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Use of temporary immersion bioreactors on *in vitro* culture of cactus pear

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This work was conducted with the objective of evaluating the performance of cactus pear cultivars on *in vitro* culture using bioreactors and the conventional method. The treatments were arranged in a 3 × 2 factorial scheme with a completely randomized design, and the three cactus pear cultivars (*Orelha de elefante mexicana*, *Miúda* and *IPA-Sertânia*) were combined with two micropropagation methods (conventional and temporary immersion bioreactors), totaling six treatments with five replicates each. After 30 days, the following variables were evaluated: cladode length, fresh explant matter mass, number of shoots and number of roots. There was a significant interaction between the cultivar factor and the micropropagation method for the cladode length and fresh matter mass, with bioreactors being the most responsive. However, regarding to the number of shoots and number of roots, there was no interaction between these factors under the same level of significance, where the conventional crop stood out. The results indicate different morphogenetic responses among the tested cultivars, and specific *in vitro* propagation protocols should be developed for each one.

Key words: Micropropagation, bioreactor, plant productivity, cactus pear cultivars.

INTRODUCTION

The cactus pear, *Opuntia* and *Nopalea* species, are an important resource for the maintenance of livestock in Brazilian semi-arid region, as well as food, guarantees water supply to animals during periods of drought (Frota

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et al., 2015). In general, cactus pear has low levels of dry matter (6.1 to 17.1%) and crude protein (2.9 to 6.0%) being rich in minerals, mainly calcium, potassium and magnesium.

According to Donato et al. (2017), the growing interest in cactus pear is due to productive security, associated to a set of measures necessary to reduce the risks of production's loss, and which enables the farmer to guarantee harvest. Already, Silva et al. (2017), said that choosing the appropriate cultivar, among other factors, can increase productive and qualitative safety, sustainability and resilience of activity, which is very important in a family farming crop.

The cactus pear cv. *Miúda* - IPA-100004 [*Nopalea cochenillifera* Salm-Dyck], *Orelha de elefante mexicana* - IPA-200016 [*Opuntia stricta* (Haw.) Haw] and *IPA-Sertânia* - IPA-200205 [*N. cochenillifera* Salm-Dyck] show good production of dry matter and are resistant to carmine scale (*Dactyloius opuntiae*), the main crop pest (Santos et al., 2006). These factors ensured the great acceptance by the rural producers and the increase in the cultivated area.

Cactus pear seedlings can be obtained from cladodes from a palm tree or through tissue culture. The latter provides uniform propagative material free of phytopathogenic agents, but they are still at a high price because of their production's cost.

Recent advances in biotechnology and plant tissue culture have provided new protocols and equipment aimed at minimizing production's costs, increasing seed multiplication *in vitro*, and reducing laboratory time. Among these equipment are bioreactors.

According to Teixeira (2002), bioreactors were developed from equipment known as fermenters, which were previously used in the cultivation of cells and microorganisms, aiming at the production of secondary metabolites and alkaloids for industrial purposes. Currently, they have been used in micropropagation of plants based on temporary or permanent immersion of vegetable tissues in liquid medium.

In the temporary immersion system the vegetable tissue is periodically bathed with the nutrient medium that is pumped into the container containing the explants and returning to the container that stores it, promotes the periodic exchange of the culture flask atmosphere (Fogaça et al., 2006). This renewal of the atmosphere provides a reduction of stressors, such as the gases accumulation.

Of the numerous advantages provided by temporary immersion bioreactors (TIB) and pointed out by Escalona et al. (1999) are renovation of the internal atmosphere, increased photosynthetic and respiration rates, improved nutrient uptake, increased plant multiplication rate and reduced crop manipulation.

There are reports of success in the use of TIB's for large-scale propagation of various species, such as banana (Lemos et al., 2001), sugarcane (Lorenzo et al., 1998), orchid (Paek et al., 2001) and pineapple (Silva et al., 2007), presenting a multiplication rate higher than conventional micropropagation.

In this sense, the objective of this work was to analyze the performance of cactus pears explants cv. *Miúda*, cv. *Orelha de elefante mexicana* and cv. *IPA-Sertânia* micropropagated in a conventional way and with use of temporary immersion bioreactors.

MATERIALS AND METHODS

The experiment was conducted at the Plant Biotechnology Laboratory of Agricultural Research Company of Minas Gerais - EPAMIG, EPAMIG Norte - Gorutuba Experimental Field, Nova Porteirinha - MG, during the year 2018.

Cactus pear explants (cv. *Miúda*, cv. *Orelha de elefante mexicana* e cv. *IPA-Sertânia*) used in the experiment were obtained from the *in vitro* establishment in MS solid medium (Murashige and Skoog, 1962) of young cladodes buds supplemented with 30 g L^{-1} sucrose, 0.1 g L^{-1} inositol, 4 mg L^{-1} benzylamine-6-purine (BAP) and 7 g L^{-1} agar with pH adjusted to 5.8. This material remained in the growth room for 60 days, or until sprouting was emitted, under irradiance of 40 µmol m^{-2} s^{-1} provided by cold white light, with 16 h of photoperiod at 25 ± 2°C. Sprouting originated was subcultured in culture medium of the same composition for 90 days, reaching the fourth stage.

In vitro culture, two methods were used, the conventional one using the solid culture medium (7 g L^{-1} agar) and the TIB's using the liquid medium with immersion of 3 min every 4 h. Explants of three cultivars of forage palm were used: cv. *Miúda*, cv. *Orelha de elefante mexicana* and cv. *IPA-Sertânia*.

The treatments were arranged in a 3 × 2 factorial scheme with a completely randomized design, and the three cactus pear cultivars were combined with the two micropropagation methods, thus totaling six treatments with five replications, in which each replicate was composed of two explants.

The explants had their length reduced to 15 mm (± 1 mm) and were then packed in temporary immersion bioreactors and in conventional subculture bottles.

In conventional micropropagation, 50 mL of MS culture medium supplemented with 30 g L^{-1} sucrose, 0.1 g L^{-1} inositol, 7 g L^{-1} agar, 0.5 mg L^{-1} of naphthalene-acetic acid (ANA) and 1 mg L^{-1} of BAP.

A culture medium of similar composition was used in the temporary immersion bioreactor, but no agar was used and 200 mg L^{-1} of ascorbic acid and 0.25 ml of Plant Preservative Mixture (PPM, Sigma) were added. The immersion time of the explants was adjusted to 3 min every 4 h. In both methods, the pH of the culture medium was adjusted to 5.8 and autoclaved at 120°C for 20 min.

The treatments were kept in a growth room, submitted to a 16-h photoperiod obtained from white LED lamps (40 µmol m^{-2} s^{-1}) and a mean temperature of 25°C for a period of 30 days. At the end of this period, the following parameters were evaluated: cladode length (mm), fresh matter mass of the explants (g), number of shoots (unit) and number of roots (unit).

The data collected were submitted to analysis of variance ($F < 0.05$) by the statistical program Sisvar 5.6 (Ferreira, 2008) and, when significant, Test F was carried out for the method and T test for the cultivars. The mean values of the fresh mass of the explants, number of roots and number of shoots were transformed to $(X + 0.5)^{0.5}$, the latter analyzed at 10% significance.

RESULTS AND DISCUSSION

Based on the results obtained, the interaction between

Table 1. Mean values of forage palm cladode length (mm), obtained at 30 days of *in vitro* culture under different micropropagation methods and cultivars.

Method	Cultivars			
	OEM	**Miúda**	**IPA-Sertânia**	**Average**
Conventional	13.7^{Ba}	14.1^{Ba}	14.3^{Aa}	14.0
TIB	17.7^{Ab}	19.8^{Aa}	14.6^{Ac}	17.3
Average	15.7	16.9	14.4	-
CV (%)	8.05	-	-	-

Means followed by the same capital letter in the column and lowercase in the row do not differ by the T test at 5% significance.

Table 2. Mean values transformed to $(X + 0.5)^{0.5}$ of the fresh mass of forage palm explants (g), obtained at 30 days of *in vitro* culture under different micropropagation methods and cultivars.

Method	Cultivars			
	OEM	**Miúda**	**IPA-Sertânia**	**Average**
Conventional	0.16^{Ba}	0.23^{Aa}	0.21^{Aa}	0.20
TIB	0.60A^{a}	0.29^{Ab}	0.15^{Ac}	0.34
Average	0.38	0.26	0.18	-
CV (%)	6.36	-	-	-

Means followed by the same capital letter in the column and lowercase in the row do not differ by the T test at 5% significance.

the factors cultivar and cultivation methods was observed when the cladode length and fresh mass were evaluated at 30 days (Tables 1 and 2).

In this study, it was observed that the use of the TIB provided better mean values for cladode length in the *Orelha de elefante mexicana* (OEM) and *Miúda* cultivars, but did not differ in the cultivar *IPA-Sertânia* (Table 1).

A similar event occurred when the fresh mass was evaluated, where OEM cultivation using bioreactor was more responsive and did not differ in the others (Table 2).

These results demonstrate that the use of bioreactors can increase the performance of cultivars *Orelha de elefante mexicana* and *Miúda* and indicate the tendency of the cultivars to respond differently to the techniques used. It is noteworthy that these belong to different species and genus and their responses *in vitro* culture are related to nutritional requirements and different morphogenetic characteristics.

Silva et al. (2017), when working with these three cactus pear cultivars comparing the growth, productivity and relationships with the meteorological variables, verified differences both morphologically and in the productive parameters. Also in this work, it was noticed that the OEM stands out as the one that produces the most fresh matter, revalidating the data obtained in the present work.

Nobel (2001) and Pimienta-Barrios et al. (2005) reported that in favorable times *Opuntia* species alter the pattern of CO_2 capture, thus promoting an increase in growth, accumulation of reserves and increase in productivity. This factor may have influenced the performance of the *Orelha de elefante mexicana*, belonging to the genus *Opuntia*, on the others under the favorable conditions provided by the bioreactor, conditioning the production of almost four times more fresh mass than when compared to the other method.

Similar results were obtained by Medeiros (2011), working with different methods of *in vitro* cultivation combined with different doses of BAP in the micropropagation of cactus pear cv. *Orelha de elefante mexicana*, which observed the highest fresh mass gain of the shoots when using the temporary immersion system with high frequency of immersion.

Silva et al. (2017) evaluated the efficiency of water use and nutrient use of these cultivars, and observed that the *Orelha de elefante mexicana* and *IPA-Sertânia* stand out in the water use efficiency considering fresh mass production. In the efficiency of nutrient use, the genotypes had similar performances, with the exception of the element magnesium and sodium that was higher for *Orelha de elefante mexicana*.

According to Taiz and Zeiger (2004), magnesium is an important structural constituent of chlorophyll and acts on the activation of photosynthesis, respiration and nucleic acid synthesis enzymes. For Frizzone et al. (2005), increasing their uptake by the plant may promote increased crop productivity. However, sodium has great importance in the stomatal activity of CAM species, which

Table 3. Mean values transformed to $(X + 0.5)^{0.5}$ of the number of roots of forage palm (units), obtained at 30 days of *in vitro* cultivation under different cultivation methods and cultivars.

Parameter	Methods		Cultivars			CV (%)
	TIB	Conventional	OEM	Miúda	IPA-Sertânia	
Number of roots	0.26^{B}	1.20^{A}	0.20^{B}	0.90^{AB}	1.10^{A}	28.72

Means followed by the same letter in the line do not differ from each other by the F test for method and T test to cultivate, both at 5% significance.

gives greater efficiency in the use of water (Taiz and Zeiger, 2004), being essential to the survival of crops such as forage palm.

When analyzing the cultivar unfolding in each cultivation method, it was observed that the cultivars did not differ using the conventional method in both cladode length and fresh mass (Tables 1 and 2). For the cultivars in the bioreactor, it was observed that these differed from each other, standing out the *Orelha de elefante mexicana* and *Miúda* in the fresh mass and length of the cladodium, respectively.

This performance is probably related to the types of culture media used in both methods. On the one hand, in the conventional cultivation, the action of the gelling agent of the culture medium and the solid state that only allows the absorption of the nutrients by parts of the explant that are in direct contact with the medium, thus suppressing the development of the explants. On the other hand, the use of the liquid medium in the bioreactor and its greater absorption by the tissue and constant renewal of the air during the period of transfer of the medium.

Other authors have already demonstrated the superiority of bioreactors in relation to the conventional technique in hybrid clones of *Eucalyptus globulus* (Correia, 2011), in banana cv. Dwarf Cavendish (Farahani and Majd, 2012), in bamboo (Ribeiro et al., 2016) and in sugarcane (Matoso et al., 2017), which confirm the data presented in the present study. The results also identify the need for an adjustment in the *in vitro* propagation protocol for the cultivars in question, as they present individuals under the same conditions.

For the number of roots, there was no significant interaction between the cultivar factors and the cultivation method, but a significant difference was observed between the methods and the cultivars alone. Conventional *in vitro* cultivation provided the highest number of roots and the most responsive cultivar was *IPA-Sertânia* (Table 3).

Bioreactors, due to the use of liquid medium, provide a greater contact of the tissue of the explants with the medium and promote a greater absorption of nutrients and phytoregulators. With the higher absorption of BAP, cytokinin responsible for cell division and shoot morphogenesis probably influenced the development of the roots due to the increase of its concentration in the tissue, inhibiting the action of auxins and suppressing root initiation.

Araújo et al. (2008), working with the *in vitro* multiplication of 'smooth cayenne' pineapple using different concentrations of BAP and ANA in the culture medium, observed that the non-use of BAP stimulated the rooting and the greater growth of the explants. For them the availability and interaction of auxins and cytokinins regulate the formation of root, shoot and callus. Taiz and Zeiger (2004) reiterate this theory by saying that plant morphogenesis is guided by the balance of auxins/cytokinins, which in high/low favor rooting and the reverse balance promotes aerial part formation.

The cultivar *IPA-Sertânia* was the most responsive to the development of roots differing from the *Orelha de elefante mexicana*, which shows that besides the cultivation method, the cultivar also influences the *in vitro* morphogenesis of forage palm, indicating the need to establish specific protocols for each one.

Similar results were observed by Frota et al. (2004), which worked with proliferation and rooting of 10 clones of cactus pear [*Opuntia ficus-indica* (L.) Mill.] under different growth regulators and found different responses to rooting and sprouting among the clones. Alves et al. (2013) also observed variability of response among the studied genotypes to benzyladenine (BA) concentrations in budding induction, thus corroborating with the results found in the present study.

According to Vasconcelos et al. (2010), in addition to the type of growth regulators, their concentration and combination with other regulators, the response of explants *in vitro* also varies among species, variety, age and physiological state of the material used.

For the number of shoots, there was no interaction between the cultivar and method factors, being these analyzed in isolation. There was a significant difference in the cultivation method factor, with conventional cultivation giving the highest number of shoots. The cultivars did not differ from each other (Table 4).

The lower contact surface between the medium and the explant in conventional culture restricts the uptake of the growth regulators present in the medium. In this sense, the lower concentration of BAP in the tissue probably benefited shoot formation, since the plant growth regulators have activity under low concentrations.

For Taiz and Zeiger (2004), the effect of cytokinins is

Table 4. Mean values transformed to $(X + 0.5)^{0.5}$ of the number of forage palm shoots (units), obtained at 30 days of *in vitro* cultivation under different cultivation methods and cultivars.

Parameter	Methods		Cultivars			CV (%)
	TIB	Conventional	OEM	Miúda	IPA-Sertânia	
Number of shoots	0.0^{B}	0.2^{A}	0.2^{A}	0.1^{A}	0.0^{A}	19.69

Means followed by the same letter in the line do not differ from each other by the F test for cultivation method and T test to cultivate, both at 10% significance.

regulated by the enzyme cytokinin oxidase present in many plant tissues. It irreversibly inactivates cytokinins when high concentrations of these hormones are found in tissues.

Similar results were obtained by Medeiros (2011), working with different methods of *in vitro* cultivation combined with different doses of BAP in the micropropagation of cactus pear cv. *Orelha de elefante mexicana*, where they observed that in the treatments that had greater availability of the cytokinin and contact with the vegetal tissue caused a decrease in the emission of shoots with the increase in the concentration of BAP. For them the reduction of the BAP availability in the medium, either by the addition of the agar or reduction in the frequency of immersion, reduces the phytotoxic effect of the regulator even with the increase of the concentration of the same in the medium.

The greater exploitation of the medium by the explants, provided in the temporary immersion system of the bioreactors, can promote reduction in the cost of production of the seedlings *in vitro*, since it induces the use of formulations of culture media with lower concentrations of the reagents. However, it is necessary to adjust the concentrations of the phytoregulators for the propagation of the cactus pear cultivars using this cultivation method.

Conclusions

Temporary immersion bioreactors are a promising alternative to the propagation of cactus pear *in vitro*. The tested cultivars presented different performances for the number of roots, cladode length and fresh mass.

The cultivar *Orelha de elefante mexicana* had the best performance using the temporary immersion bioreactors.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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