data not shown). These results may be related to a reduction in water potential and a consequent reduction in the capacity of the explants to absorb nutrients. Earlier experiments had indicated that evapotranspiration was greater in tubes with cotton plugs.

Experiment II – Explant Type

These experiments demonstrated a highly significant double interaction (type of explant x orientation of the explant) determining the numbers of shoots per explant (*P*d" 0.01) and a significant triple interaction (type of explant x orientation of the explant x types of seals) determining the percentages of responsive explants (*P*d" 0.05). No isolated effect was observed related to the type of test tube cap, although explant type and orientation considered independently demonstrated highly significant differences in terms of the variables examined (Table 2).

The percentages of responsive explants among hypocotyl segments inoculated either vertically or horizontally were not affected by the type of test tube cap used. On the other hand, a significant reduction in the percentage of responsive explants was observed among epicotyls inoculated vertically into test tubes with cotton plugs (Table 3).

In a similar manner, more shoots per explant were observed among hypocotyl than among epicotyl segments, especially when the former were inoculated vertically (Table 4). The best results were obtained using hypocotyl segments (8.30 shoots/segment) – yielding six times more shoots/segment than epicotyl segments inoculated vertically (1.27). These results demonstrate that hypocotyl explants can be successfully used for *in vitro* multiplication of *A. squamosa*. MOURA *et al*. (2001), on the other hand, reported larger numbers of shoots when using epicotyl segments of *Citrus* ('Valencia' oranges) that were longitudinally sectioned and inoculated upright into the culture medium. Larger numbers of shoots per explant were also observed in *Vigna subterranea* (L.) Verdc. when epicotyl segments were used (KONÉ *et al*., 2007). CUI *et al*. (2004), however, reported results similar to those observed in the present work during *in vitro* culture of *Antirrhinum majus*, as they obtained more than ten shoots per explant using hypocotyl segments.

Shoot generation from hypocotyl segments cultivated *in vitro* has been reported in diverse species of *Annona*, such as *A. cherimola* cv. 'Concha Lisa' (JORDAN, 1988), *A. squamosa* (LEMOS & BLAKE, 1996), *A. muricata*

Table 2. Analysis of variance summary for the average numbers of shoots per explant and the percentages of responsive explants of hypocotyl and epicotyl segments of *Annona squamosa* L. inoculated in different orientations and in test tubes with different cap types.

Source	DF	Mean Square	
		Numbers of shoots [y]	Responsive explants [z]
Type of explant (A)	1	16.8695**	27.7063**
Explant orientation (B)	1	1.4138**	56.6411**
Type of cap (C)	2	0.0367^{ns}	0.1351^{ns}
A x B	1	1.6019**	0.0785^{ns}
A x C	2	0.1300^{ns}	3.5173^{ns}
B x C	2	0.1134^{ns}	23.8401**
A x B x C	2	0.0559^{ns}	11.1168*
Resíduo	36	0.1353	3.1871
C.V. (%)		17.68	26.54

(**) or (*): Significant by the F Test to *P*d"- 0.01 or *P*d"- 0.05, respectively. ns: not significant.
[y], [z]: original data transformed by $\sqrt{x+1}$ or by arc-sine $\sqrt{\%}$, respectively.

Table 3. Percentage of responsive explants derived from hypocotyls and epicotyls of *Annona squamosa* L. inoculated in different orientations and in test tubes with different cap types (PP+PVC= plastic cap plus PVC film; PC= plastic cap only; CC=cap cotton).

Type of explant	Explant Orientation					
	Vertical			Horizontal		
	PP+PVC	PC	CC	PP+PVC	PC	CC
Hypocotyl	83.35a[z]A[y]	75.00 aA	66.70 aA	41.62 aA	50.00 aA	58.35 aA
Epicotyl	66.67 aA	66.70 aA	24.97 bB	24.97 aA	16.65 aB	24.97 aA

[z]Averages followed by the same lower case letter in each column indicates that they do not differ at a 5% probability level by the Tukey test. [y]Averages followed by the same upper case letter in each line, in each explant orientation, indicates that they do not differ at a 5% probability level by the Tukey test.

Table 4. Average number of shoots per explant derived from hypocotyls and epicotyls of *Annona squamosa* L. inoculated in different orientations.

Type of explant	Explant orientation	
	Vertical	Horizontal
Hypocotyl	8,30 A[z]a[y]	4,49 B a
Epicotyl	1,27 A b	1,36 A b

[z]Averages followed by the same lower case letter in each column indicates that they do not differ at a 5% probability level by the Tukey test.
[y]Averages followed by the same upper case letter in each line, in each explant orientation, indicates that they do not differ at a 5% probability level by the Tukey test.

(BEJOY & HARIHARAN, 1992), and atemoya cv. 'African Pride' (RASAI *et al*., 1994). The average number of shootss per explant (6.80) encountered in the present work was superior to that reported by BEJOY & HARIHARAN (1992) for *A. muricata* – an average of 4.8 shoots per explant in MS culture medium supplemented with 8.87 µM BAP and 0.54 µM ANA (naphthalene acetic acid).

In addition to the results described above, a direct relation was noted between the numbers of shoots and sprouts along epicotyl segments very near the cotyledon node. This relationship between the distance to the cotyledon node and the number of shoots was also reported for *Troyer citrange* (*Citrus sinerensis* x *Poncirus trifoliata*) by MOREIRA-DIAS *et al*. (2001). According to CUTTER (1986), sectioning regions near meristem and parenchyma tissue stimulates cell division. However, no reason has yet been established for this loss of shoot generating capacity as the distance to the cotyledon node increases. GUNKEL *et al*. (1972) demonstrated the importance of phytoregulator transport in the vascular tissue in annulling stem polarity by adding growth regulators to the culture medium. Based on this information, it can be inferred that the influence of polarity on morphogenetic potential is probably linked to phytoregulator transport.

CONCLUSIONS

The utilization of vertically (natural polarity) inoculated hypocotyl and epicotyl segments constitutes the most efficient method for obtaining shoots in *A. squamosa*.

Inoculating explants horizontally reduces potential organogenic expression.

The type of cap used to close the test tubes does not affect the morphogenetic capacity of *A. squamosa*.

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