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Full Length Research Paper

In vitro cultivation of *Anacardium othonianum* Rizz.: Effects of growth regulator, explant orientation and lighting

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***Anacardium othonianum* Rizz. is a Brazilian savannah native species, commonly known as caju-de-árvore-do-cerrado. Its usual reproduction is by seeds or asexually; however, its use in forest programs or for commercial orchards demands continuous and large scale seedling production. This study evaluated the effect of naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP), the position of the explants and the effect of lighting in the regeneration of *A. othonianum* Rizz. stem segments. The Murashige and Skoog medium (MS) medium was used in the first test, amended with nine combinations of NAA (0, 5.37 and 10.74 µM) and BAP (0, 4.44 and 8.88 µM) in a completely randomized design. While the second test was arranged as a 2 × 2 factorial, evaluating the orientation of the explants (upright or horizontal) and incubation condition (absence or presence of light). The number of buds, the number and average length of leaves and the number of plantlets were evaluated after 30 and 60 days of growth. The results obtained indicate that the concentrations of BAP and NAA used had no effect on the multiplication of *A. othonianum* Rizz. stem segments, and the best morpho-physiologic *in vitro* response was obtained with horizontal segments in the presence of light.**

Key words: Growth medium, micropropagation, savannah plants.

INTRODUCTION

The savannah vegetation has peculiar characteristics that make it unique among all other vegetation types, which indicate the importance of basic studies for better understanding of the adaptation mechanisms presented by species of this ecosystem. Within the great diversity of this ecosystem, there are fruiting species with potential for use in traditional systems, such as *Anacardium othonianum* Rizz., commonly known as caju-de-árvore-do-cerrado. *A. othonianum* Rizz. has oily fruits that are edible and tasty after roasting, with pericarp identical to cashew's (*Anacardium occidentale* L.) and from which an oil-resin is extracted, which is used to heal skin illnesses. The fleshy peduncle is tasty, acid and cooling, considered as anti-syphilitic. The stem bark is used as anti-diarrheic, as infusion or decoction. The flowers are expectorant, and used as infusion (Silva et al., 2001).

Anacardium species are perennial and predominantly allogamic, with high heterozygosis degree in the natural populations, since the natural propagation process is through seeds (Araújo and Silva, 1995). It is possible that plants in the genus *Anacardium* propagate sexually and asexually (forks, buds and stem nodes), however, its use in forest programs or for commercial orchards demands continuous and large scale seedling production. In

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natural conditions or in nurseries, germination is irregular and slow, and these limitations hinder the rational production of seedlings, as well as the use as fruit crops and in silviculture (Valle Filho, 1991; Oliveira, 2008). Therefore, micropropagation using tissue culture techniques has been a valuable instrument in the propagation of some species of the Anacardiaceae (Boggetti et al., 1999; Das et al., 1999; Mneney and Mantell, 2002; Sansberro et al., 2003; Prakash and Staden, 2008). However, multiplication in commercial scale demands better knowledge of the factors controlling *in vitro* morphogenesis and limiting multiplication and rooting rates.

Conventional systems of morphogenetic responses can be improved by *in vitro* manipulation of determination factors. Several factors can affect the regeneration potential of a given species and the need to select the medium type and the addition of a growth regulator, the type of explant and the environment surrounding it is completely dependent of the desired goal, since their effects are quite distinct. Specific tests are indispensable to establish an ideal protocol with effective combinations and concentrations to induce the expected response (Silva et al., 2005; Fogaça et al., 2007). Despite the economic importance of *A. othonianum* Rizz. for the region, there are no reports about its *in vitro* cultivation. Therefore, this study evaluated the basic procedures such as the relation of auxin/cytokinin, explant orientation and the effect of lighting in the regeneration of stem segments of *A. othonianum* Rizz.

MATERIALS AND METHODS

The tests were done at the Savannah Tissue Culture Laboratory at the Instituto Federal Goiano, Campus de Rio Verde, GO.

Plant materials and asepsis

Ripe fruits of caju-de-árvore-do-cerrado (*A. othonianum* Rizz.) were collected in October 2008, at a farm in Gameleira, county of Montes Claros, GO, located at 16° 06′ 20″ S, 51° 17′ 11″ W at 592 m above sea level. The exsiccate is deposited at the Herbário Jataiense, from Universidade Federal de Goiás, Campus Jataí, under the collection number 3793. The fruits were pulped manually, followed by rinsing in tap water to remove the residues. Subsequently, the seeds were left over paper towels at room temperature to remove excess moisture. The seeds were selected and those malformed were culled, while the ones used in the experiments were treated with the fungicide Vitavax-Thiram® [active ingredient; carboxin + thiram: 200 + 200 g L^{-1}], at 300 ml fungicide for 100 kg seeds. Treated seeds were dehydrated in direct contact with silica gel in plastic trays (35 × 30 × 8 cm) until reaching 13% moisture contents. Finally, the seeds were packaged in plastic bags and stored at 18°C.

Stored seeds were removed in groups of 100 and germinated in plastic trays (50 × 35 × 8 cm), containing washed sand as substrate. The trays were kept under controlled environment with average temperature of 25.61°C and 58.18% air moisture. Phytosanitary control was done with sprays of the systemic fungicide Derosal® commercial product at 0.2%, 24 h before explant collection. Nutrient solution made of the salts from MS medium (Murashige and Skoog, 1962) was applied to the seedlings every two weeks. 30 days after sowing, when seedlings were about 4 cm long, the apical meristems were pruned. The node segments were removed from these meristems and used as explant sources.

Explant disinfestation

The node segments removed from the seedlings were maintained in containers with tap water and three drops of a neutral detergent for 20 min. Subsequently, the segments were immersed in alcohol 70% (v/v) for 30 seconds, followed by immersion in sodium hypochloride solution (20%) for 15 min. Finally, the explants were rinsed three times in a flow hood in sterile distilled water.

In vitro establishment

The 2 cm long node segments were inoculated in test tubes (25 × 150 mm) containing 10 ml culture medium. The medium MS (50%), supplemented with 3% sucrose, 2 g L^{-1} active charcoal and 30 µM BAP (6-benzylaminopurine) was used. Preliminary experiments (data not shown), indicated that better explant growth was observed with this concentration of BAP. Medium pH was adjusted to 5.7 ± 0.3 before autoclaving. Inoculated test tubes were kept in darkness in a growth room at 25 ± 3°C, for 30 days. After this period of incubation in darkness, the explants were transferred to a new growth medium identical to the first ones, and maintained at 25 ± 3°C, under 16 h light, with photosynthesis active radiation of 45 to 55 µmol m^{-2} s^{-1} for 30 days. Subsequently, after 60 days of *in vitro* cultivation, the evaluations as described in Tests I and II were performed.

Test (I): Evaluation of the proportion of auxin/cytokinin in the regeneration of *A. othonianum* Rizz. node segments

Two centimeters long node segments, containing two buds were used as explant sources. These segments were grown in test tubes (25 × 150 mm) containing solid MS medium amended with 2 g L^{-1} active charcoal and different concentrations of auxin and cytokinin. Nine combinations of naphthaleneacetic acid (NAA) and BAP were evaluated (µM): (T1) 0 NAA + 0 BAP; (T2) 0 NAA + 4.44 BAP; (T3) 0 NAA + 8.88 BAP; (T4) 5.37 NAA + 0 BAP; (T5) 5.37 NAA + 4.44 BAP; (T6) 5.37 NAA + 8.88 BAP; (T7) 10.74 NAA + 0 BAP; (T8) 10.74 NAA + 4.44 BAP; (T9) 10.74 NAA + 8.88 BAP. The cultures were maintained at 25 ± 3°C under 16 h lighting, with photosynthesis active radiation of 45 to 55 µmol m^{-2} s^{-1}.

The number of buds and leaves per explant and their average length were evaluated after 30 days of incubation. The plantlets obtained during this period were evaluated, excised and inoculated in the same medium from which they came from; 30 days later, new evaluations similar to the first one were done. Therefore, evaluations were done at 30 and 60 days after the first inoculation. The experimental design was completely randomized, with 25 repetitions consisting of one test tube each. The data on number of buds and leaves were submitted to analysis of variance using the software R (Development Core Team, 2009), and the averages compared by contrasts of non generalized linear models. The average length of plantlets and leaves were submitted to analysis of variance and the averages compared by the Scott Knott test at 5% probability, using the software SISVAR (Ferreira, 2003).

TEST (II): Evaluation of explant orientation and lighting conditions on the regeneration of *A. othonianum* Rizz. segment nodes

Two centimeters long node segments, containing two buds were

used as explant sources. These segments were grown in test tubes (25 × 150 mm) containing solid MS (50%) medium amended with 2 g L^{-1} active charcoal, 30 µM BAP and pH adjusted to 5.7 ± 0.3 before autoclaving. The node segments were inoculated in the culture medium in two orientations: 1) horizontally on the medium surface, and 2) vertically (normal position), with the base end inserted in the medium at a depth of 2 to 3 mm. The cultures were maintained at 25 ± 3°C under 16 h lighting, with photosynthesis active radiation of 45 to 55 µmol m^{-2} s^{-1}.

The number of buds and the number of leaves per explant and their average length were evaluated after 30 days of incubation. The plantlets obtained during this period were evaluated, excised and inoculated in the same medium from which they came from; 30 days later, new evaluations similar to the first one were done. Therefore, evaluations were done at 30 and 60 days after the first inoculation. The experimental design was completely randomized, in a 2 × 2 factorial (explant orientation × lighting) with 25 repetitions, consisting of one test tube each. The data on number of buds and leaves were submitted to analysis of variance using the software R (Development Core Team, 2009), and the averages compared by contrasts of non generalized linear models. The average length of plantlets and leaves were submitted to analysis of variance and the averages compared by the Scott Knott test at 5% probability, using the software SISVAR (Ferreira, 2003).

RESULTS AND DISCUSSION

TEST (I): Evaluation of the proportion auxin/cytokinin in the regeneration of *A. othonianum* Rizz. Node segments

Regeneration of axillary buds from node segments into plants was observed in all treatments. All node segments used regenerated into well formed, vigorous plantlets, with the dark green color characteristic of the mother plant. No morphological alterations were visually observed in the plantlets, nor oxidized or without roots. Also, no clusters or calli were observed (Figure 1). Moreover, the analyses of variance done at 30 and 60 days indicated significant effects of growth regulators on the characteristics evaluated (Figure 2 and Table 1).

The treatment without growth regulator allowed similar growth of explants as the treatments with growth regulators. The non-significant response of the use of NAA and BAP for the characteristics evaluated and in these specific conditions, indicated that the concen-trations used were not enough to supply eventual deficiencies in the endogenous content of plant hormones in the explants. Therefore, the expected multiplication rate was not observed, although this led to increased length without multiple sprouting.

TEST (II): Evaluation of explant orientation and lighting conditions on the regeneration of *A. othonianum* Rizz. segment nodes

The interaction explant position vs. lighting was significant only for leaf length (P≤ 0.05) during the first 30 days growth. Greater leaf length was observed in the presence of light when the explants were inoculated horizontally, with an average of 0.99 leaves per explant (Table 2). The effect of explant orientation was significant for plantlet and leaf number at 30 days (P≤ 0.05). The node segments inoculated horizontally presented longer leaves (0.49 cm) and plantlets (2.39 cm), which were significantly different from those inoculated upright, with averages of 0.08 and 2.02 cm, respectively (Table 2). No differences were observed at 60 days between the treatments. The average leaf length was 0.93 cm for the upright plantlets and 0.99 cm for the horizontal ones. The average plantlet size was 2.58 cm for the upright orientation and 2.77 cm for the horizontal ones.

Furthermore, significant differences were observed of the lighting effect on leaf and plantlet sizes at 30 days. Leaves and plantlets grew more under light, with an average of 0.58 and 2.39 cm, respectively. The same performance was observed at 60 days for both variables, with averages of 1.87 and 3.30 cm, respectively (Table 2). The contrast analysis between treatments (Table 3) indicated a significant effect at 30 days between explant orientation and lighting. The effect of horizontal orientation in the presence of light was greater for the number of leaves. However, no significant effect was observed for all other contrasts. Meanwhile, significant effect was observed at 60 days for the contrast lighting and explant orientation for the number of leaves. The effect of light was greater than its absence for the explants grown upright. No significant effect was observed for all other contrasts (Table 3).

Plantlet regeneration was observed from explants grown in both orientations, although no leaf formation was observed in those kept in darkness; moreover, these presented white color, resulting from the lack of light and etiolation. Plantlets grown under light were well formed and vigorous, with no morphological change or calli formation (Figure 3). In general, better results were obtained from explants inoculated horizontally and grown under light, both at 30 and 60 days (Tables 2 and 3). Explant orientation had no effect on growth after 60 days, although at this stage, it is more interesting to grow the explants upright for the speed of inoculation since the explants are bigger and more vigorous, besides the limitations imposed by the test tube, which restricts growth space and hinders leaf growth, as shown by the better result obtained for the number of leaves in the upright treatment in the presence of light (Table 3). Several authors report that explants grown horizontally are more effective due to the greater contact with the culture medium, favoring the absorption of nutrients (Erig and Schuch, 2002; Silva et al., 2005; Pereira et al., 2006; Erig and Schuch, 2006; Santos et al., 2009). This superiority of the treatment is also related to breaking the apical dominance induced by the vegetative meristem, inhibiting auxin translocation and, consequently, stimulating the growth of lateral buds (Erig and Schuch, 2006; Santana et al., 2009).

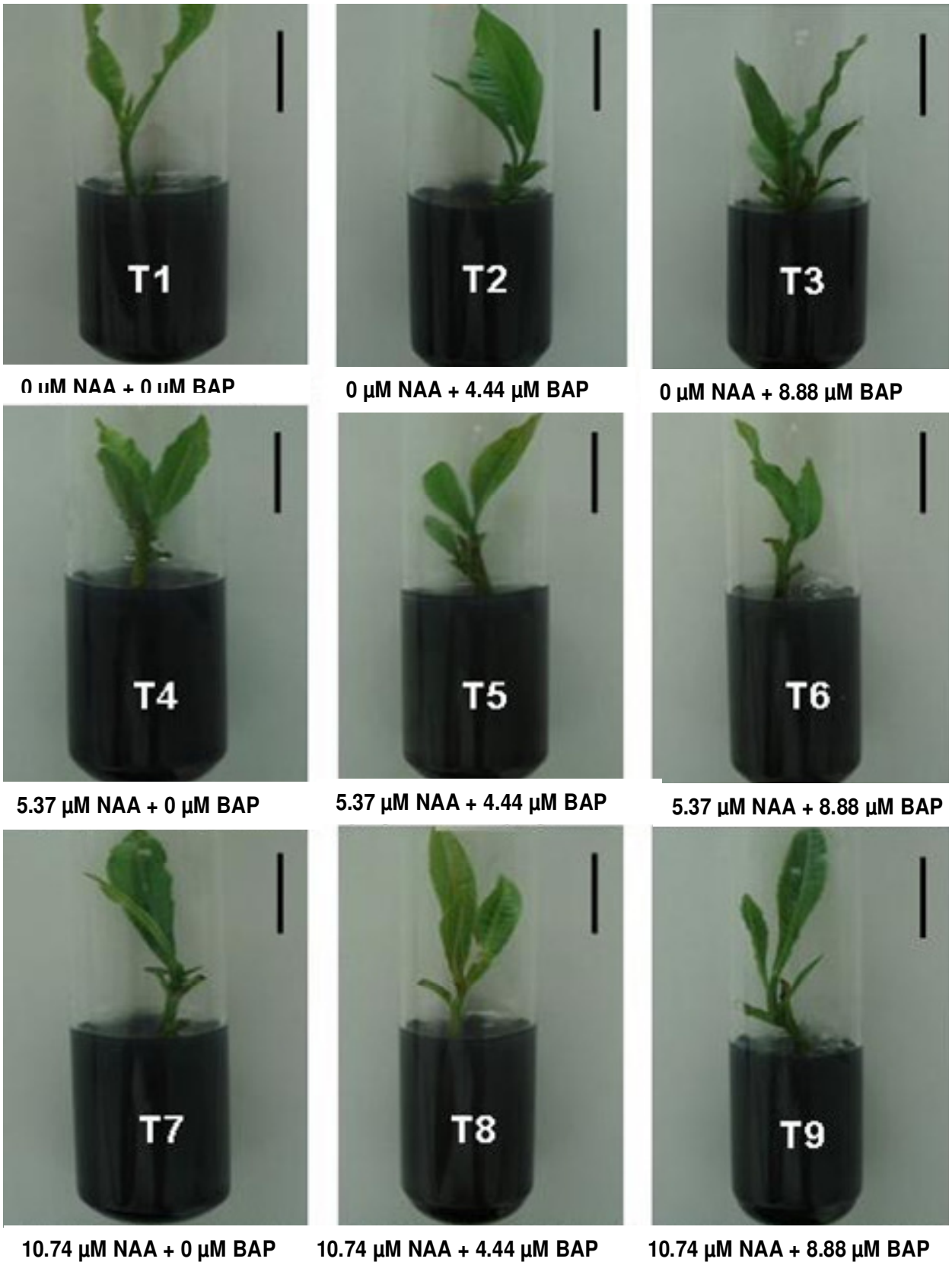

Figure 1. *Anacardium othonianum* Rizz. plantlets from *in vitro* growth (60 days old). Treatments (µM): T1, 0NAA + 0BAP; T2, 0NAA + 4.44BAP; T3, 0NAA + 8.88BAP; T4, 5.37NAA + 0BAP; T5, 5.37NAA + 4.44BAP; T6, 5.37NAA + 8.88BAP; T7, 10.74NAA + 0BAP; T8, 10.74NAA + 4.44BAP; T9, 10.74NAA + 8.88BAP. Bar = 10 mm.

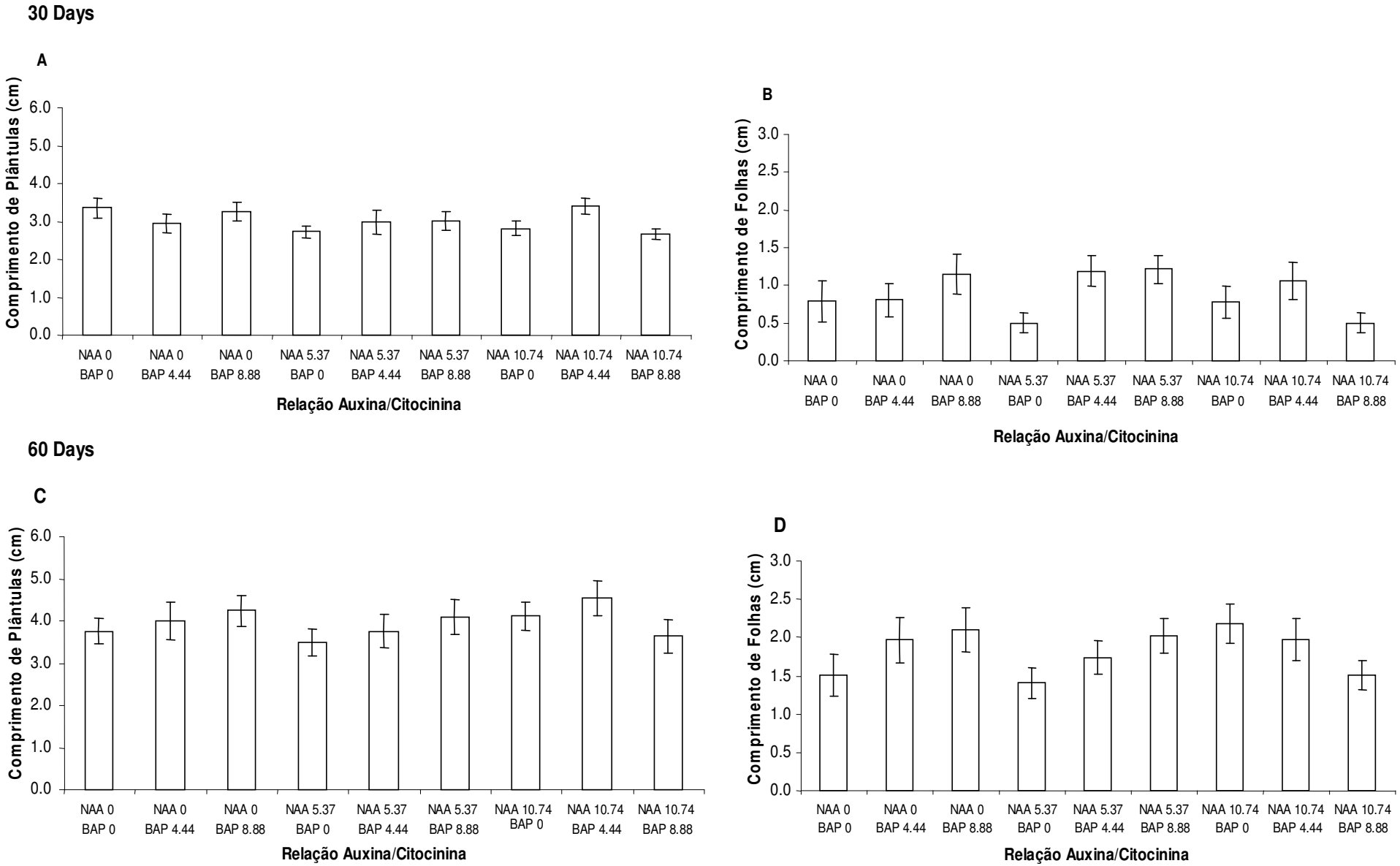

Figure 2. (A) Average plantlet size, (B) average leaf length, at 30 days *in vitro* growth; (C) Average plantlet size, (D) average leaf length, at 60 days *in vitro* growth, as a function of the relation auxin/cytokinin, in μM.

Table 1. Contrast analysis between treatments for the number of leaves and buds of *Anacardium othonianum* Rizz. at 30 and 60 days *in vitro* growth, as a function of the relation auxin/cytokinin.

Contrast	Day	Variable	Estimate	Standard error	Pr(>|z|)
T1 vs. T2, T3, T4, T7, T5, T6, T8, T9	30	Number leaves	0.13	0.15	$0.36^{n.s}$
		Number buds	0.36	0.18	$0.45^{n.s}$
	60	Number leaves	0.19	0.10	$0.44^{n.s}$
		Number buds	0.14	0.17	$0.40^{n.s}$

Treatments (µM): T1, 0NAA + 0BAP; T2, 0NAA + 4.44BAP; T3, 0NAA + 8.88BAP; T4, 5.37NAA + 0BAP; T5, 5.37NAA + 4.44BAP; T6, 5.37NAA + 8.88BAP; T7, 10.74NAA + 0BAP; T8, 10.74NAA + 4.44BAP; T9, 10.74NAA + 8.88BAP. ns, Non significant.

Table 2. Average length of plantlets and leaves of *Anacardium othonianum* Rizz. at 30 and 60 days *in vitro* growth, as a function of explant orientation and lighting.

Variable (cm)	Orientation	Lighting		Average
		Presence	Absence	
30 days growth				
Leaf length	Vertical	0.17^{bA}	0.00^{aA}	0.08^{b}
	Horizontal	0.99^{aA}	0.00^{aB}	0.49^{a}
Average		0.58^{A}	0.00^{B}	
Plantlet length	Vertical	2.10	1.94	2.02^{b}
	Horizontal	2.68	2.10	2.39^{a}
Average		2.39^{A}	2.02^{B}	
60 days growth				
Leaf length	Vertical	1.77	0.10	0.93^{a}
	Horizontal	1.98	0.00	0.99^{a}
Average		1.87^{A}	0.05^{B}	
Plantlet length	Vertical	3.24	1.92	2.58^{a}
	Horizontal	3.36	2.18	2.77^{a}
Average		3.30^{A}	2.05^{B}	

*Averages followed by the same capital letter in each row, and the same small cap letter in each column, are not significantly different by the Scott Knott test at 5% probability.

Table 3. Contrast analysis between treatments for the number of leaves and sprouts of *Anacardium othonianum* Rizz. at 30 and 60 days *in vitro* growth, as a function of explant orientation and incubation conditions.

Fixed Contrast	Contrast	Estimate	Standard error	Pr(>|z|)
30 days growth			**Number of leaves**	
Lighting	OV+P vs. OV+A	18.87	0.37	$0.99^{n.s}$
(P/A)	OH+P vs. OH+A	20.28	0.37	$0.99^{n.s}$
Orientation	P+OV vs. P+OH	-1.15	0.46	0.01*
(OV/OH)	A+OV vs. A+OH	-5.35	0.43	$1.00^{n.s}$
			Number of buds	
Lighting	OV+P vs. OV+A	18.16	0.32	$0.99^{n.s}$
(P/A)	OH+P vs. OH+A	-0.13	0.51	$0.79^{n.s}$
Orientation	P+OV vs. P+OH	0.13	0.51	$0.79^{n.s}$
(OV/OH)	A+OV vs. A+OH	-18.16	0.32	$0.99^{n.s}$

Table 3. Contiune

60 days growth			**Number of leaves**	
Lighting	OV+P vs. OV+A	2.39	0.60	0.00*
(P/A)	OH+P vs. OH+A	19.77	0.32	$0.99^{n.s}$
Orientation	P+OV vs. P+OH	-0.19	0.23	$0.41^{n.s}$
(OV/OH)	A+OV vs. A+OH	17.18	0.32	$0.99^{n.s}$
			Number of buds	
Lighting	OV+P vs. OV+A	0.06	0.34	$0.86^{n.s}$
(P/A)	OH+P vs. OH+A	-0.64	0.37	$0.08^{n.s}$
Orientation	P+OV vs. P+OH	0.43	0.38	$0.26^{n.s}$
(OV/OH)	A+OV vs. A+OH	-0.27	0.33	$0.41^{n.s}$

OV, Vertical orientation; OH, horizontal orientation; P, light presence; A, absence of light. Rio Verde-GO, 2009. *Significant at 5% probability. ns, Non significant.

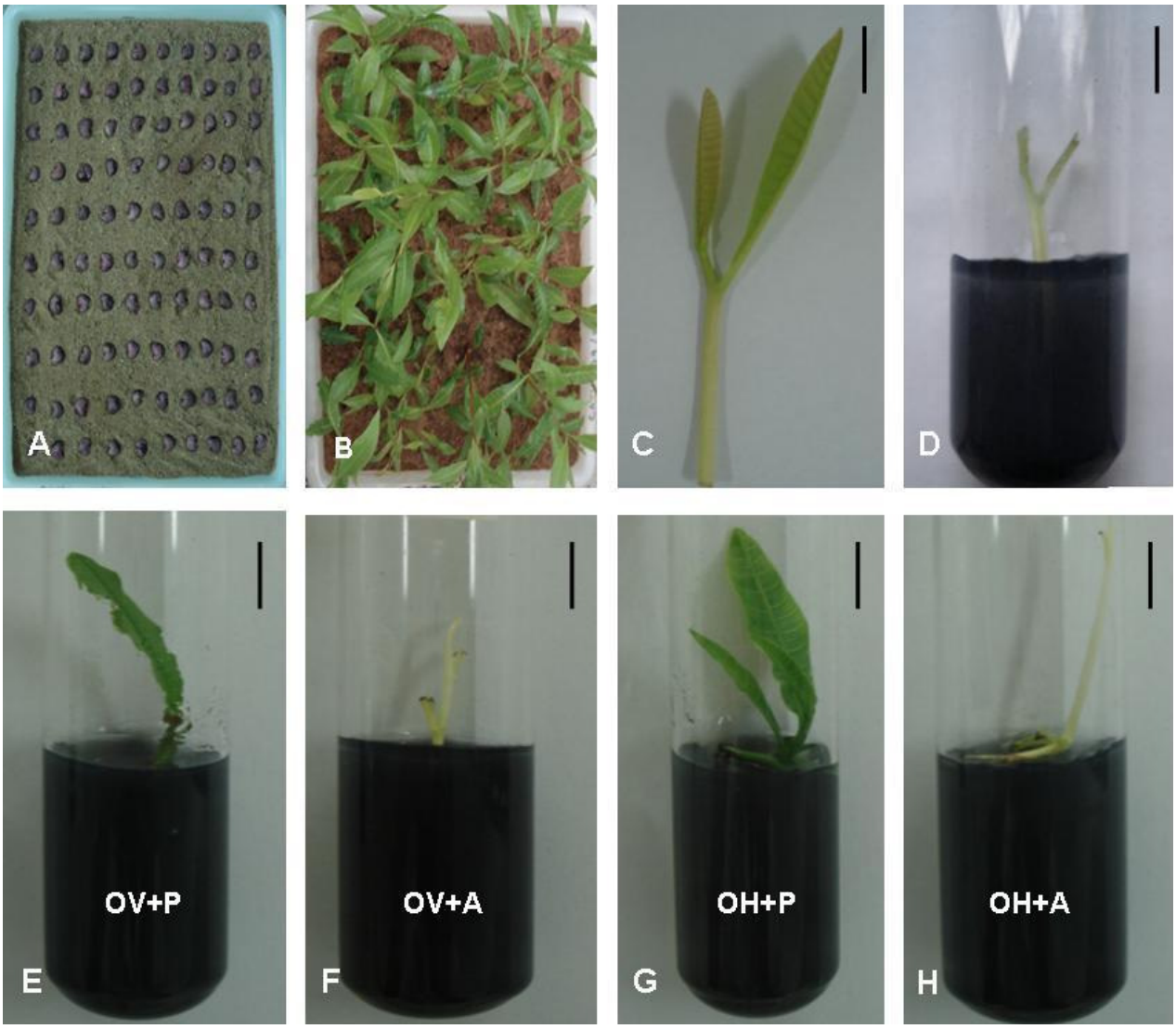

Figure 3. Seeds cultivated in trays (A); plants germinated in trays (B); removed apex of plants germinated in trays (C); nodal segment established *in vitro* (D); plantlet of *Othonianum Rizz.*, to the 60 days of culture (E to H). OV, Vertical orientation; OH, horizontal orientation; P, light presence; A, absence of light. Bar = 10 mm.

Light has a pronounced effect on plant growth and development and can change certain morphological characteristics. Plant growth in darkness is suggested because plant internode elongate, thus separating the nodes which under light, are near each other. Node separation for micropropagation aids development of axillary buds and the manipulation of regenerated plantlets (Pereira et al., 2008). Another important contribution of growing plants in darkness is related to the removal of phenolic substances, which is very common in woody species. However, plant growth in darkness need to be adjusted to each species requirements, since they differ genetically, and different results can be observed under the same cultivation conditions. However, the use of light *in vitro* is questioned by several authors such as Santos et al. (2009), who reported for pequizeiro (*Caryocar brasiliense* A. St.-Hil.) that the buds developed better under light and were longer when grown in darkness. Similar results were reported by Silva et al. (2008) who demonstrated the beneficial effect of light in the micropropagation of sour orange, from epicotyl segments. Opposite results were, however, found by Silva et al. (2005) for cultivation of Cleopatra tangerine (*Citrus reshni* Hort. ex Tan), when maximum *in vitro* production of adventitious buds was obtained in darkness. Similarly, Duran-Vila et al. (1992) also observed that cultivation of explants of orange pineapple (*Citrus sinensis* (L.) Osb.) in darkness increased the regeneration of buds and sprouts.

Conclusion

The concentrations of BAP and NAA used had no effect on the multiplication of segment nodes of *A. othonianum* Rizz. The best *in vitro* morpho-physiologic response was obtained when the node segments were inoculated horizontally and grown in the presence of light.

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