



## ***In vitro* cultivation of *Anacardium othonianum* Rizz.: effects of salt concentration and culture medium volume**

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**ABSTRACT.** *Anacardium othonianum* Rizz. is a medicinal plant species that is native to the Brazilian savannah. Adult plants are different from other genus members in this ecosystem due to their size, and efforts to locate the plants may lead to their extraction from the savannah and, frequently, plant death. Micropropagation has played a significant role in the propagation and preservation of specimens of several plant species; therefore, this study evaluated the effect of various salt concentrations and culture medium volumes on the *in vitro* cultivation of *A. othonianum* Rizz. Trial (I) evaluated two culture media (MS and WPM) and three salt concentrations (100, 50 and 25%) in a completely randomized design. Trial (II) evaluated two culture media and salt concentrations, MS (50%) and WPM (100%), and five medium volumes (10, 15, 20, 25 and 30 mL per test tube) as a 2 x 5 factorial in a completely randomized design. After 30 and 60 days of growth, the percentage of plantlet survival, average plantlet and leaf lengths and the average number of leaves and buds per explant were evaluated. The MS (50 and 25%) media and WPM (100 and 50%) media were the most effective for plantlet regeneration. The best responses were observed in 15- and 25-mL volumes of the MS (50%) medium. Therefore, the use of a 15-mL volume is suggested for greater medium economy.

**Keywords:** cashew, tissue culture, *in vitro* propagation.

## **Cultivo *in vitro* de *Anacardium othonianum* Rizz.: efeito da variação das concentrações dos sais e volumes do meio de cultura**

**RESUMO.** O *Anacardium othonianum* Rizz. é uma espécie frutífera e medicinal nativa do Cerrado brasileiro. As plantas adultas distinguem-se das demais espécies do gênero existente nesse bioma em função do seu porte arbóreo. Sua exploração ocorre de forma extrativista e muitas vezes em caráter predatório. Sob esse contexto, a micropropagação tem dado significativas contribuições na propagação e preservação de caracteres de interesse em diversas espécies de plantas e, desta forma, o objetivo deste trabalho foi avaliar os efeitos de diferentes concentrações de sais e volumes do meio de cultura no cultivo *in vitro* de *A. othonianum* Rizz. No ensaio (I), testou-se dois meios de cultura (MS e WPM) e três concentrações de sais (100, 50 e 25%) dispostos em delineamento inteiramente casualizado. No ensaio (II), testou-se dois meios de cultura MS (50%) e WPM (100%) e cinco volumes de meio (10, 15, 20, 25 e 30 mL por tubo de ensaio) utilizando delineamento inteiramente casualizado, conduzido em arranjo fatorial 2 x 5. Após 30 e 60 dias de cultivo avaliou-se: porcentagem de sobrevivência de plântulas, comprimento médio de plântulas e folhas e, número médio de folhas e gemas por explante. Concluiu-se que os meios MS (50 e 25%) e WPM (100 e 50%) foram os mais eficientes na regeneração de plântulas. Foram observadas as melhores respostas no meio MS (50%) com volumes de 15 e 25 mL, desta forma sugere-se a utilização do volume de 15 mL visando maior economia.

**Palavras-chave:** caju, cultura de tecidos, propagação *in vitro*.

### **Introduction**

The native fruit species of Brazil are genetically diverse and are an important part of the agricultural sector of the country, playing important roles in the country's economic, social and agricultural systems (BASTOS, 2007).

*Anacardium othonianum* Rizz. is native to the Brazilian Savannah and is commonly known as the

caju-de-árvore-do-cerrado. Adult plants differ from genus members in the ecosystem because of their size. Plant height and canopy diameter vary from three to four meters. The pseudo-fruit is pear-shaped and varies in color from yellow to red with a whitish-yellow interior. The species is regionally important, having medicinal properties and widespread acceptance as a food product. However, efforts to locate the

species may lead to their extraction from the savannah and, frequently, plant death (SILVA et al., 2001).

Micropropagation has contributed significantly to the propagation and preservation of specimens of several plant species. Although this process involves a number of stages, the micropropagation protocol, once defined for a given species, can be optimized to obtain high-quality plantlets at a low cost of production. Micropropagation studies of little-known species must define the culture medium to be used because culture media, besides supplying essential elements for growth, also control the pattern of *in vitro* development (GRATTAPAGLIA; MACHADO, 1998).

A variety of culture media have been used for the regeneration of species from various genera. Some of these media were specifically developed to supply the particular demands of the species studied, such as the basic culture medium MS of Murashige and Skoog (1962), which was initially developed for *Nicotiana tabacum* pith tissue, and the Woody Plant Medium (WPM), which was made by Lloyd and McCown (1980) for the propagation of woody plants.

The salt and sugar concentrations within the media have a nutritive effect during *in vitro* growth, but also affect cell growth and morphogenesis, due to their osmotic properties (GEORGE, 1996).

Several formulations of basic media have been used for *in vitro* cultivation. There is no standard formula, but MS medium (MURASHIGE; SKOOG, 1962) has been successfully used, with various modifications and dilutions, for several species. However, for woody species, MS medium does not have the same effect, and compositions with more dilute macronutrients yield better growth and development (GRATTAPAGLIA; MACHADO, 1998). The growth medium WPM (LLOYD; MCCOWN, 1980), for example, contains 25% of the nitrate and ammonia ions found in MS medium, but contains more potassium and sulfate ions, and has been widely and successfully used for the micropropagation of woody species (PASQUAL, 2001).

This work evaluated the effect of various salt concentrations and culture volumes on *in vitro* growth of *A. othonianum* Rizz. in preparation for basic micropropagation studies to preserve the species and clone its elite genotypes.

## Material and methods

### Vegetable Material

The vegetable material used for *in vitro* propagation was removed from *A. othonianum* Rizz. fruits that were collected in October 2008 at the Gameleira farm, which is located in Montes Claros county, GO, with geographical coordinates of 16° 06' 20" S, 51° 17' 11"

W at 592 m above sea level. The plant exsiccate was deposited at the Herbário Jataiense of the Universidade Federal de Goiás, Campus Jataí, under the collection number 3793. The trials were performed in the Savannah Vegetable Tissue Culture Laboratory (Laboratório de Cultura de Tecidos Vegetais Cerrados) of the Instituto Federal Goiano, Campus Rio Verde, Goiás State.

The fruits were hand-pulped and were subsequently washed in tap water. Surface moisture was removed by blotting the seeds with paper towels at room temperature, and malformed seeds were culled. The seeds used were treated with the fungicide Vitavax-Thiram® [Active ingredient (carboxin + thiram): 200 + 200 g L<sup>-1</sup>] at 300 mL fungicide per 100 kg of seeds and were dried in direct contact with silica gel in plastic trays (35 x 30 x 8 cm) until reaching a moisture level of 13%. Subsequently, seeds were packaged in plastic bags and stored at 18°C.

Stored seeds were germinated in groups of 100 in plastic trays (50 x 35 x 8 cm) containing washed sand as a substrate. The trays were maintained in a controlled environment with an average temperature of 25.6°C and a relative moisture of 58.2%. Phytosanitary control was maintained by spraying the systemic fungicide Derosal® at a 0.2% concentration 24 hours before collecting the explants. The seedlings were fertilized every two weeks with a nutrient solution prepared with salts derived from the medium MS (MURASHIGE; SKOOG, 1962).

Thirty days after sowing, when the seedlings were approximately 4 cm long, the nodal segments were removed and used as the explant source.

### *In vitro* establishment

Nodal segments were removed from elite plants and immersed for 20 minutes in water containing three drops of neutral detergent. Subsequently, the nodal segments were immersed for 30 seconds in 70% alcohol (v v<sup>-1</sup>) and for 15 minutes in 20% sodium hypochlorite. A triple rinse was performed with distilled and autoclaved water in a flow hood.

The medium used for establishment of explants was MS (50%), solidified with 4 g L<sup>-1</sup> agar and modified with 3% sucrose, 2 g L<sup>-1</sup> active charcoal and 30 µM BAP (6-benzylaminopurine). Preliminary studies (data not shown) indicated that this concentration of BAP resulted in better explant growth. The pH was adjusted to 5.7 ± 0.3 before autoclaving. Approximately 2 cm of each nodal segment were excised and inoculated in test tubes (25 x 150 mm) containing 10 mL culture medium. Inoculated tubes were kept in the dark in a growth room at 25 ± 3°C for 30 days. After the period in darkness, the explants were transferred to fresh culture

medium and maintained under 16 hours of light per day for 30 days at  $25 \pm 3^\circ\text{C}$  with photosynthetic active radiation of  $45\text{--}55 \mu\text{mol m}^{-2} \text{s}^{-1}$ . The growth evaluations described in Trials I and II were made after 60 days of *in vitro* growth.

**Trial (I) Evaluation of the effect of various growth-media salt concentrations on the regeneration of *A. othonianum* Rizz. nodal segments.**

Nodal segments that were approximately 2 cm long and contained two buds were used as explant sources. These segments were grown in test tubes (25 x 150 mm) containing 20 mL of growth media. MS and WPM culture media (LLOYD; McCOWN, 1980) containing various salt concentrations (100, 50 or 25%) were used. The media were modified with  $2 \text{ g L}^{-1}$  active charcoal and  $30 \mu\text{M}$  BAP; the pH was adjusted to  $5.7 \pm 0.3$  before autoclaving, and the nodal segments were planted after cooling and solidification of the media. The cultures were maintained under 16 hours of light at  $25 \pm 3^\circ\text{C}$  with photosynthetic active radiation of  $45\text{--}55 \mu\text{mol m}^{-2} \text{s}^{-1}$ .

Thirty days later, the survival percentage, the average number of buds and the number and average lengths of leaves and plantlets were evaluated, and the plantlets were excised and inoculated in fresh media. The same variables were evaluated after another 30 days of growth. Therefore, evaluations were made at 30 and 60 days.

The experimental design was completely randomized, with six treatments and 25 repetitions of one test tube each, for a total of 150 experimental units at each growth time point (30 and 60 days). The data for the number of buds and leaves were submitted to analysis of variance using the software R (R DEVELOPMENT CORE TEAM, 2009), and the averages were compared by contrast using non-generalized linear models. The average lengths of plantlets and leaves and the survival percentages were evaluated by analysis of variance, and the averages were compared by the Scott Knott test at 5% probability with the software SISVAR (FERREIRA, 2003).

**Trial (II) Evaluation of the effect of growth medium volume on regeneration of *A. othonianum* Rizz. nodal segments**

The two best media, based on the results of Trial (I), were selected. The media pH was adjusted to  $5.7 \pm 0.3$  before autoclaving. Five volumes (10, 15, 20, 25 or 30 mL) were evaluated for each culture medium.

Two-centimeter-long nodal segments containing two buds each were used as explants and were grown in test tubes (25 x 150 mm). The cultures were kept under 16 hours of light at  $25 \pm 3^\circ\text{C}$  with photosynthetic active radiation of  $45\text{--}55 \mu\text{mol m}^{-2} \text{s}^{-1}$ .

Thirty days later, the survival percentage, the average number of buds and the number and average lengths of leaves and plantlets were evaluated, and the plantlets were excised and inoculated in fresh media. The same variables were evaluated again after another 30 days of growth. Therefore, evaluations were made at 30 and 60 days.

The experimental design was completely randomized as a 2 x 5 factorial (culture media x media volume), and each treatment consisted of 25 repetitions of one test tube, for a total of 250 experimental units for each growth period. The data for the number of buds and leaves were submitted to analysis of variance using the software R (R DEVELOPMENT CORE TEAM, 2009), and the averages were compared by contrast using non-generalized linear models. The average lengths of plantlets and leaves and the survival percentages were evaluated by analysis of variance, and the averages were compared by the Scott Knott test at 5% probability with the software SISVAR (FERREIRA, 2003).

## Results and discussion

**Trial (I) Evaluation of the effect of various growth media salt concentrations on the regeneration of *A. othonianum* Rizz. nodal segments**

After 30 days of growth, the nodal segments regenerated less vigorous plantlets (with a yellowish color) in the MS (100%) medium than did nodal segments regenerated in the other media, which presented well-formed, vigorous, dark-green plantlets. No oxidation, root or calli formation was observed. The MS (100%) medium was significantly different from all other conditions, as determined by the Scott Knott test, with 84% plantlet survival (Table 1).

Differences in plantlet lengths and leaves were observed at 30 days (Figure 1). The MS (50 and 25%) media and WPM (100 and 50%) yielded greater growth with lengths varying from 3.17 to 3.72 cm. The average leaf lengths were 0.97, 1.00 and 0.88 cm in the media MS (50%), MS (25%) and WPM (100%), respectively.

**Table 1.** Percentage of *A. othonianum* Rizz. plantlet survival after 30 and 60 days of *in vitro* growth under various concentrations of MS and WPM media.

Culture medium	Plantlet survival (%)	
	30 Days	60 Days
MS-100%	84 B*	12 B
MS-50%	100 A	96 A
MS-25%	100 A	100 A
WPM-100%	100 A	100 A
WPM-50%	100 A	100 A
WPM-25%	100 A	100 A

\*Averages followed by the same letter in each column do not differ by the Scott Knott test at 5% probability.

After 60 days of growth, most plantlets grown in MS (100%) were dehydrated. The survival percentage was only 12%, differing from all other experimental conditions (Table 1).

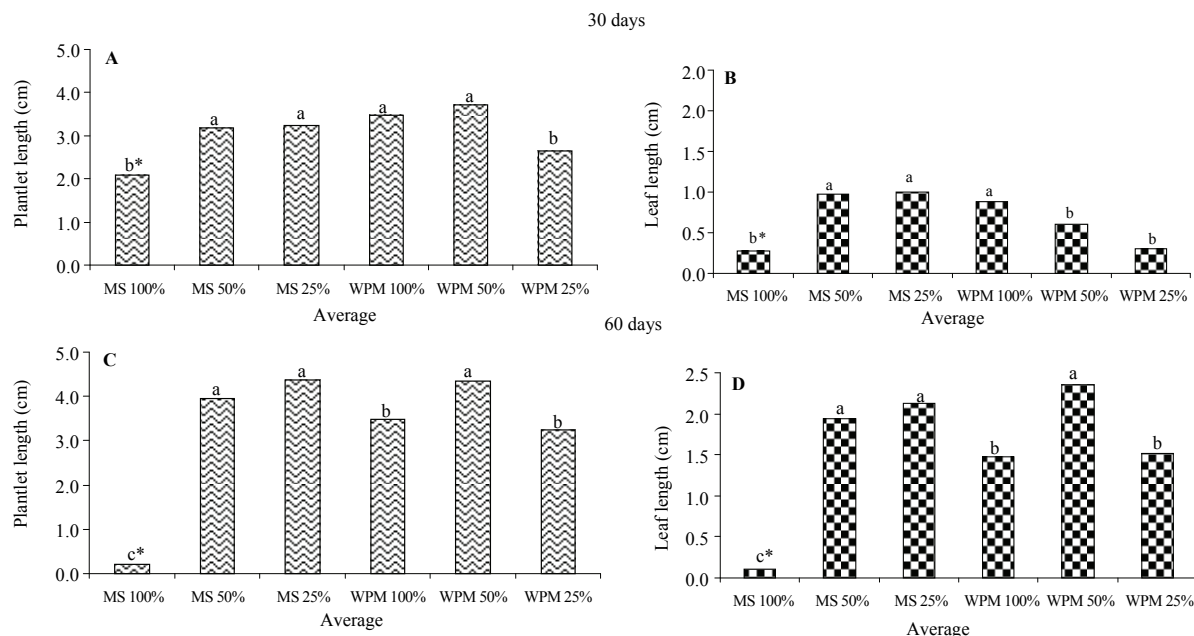
Differences among the variables were observed after 60 days of growth (Figures 1 and 2). Media MS (50%), MS (25%) and WPM (50%) yielded the greatest average plantlet lengths (3.94, 4.36 and 4.34 cm, respectively) and leaf lengths (1.94, 2.13 and 2.36 cm, respectively).

A significant effect on the number of leaves was determined by contrast analysis at 30 days (Table 2). MS (50 and 25%) and WPM (100 and 50%) media had a greater effect than did other media concentrations. No bud formation was observed.

Significant differences were observed by contrast analysis in leaf number and buds after 60 days of

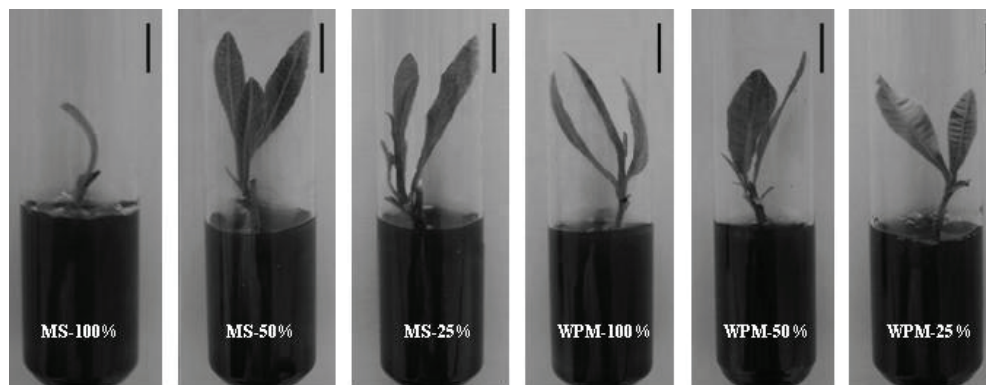
growth (Table 2). The WPM medium had a greater effect than the MS medium for the three concentrations evaluated. The MS (50 and 25%) media had a greater effect than did MS (100%). In contrast, the WPM (100%) medium yielded a significant difference only in the number of leaves.

The MS (100%) medium was the least effective medium with respect to the characteristics evaluated in Trial (I). The MS medium has a higher salt concentration than the WPM culture medium, which appears to have a negative effect on the regeneration of *A. othonianum* Rizz. nodal segments. However, several authors have observed that the best regeneration and *in vitro* growth of *A. occidentale* L. occurs in the MS (100%) medium (ALIYU; AWOPETU, 2005; ANANTHAKRISHNAN et al., 2002; DAS et al., 1996).



**Figure 1.** Average plantlet lengths (A and C) and average leaf lengths (B and D) of *A. othonianum* Rizz. after 30 and 60 days, respectively, in various concentrations of MS and WPM media.

\*Averages followed by the same letter do not differ by the Scott Knott test at 5% probability.



**Figure 2.** Plantlets of *A. othonianum* Rizz. after 60 days of *in vitro* growth in the indicated concentrations of MS and WPM media. 2009. Bar = 10 mm.

**Table 2.** Contrast analyses for the average number of leaves and buds of *A. othonianum* Rizz. after 30 and 60 days of growth in various concentrations of MS and WPM media. 2009.

30 days of growth			
Contrast	Estimate	Standard Error	Pr (>  z )
	Number of leaves		
MS-100+MS-50+MS-25 vs. WPM-100+WPM-50+WPM-25	0.16	0.18	0.37 <sup>ns</sup>
MS-100 vs. MS-50+MS-25	-2.08	0.62	0.00 *
WPM-100 vs. WPM-50+WPM-25	1.26	0.52	0.01 *
MS-50 vs. MS-25	0.00	0.24	1.00 <sup>ns</sup>
WPM-50 vs. WPM-25	1.20	0.38	0.00 *
60 days of growth			
Contrast	Estimate	Standard Error	Pr (>  z )
	Number of leaves		
MS-100+MS-50+MS-25 vs. WPM-100+WPM-50+WPM-25	-0.82	0.26	0.00 *
MS-100 vs. MS-50+MS-25	-2.99	0.71	0.00 *
WPM-100 vs. WPM-50+WPM-25	0.41	0.20	0.04 *
MS-50 vs. MS-25	-0.20	0.22	0.37 <sup>ns</sup>
WPM-50 vs. WPM-25	0.47	0.26	0.07 <sup>ns</sup>
Number of buds			
MS-100+MS-50+MS-25 vs. WPM-100+WPM-50+WPM-25	-1.07	0.39	0.00 *
MS-100 vs. MS-50+MS-25	-2.29	1.02	0.02 *
WPM-100 vs. WPM-50+WPM-25	-0.18	0.34	0.59 <sup>ns</sup>
MS-50 vs. MS-25	-0.20	0.44	0.65 <sup>ns</sup>
WPM-50 vs. WPM-25	-0.20	0.37	0.57 <sup>ns</sup>

\*significant at 5% probability; <sup>ns</sup> not significant.

Salt reduction is generally beneficial for the culture growth of woody species. This was confirmed in Trial (I) by reducing the salt concentration of the MS medium or by using the WPM medium; however, the WPM (25%) medium was not effective for supplying the plantlets' needs and promoting satisfactory growth. Other authors have also observed that reducing the salt concentrations of culture media favored *in vitro* growth of *A. occidentale* (LÉDO et al., 2007; RADMANN et al., 2009a and b; THIMMAPPAIAH et al., 2002).

Our experiments have shown that the MS (50 and 25%) media and the WPM (100 and 50%) media were the most effective in Trial (I) for the regeneration and growth of nodal segments of *A. othonianum* Rizz.

**Trial (II) Evaluation of the effect of the growth-medium volume on the regeneration of *A. othonianum* Rizz. nodal segments**

Consequently, Trial (II) evaluated the MS (50%) medium and the WPM (100%) medium, each supplemented with 2 g L<sup>-1</sup> active charcoal and 30 µM BAP.

The type of media and media volume each had significant effects on the average leaf length, but these effects were only observed when the nodal segments were grown for 30 days (Table 3). MS medium volumes of 15, 20 and 25 mL promoted greater leaf growth (3.03, 3.29 and 3.17 cm, respectively) than did volumes of 10 and 30 mL (2.19 and 2.54 cm, respectively). The 10-mL volume promoted significant leaf growth in the WPM medium (3.46 cm), while in the MS medium, the average was 2.19 cm (Table 3).

The culture media alone had no significant effect on plantlet and leaf lengths after 30 days (Table 3). The average plantlet length was 4.70 cm for the MS

medium and 4.50 cm for the WPM medium. The average leaf length was 2.85 cm for MS and 2.89 cm for WPM. Significant differences were found after 60 days for plantlet lengths only, with averages of 5.46 cm for medium MS and 4.69 cm for WPM. Volume alone did not have a significant effect on either plantlet or leaf lengths at 30 and 60 days (Table 3).

Contrast analysis of the varying conditions (Table 4) indicated a significant difference between the two media at each volume after 30 days of growth. The effect of the MS medium on the number of leaves was greatest at 30 mL. The MS medium also had a greater effect on the number of buds at volumes of 10, 15 and 25 mL. None of the other contrasts were significant at 30 days.

After 60 days, significant effects were observed when the culture media were contrasted at each volume. The MS medium mediated an increase in the number of leaves at volumes of 15, 25 and 30 mL. A greater number of buds were found in 10, 15 and 25 mL volumes of MS. None of the other contrasts were significant (Table 4).

Both culture media mediated the formation of vigorous plantlets at all volumes tested, with no calli and no root formation from the nodal segments (Figure 3).

The internal space available for explant growth has a significant effect on the *in vitro* development of explants. In addition to the medium volume, this space varies with container shape and volume. These variables are infrequently analyzed, although they affect the air volume within the container and the depth of the growth medium in it. These factors also affect the gas composition within the container and, consequently, culture growth and development (PEREIRA et al., 2006).

**Table 3.** Average plantlet and leaf lengths of *A. othonianum* Rizz. after 30 and 60 days of growth in various volumes of culture media. 2009.

		30 days growth					
Variable (cm)	Media	Volume (mL)					Average
		10	15	20	25	30	
Plantlet length	MS	4.18*	5.22	4.98	5.02	4.12	4.70 a
	WPM	4.80	4.60	4.54	4.44	4.16	4.50 a
Average		4.49 A	4.91 A	4.76 A	4.73 A	4.14 A	
Leaf length	MS	2.19 b B	3.03 a A	3.29 a A	3.17 a A	2.54 a B	2.85 a
	WPM	3.46 a A	2.91 a A	2.67 a A	2.83 a A	2.57 a A	2.89 a
Average		2.83 A	2.97 A	2.98 A	3.00 A	2.55 A	

		60 days growth					
Variable (cm)	Media	Volume (mL)					Average
		10	15	20	25	30	
Plantlet length	MS	4.90	6.16	5.68	5.64	4.94	5.46 a
	WPM	5.14	4.92	5.10	4.36	3.96	4.69 b
Average		5.02 A	5.54 A	5.39 A	5.00 A	4.45 A	
Leaf length	MS	2.72	3.48	3.26	3.08	2.70	3.05 a
	WPM	3.05	3.02	2.95	2.67	2.33	2.81 a
Average		2.89 A	3.25 A	3.10 A	2.87 A	2.52 A	

\*Averages followed by the same uppercase letter in each row and the same lowercase letter in each column are not different by the Scott Knott test at 5% probability.

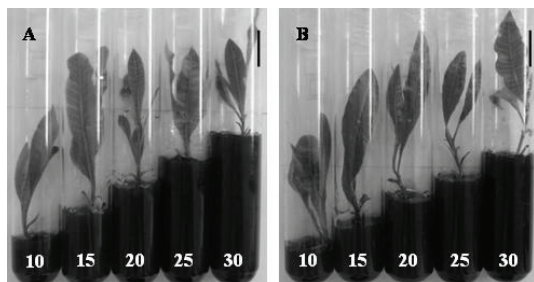
**Table 4.** Contrast analysis of the numbers of leaves and buds of *A. othonianum* Rizz. after 30 and 60 days of growth in various volumes of culture media. 2009.

		30 days growth			
Fixed Contrast	Contrast	Estimate	Standard Error	Pr(>  z )	
					Number of leaves
Volumes (mL)	MS-10 vs. WPM-10	0.22	0.24	0.34 <sup>ns</sup>	
	MS-15 vs. WPM-15	0.36	0.23	0.13 <sup>ns</sup>	
	MS-20 vs. WPM-20	0.08	0.24	0.71 <sup>ns</sup>	
	MS-25 vs. WPM-25	0.33	0.26	0.19 <sup>ns</sup>	
	MS-30 vs. WPM-30	0.55	0.25	0.02*	
Number of buds					
Volumes (mL)	MS-10 vs. WPM-10	1.38	0.55	0.01*	
	MS-15 vs. WPM-15	1.16	0.51	0.02*	
	MS-20 vs. WPM-20	0.53	0.47	0.25 <sup>ns</sup>	
	MS-25 vs. WPM-25	1.87	0.75	0.01*	
	MS-30 vs. WPM-30	0.69	0.46	0.13 <sup>ns</sup>	

		60 days growth			
Fixed Contrast	Contrast	Estimate	Standard Error	Pr(>  z )	
					Number of leaves
Volumes (mL)	MS-10 vs. WPM-10	0.24	0.18	0.19 <sup>ns</sup>	
	MS-15 vs. WPM-15	0.45	0.19	0.01*	
	MS-20 vs. WPM-20	0.16	0.18	0.36 <sup>ns</sup>	
	MS-25 vs. WPM-25	0.53	0.19	0.00*	
	MS-30 vs. WPM-30	0.72	0.21	0.00*	
Number of buds					
Volumes (mL)	MS-10 vs. WPM-10	1.84	0.62	0.00*	
	MS-15 vs. WPM-15	2.14	0.74	0.00*	
	MS-20 vs. WPM-20	0.40	0.52	0.44 <sup>ns</sup>	
	MS-25 vs. WPM-25	2.19	1.05	0.03*	
	MS-30 vs. WPM-30	0.31	0.46	0.49 <sup>ns</sup>	

\*significant at 5% probability; <sup>ns</sup>not significant.

**Figure 3.** Plantlets of *A. othonianum* Rizz. after 60 days of *in vitro* growth. Numbers indicate media volume in mL. MS (50%) medium (A); WPM medium (B). 2009. Bar = 10 mm.

Previous studies have analyzed the ideal volume of culture medium for the growth of various species

(CARVALHO et al., 1995; PEREIRA et al., 2006; REIS et al., 2004, 2007). Other studies have also evaluated various container volumes for their effect on plant growth (CAMOLESI et al., 2010; NICOLOSO; ERIG, 2002).

In summary, the MS (50%) medium showed greater potential than the WPM medium for supporting plantlet growth. Volumes of 15 and 25 mL yielded the best responses; the use of the 15 mL medium volume would, therefore, be the most economical.

### Conclusion

The MS (50 and 25%) media and the WPM (100 and 50%) media were most effective for

*Anacardium othonianum* Rizz. plantlet regeneration. The best plantlet regeneration was observed in the MS (50%) medium in volumes of 15 and 25 mL; therefore, the use of the 15-mL volume is suggested for maximal economy.

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