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EFFECT OF GROWTH REGULATORS ON THE ORGANOGENESIS AND MULTIPLICATION OF *ORTEGOCACTUS MACDOUGALLII* ALEXANDER

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Abstract

Ortegocactus macdougallii is highly appreciated ornamental and endemic Mexican cactus recently classified as a threatened plant species by the Government of Mexico. In order to rescue this plant from the risk of extinction and because conventional systems produce low propagation rates, we established a reliable and efficient method of micropropagation based in tubercle culture. During the establishment stage, varies concentracions of the combination of NAA (1.3, 4.4, and 13.3 μM) and BA (1.3, 4.4, and 13.3 μM) induced callus and shoot formation when explants (tubercles and axillary meristems or areoles dissected from adult plants) were incubated on Murashige and Skoog medium. In adition to this, the NAA free variants of the medium stimulated the rooting during the establishment stage, in particular the treatments including low and medium concentrations of BA (1.3 and 4.4 μM). During shoot proliferation, treatments including NAA (4.4 μM) and BA at 13.3, 19.9 or 26.6 μM produced between 5 and 6 shoots per explant; however, shoots obtained on medium with NAA 4.4 and BA 13.3 μM were statistically higher (5 mm). Rooting was produced on quarter- or half-strength MS medium but half-strength MS medium supplemented with IBA (1.6 or 4.9 μM) resulted in higher percent of rooted shoots (75%) and mean root number (8).

Key words: Cactaceae, cacti, *in vitro* propagation, micropropagation

INTRODUCTION

Ortegocactus macdougallii is a highly appreciated ornamental cactus, endemic to Mexico, and distributed in a small area located in the municipality of San José Lachiguiri in the State of Oaxaca (Alexander 1961, Weightman 2004). It is the only species of the monotypic genus *Ortegocactus* (Pilbeam and Weightman 2006, Weightman 2004). Unfortunately, native populations are threatened by extinction as a result of soil degradation and human over-collection of adult plants, their fruits, seeds, and seedlings (Pilbeam and Weightman 2006). In addition to this, *Ortegocactus macdougallii* has very slow growth rates, reduced sprout production, low germination and self-incompatibility that results in poor fruit and seed set, and low survival of seedlings growing in natural habitats, which makes the future of this plant species a critical issue. Therefore, the Mexican Government recently classified *Ortegocactus macdougallii* as a threatened plant species (Nom-059-Semarnat 2010), and development of successful large-scale propagation is among the urgent measures for its conservation.

The *in vitro* propagation of cacti species reduces the time for shoot production (Ault and Blackmon 1987) and could assist for mass production (Escobar et al. 1986, Ali et al. 2001, Velázquez-Enciso and Soltero-Quintana 2001, Estrada-Luna et al. 2008) of genetically identical (Pérez-Molphe-Balch and Dávila-Figueroroa 2002, Wyka et al. 2006) and healthy plants for the market (Estrada-Luna et al. 2002). Also, it could rescue their

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threatened populations (Clayton et al. 1990, Giusti et al. 2002, Dávila-Figueroa et al. 2005, Ramírez-Malagón et al. 2007) and could be useful for the establishment of germplasm banks (Dávila-Figueroa et al. 2005, Cardarelli et al. 2010). Micropropagation, in particular, has been studied extensively and used for the successful cloning of more than 50 different cacti, including at least 35 threatened or endangered species (Clayton et al. 1990, Fay and Gratton 1992).

Cacti can be tissue-cultured by areole activation (axillary shoot formation) (Mauseth 1977, Machado and Prioli 1996, Wyka et al. 2006), adventitious shoots formation (Minocha and Mehra 1974), and somatic embryogenesis induced from callus culture (Infante 1992, Stuppy and Nagl 1992, Ali et al. 2001, Gomes et al. 2006, Angulo-Bejarano and Paredes-López 2011). Shoot neo-organogenesis through adventitious shoots, in which shoots are generated directly from explants without nodes has not been reported yet. Depending on the availability of biological materials, the cultures may be initiated from seedlings (Choreño-Tapia et al. 2002, Moebius-Goldammer et al. 2003, Gómez-Juárez et al. 2006), isolated areoles, mammillae, tubercles or flower sections derived from adult plants (Rubluo et al. 2002, Estrada-Luna et al. 2008, Wyka 2008).

The objectives of the present study were to test the effect of N^6-benzyladenine (BA), α-naphthaleneacetic acid (NAA) and their combination on shoot induction and optimum proliferation and to determine the effect of medium strength and indole-3-butyric acid (IBA) on adventitious root formation in the regenerated shoots.

MATERIALS AND METHODS

Plant material

Healthy adult plants of *Ortegocactus macdougallii* seven-year old and 8 cm high, obtained from a local nursery retailer were used as experimental material. For a period of 12 months, they were grown in a greenhouse with a maximum photosynthetic photon flux density (PPFD) of 1,000 μmol m^2 s^{-1}, an average of day/night temperature of 30/20°C, and fertilization provided with the commercial fertilizer Peters Professional 20%-20%-20% (N-P-K) (Scotts-Sierra Horticultural Products Co., Marysville, OH, USA), once a month. The stock plants were treated with pesticides Confidor®(Bayer) and Agrimec® (Syngenta) to control whiteflies and mites, respectively.

Young shoots (0.5-1.5 cm in length) were excised from the stock plants, washed with running tap water, and cleaned by soaking and mechanic agitation for 15 min in a solution containing a commercial detergent (Liqui-Nox, Alconox Inc., New York). The shoots were consecutively disinfected in ethanol (70%, v/v) for 10 min and commercial bleach (20%, v/v) [Clorox (6% NaOCl)] plus Tween-20 (Sigma-Aldrich Chemical) (0.1%, v/v), for 20 min. Finally, they were washed three times with de-ionized sterile water. Single or multiple tubercles, including one to three areoles, were dissected and used as initial explants.

Micropropagation

For the culture establishment, we tested MS (Murashige and Skoog 1962) medium supplemented with NAA (Sigma-Aldrich Chemical) and BA (Sigma-Aldrich Chemical), which were supplied alone or in combinations at different concentrations including 0, 1.3, 4.4, and 13.3 μM. Each treatment was represented by six explants. After 116 days of culture, mean number of shoots, explants producing calluses (%), rooted explants (%), root number, and root length (mm) were determined. The data for explants producing calluses and rooted explants were not statistical analysed because Ortegocactus macdougallii is endongered species and limited quantity of initial explants was possible to be obtained.

For the multiplication stage, we run an experiment using MS medium supplemented with combination of NAA (4.4 μM) and BA (13.3 μM, 19.9 μM or 26.6 μM). The experiment was set up to compare the best treatment obtained from the initial cultures (NAA 4.4 μM and BA 13.3 μM) against two new BA concentrations (19.9 μM and 26.6 μM). Each treatment was represented by eight culture flasks containing six explants. The experiment was repeated at two times. After 110 days of culture, mean number of shoots, shoot length (mm), shoot fresh weight (mg), rooted explants (%) root number, and root length (mm) were recorded. Data in percentage were arcsin transformated before statistical analysis.

Rooting

Half-strength MS medium, supplemented with 1.6 or 4.9 μM IBA, was used for the experiments. The plant growth regulator-free, quarter- or half-strength MS medium was used as control. Each treatment was represented by eight culture flasks containing three explants each. After 50 days of culture, the percent of explants producing roots, mean number of roots, and root length (mm) were determined. Data in percentage were arcsine transformed before the statistical analysis.

Culture conditions

All variants of the medium were supplemented with 5% sucrose and 0.7% bacto-agar. The pH was adjusted to 5.7 before autoclaving at 121°C for 20 min. The cultures were grown in a cultivation chamber at 27 ± 3ºC with 16 h of cool white fluorescent light that provided about 50 μmol m^2 s^{-1} of PPFD.

Statistical data analysis

The data of all experiments were subjected to one-way analysis of variance (ANOVA), followed by a Tukey mean separation test at $p \leq 0.05$.

RESULTS

The initial cultures revealed very low rates of contamination (4%) and no tissue oxidation (data not shown). After 116 days of culture initiation, callus and organ formation (axillary and adventitious shoots, and adventitious roots) emerged. Calluses were induced in all variants of the medium containing NAA, irrespective of the concentration and the presence of cytokinin. Calluses were soft and friable with crystalline appearance with red or green color (Fig. 1A). Later, they became hard and compact, showing very slow growth rates. ANOVA detected statistically significant difference only between the variant with and without NAA (Table 1).

During the establishment stage, the explant response for shoot formation was considered to be very poor, because only two of the twelve treaments evaluated (1.3 µM NAA + 13.3 µM BA and 4.4 µM NAA and 13.3 µM BA), induced shoot differentiation and, from these treatments, four explants produced a total of 17 adventitious and axillary shoots. Stereoscopic observations of the cultures showed direct adventitious bud formation and axillary meristem activation of the areoles (breaking areole dormancy) as the ways to produce shoots from the explants (tubercles). The adventitious shoot primordia were differentiated in the

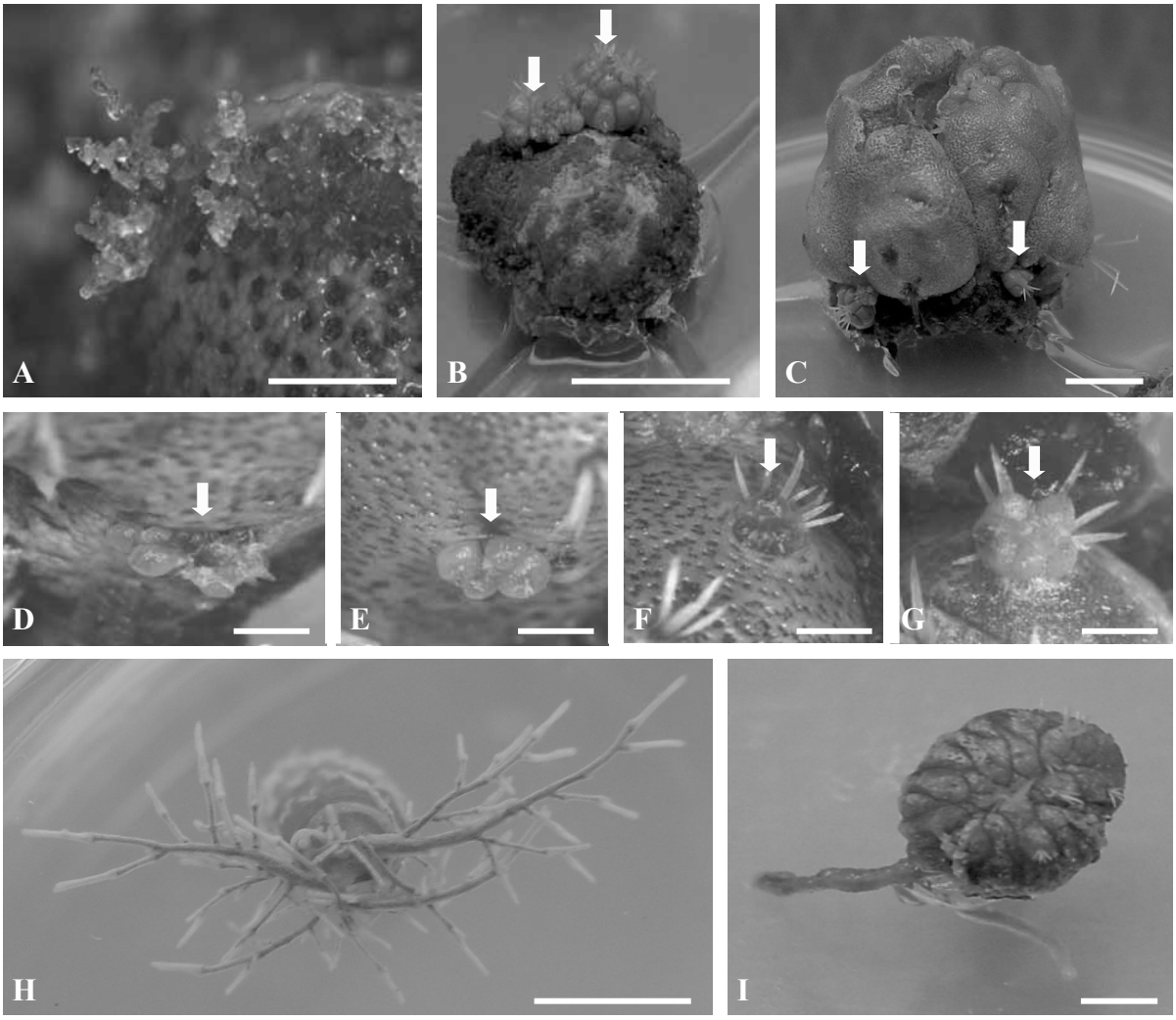

Fig. 1. Effect of growth regulators on explants of *Ortegocactus macdougallii* during micropropagation. A) Explant with a growing red callus (bar = 1 mm), B) Adventitious shoots developing on the edge of the explant (arrows) (bar = 10 mm), C) Adventitious shoots differentiated on the base of the explant (arrows) (bar = 10 mm), D) Adventitious bud initials after 54 days of culture (bar = 2 mm), E) Young emerging adventitious shoots (bar = 2 mm), F) Bud initials from areole activation after 54 days of culture (bar = 2 mm). G) Young emerging axillary shoot (bar = 2 mm), H) Adventitious roots differentiated after 50 days of culture (bar = 10 mm), I) Regenerated plantlet (bar = 5 mm).

Table 1. Effect of cytokinin and auxin on *in vitro* cultured explants of *Ortegocactus macdougallii*.

Treatment (μM)	Explants producing calluses (%)	Explants producing roots (%)	Root number	Root length (mm)
BA 1.3 + NAA 0.0	0.00	66.67	5.00 ± 0.71 b	11.33 ± 2.60 a
BA 4.4 + NAA 0.0	0.00	83.33	9.00 ± 1.47 a	10.28 ± 1.25 ab
BA 13.3 + NAA 0.0	0.00	33.33	4.25 ± 0.95 b	4.48 ± 1.11 cd
BA 1.3 + NAA 1.3	50.00	0.00	0.00 ± 0.00 c	0.00 ± 0.00 d
BA 4.4 + NAA 1.3	75.00	0.00	0.00 ± 0.00 c	0.00 ± 0.00 d
BA 13.3 + NAA 1.3	100.00	0.00	0.00 ± 0.00 c	0.00 ± 0.00 d
BA 1.3 + NAA 4.4	100.00	50.00	8.25 ± 0.63 a	4.10 ± 0.90 cd
BA 4.4 + NAA 4.4	100.00	0.00	0.00 ± 0.00 c	0.00 ± 0.00 d
BA 13.3 + NAA 4.4	100.00	0.00	0.00 ± 0.00 c	0.00 ± 0.00 d
BA 1.3 + NAA 13.3	50.00	100.00	8.50 ± 0.96 a	6.38 ± 0.90 bc
BA 4.4 + NAA 13.3	100.00	0.00	0.00 ± 0.00 c	0.00 ± 0.00 d
BA 13.3 + NAA 13.3	100.00	0.00	0.00 ± 0.00 c	0.00 ± 0.00 d
MSD	23.7203	4.8080	3.1334	3.1582

Means ± SE within a column followed by the same letter are not significantly different according to the Tukey mean separation test ($p < 0.05$). MSD - Minimum significant difference.

surface of the explants in areas delimiting tubercles, at the edge of the explants or at the basal zone of explants (Fig. 1B,C), where no areoles were present.

After induction and early stages of cell differentiation, the primordia of adventitious buds looked like a disorganized group of structures (Fig. 1D). However, five weeks later, the primordia grew-up to become organized structures with tiny tubercles colored with the typical olive green observed in young and adult plants (Fig. 1E). The shoots regenerated from axillary meristem activation of areoles were also evident after 56 days of the *in vitro* culture (Fig. 1F), which after five weeks grew-up to produce the shoots. The treatments supplemented only with BA (1.3, 4.4, and 13.3 μM) were not able to break the dormancy of axillary buds.

It was found that all NAA free variants of the medium stimulate the rooting during the establishment stage, in particular the treatments including low and medium concentrations of BA (1.3 and 4.4 μM). The explants cultivated in low concentrations of BA (1.3 μM) in presence of increased concentrations of NAA (4.4 and 13.3 μM), produced adventitious roots in addition to the calluses. The percent of explants producing roots varied according the variant of the medium used but the higher value was obtained with low concentration of BA (1.3 μM) and medium concentration of NAA (4.4 μM). The root number and root length showed differences with statistical significance among treatments. The superior treatments for root number were 0 μM NAA and 4.4 μM BA, 13.3 μM NAA and 1.3 μM BA, 4.4 μM NAA and 1.3 μM BA, which produced 9, 8.5 and 8.25 roots per explant, respectively. The ANOVA detected statisticaly significant differences among treatments, in which low concentrations of BA (1.3 and 4.4 μM) resulted in higher average lengths (11.33 and 10.28 mm, respectively), as compared to the other treatments (Table 1).

Our results showed that the shoot multiplication stage seems to be controlled by an adequate balance of auxin and cytokinin, in which the optimum concentration of NAA was 4.4 μM in combination with increased concentrations of BA (13.3, 19.9, and 26.6 μM). The shoot number ranged between 5.33 and 5.67 and the shoot fresh weight ranged from 327.64 to 388.62 mg, however, the ANOVAs and the mean separation tests did not show significant differences among the treatments for these two variables. For the shoot length, the treatment of 4.4 μM NAA and 13.3 μM BA produced

Table 2. Effect of cytokinin and auxin on shoot proliferation during multiplication stage of *Ortegocactus macdougallii*.

Treatment (μM)	Shoot number	Shoot length (mm)	Shoot fresh weight (mg)
BA 13.3 + NAA 4.4	5.67 ± 0.49 a	4.78 ± 0.17 a	340.48 ± 47.06 a
BA 19.9 + NAA 4.4	5.83 ± 0.65 a	3.93 ± 0.26 b	388.62 ± 36.79 a
BA 26.6 + NAA 4.4	5.33 ± 0.72 a	3.67 ± 0.15 b	327.64 ± 26.17 a
MSD	2.3051	0.7309	138.1700

Means ± SE within a column followed by the same letter are not significantly different according to the Tukey mean separation test ($p < 0.05$). MSD - Minimum significant difference.

Table 3. Effect of cytokinin and auxin during multiplication stage on adventitious root formation of regenerated shoots of *Ortegocactus macdougallii*.

Treatment (μM)	Explants producing roots (%)	Root number	Root length (mm)
BA 13.3 + NAA 4.4	38.83 ± 5.57a	1.25 ± 0.24 a	7.71 ± 1.15 a
BA 19.9 + NAA 4.4	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b
BA 26.6 + NAA 4.4	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b
MSD	3.673	0.5125	2.4408

Means ± SE within a column followed by the same letter are not significantly different according to the Tukey mean separation test ($p < 0.05$). MSD - Minimum significant difference.

significantly higher shoots (4.78 mm) than the other two treatments (Table 2). It is interesting to note that increased concentrations of BA (19.9 μM and 26.6 μM) produced cristate or monstrous shoots (fasciated shoots). Given that *Ortegocactus macdougallii* is a slow growing plant species, the adequate time for shoot formation was considered to be 110 days.

As observed with the initial cultures, shoot formation during multiplication occurred through areole activation and direct *de novo* differentiation (Fig. 1B-G). In addition to shoot proliferation, these treatments also induced adventitious root formation in some explants. The statistical analysis of data obtained for explants producing roots, root number and root length showed highly significant difference among the treatments. Treatment with 4.4 μM NAA and 13.3 μM BA was the only one that induced roots (Table 3). However, the mean number of formed roots (1.25) can be considered to be a poor response.

Adventitious root formation was observed after 50 days of cultures in all treatments studied. ANOVA showed statistically significant differences among treatments in the percentage of rooted explants and root number. The low (1.6 μM) and medium (4.9 μM) concentrations of IBA in the medium significantly improved the rooted explants and root number. The highest concentration of IBA (4.9 μM) resulted in a higher percentage of rooted shoots (75%) and enhanced the mean root number (9.9), compared with the other treatments (Table 4). In contrast to the observations made during the establishment stage in which adventitious roots were produced after 110 days of culture, in this case, the root primordia were visible after 30 days and 20 days later highly differentiated and elongated roots were observed (Fig. 1H) in complete regenerated plantlets (Fig. 1I). The regenerated shoots of *Ortegocactus macdougallii* differentiated adventitious roots without the induction of calluses.

DISCUSSION

Our study demonstrated that BA in combination with NAA in an adequate balance was able to control and promote callus, shoots, and adventitious roots formation. These responses were induced during initial cultures and subsequent multiplication. Callogenesis was induced by auxins (NAA), irrespective of the concentration and the presence of cytokinin (BA). Particularly, the auxin concentration requirements for *Ortegocactus macdougalli* were relatively low and the optimum responses were in agreement with the results reported for most cacti, which are between 2.23 and 22.29 μM NAA (Vyskot and Jará 1984, Choreño-Tapia et al. 2002, Wyka et al. 2006, Karimi-Narges and Ebrahimi-Morteza 2010, Ruvalcaba-Ruiz et al. 2010). However, other species such as *Cephalocereus senilis* responded better to higher concentrations (Nava-Esparza and Yañez 1984).

In vitro shoot formation of *Ortegocactus macdougallii* was induced only by the auxin-cytokinin interaction. However, the shoots were induced through direct adventitious shoots formation and activation of axillary pre-formed meristems present in dormant

Table 4. Effect of strength of MS medium and auxin concentration on adventitious root formation of regenerated shoots of *Ortegocactus macdougallii*.

Treatment	Root formation (%)	Root number	Root length (mm)
Quarter-strength MS medium	58.34 ± 8.34 ab	4.17 ± 0.48 b	11.32 ± 1.95 a
Half-strength MS medium	37.49 ± 11.68 b	5.00 ± 1.37 b	9.96 ± 3.05 a
Half-strength MS medium + 1.6 mM IBA	45.83 ± 6.11 ab	6.33 ± 0.77 ab	8.99 ± 0.91 a
Half-strength MS medium + 4.9 mM IBA	75.03 ± 5.45 a	9.92 ± 1.91 a	8.37 ± 1.56 a
MSD	3.9990	4.9854	7.9985

Means ± SE within a column followed by the same letter are not significantly different according to the Tukey mean separation test ($p < 0.05$). MSD - Minimum significant difference.

areoles (axillary shoots). According to Hartmann et al. (2011), adventitious bud formation is induced through a dedifferentiation process in which a single parenchyma cell located either in the epidermis or just below the surface of the stem develops into a shoot system with no callus production. The process involves the capability of previously developed and highly differentiated cells to initiate cell divisions and form a new meristematic growing point. These later become organized to produce single or multiple young shoots located in different regions of explants: between two tubercles, in areas where no areoles (axillary buds meristems) were present, or at the base of the explants (Fig. 1B-E). We took these observations as evidence that the shoots do not originate from pre-existing meristems. The axillary shoots came from the activation of axillary meristems by breaking the dormancy of pre-existing meristems in the areoles. In terms of clonal propagation, this way of producing shoots is preferred because it maintains genetic stability. In contrast, *de novo* differentiation sometimes promotes off-type aberrant shoots and genetic variation (Machado and Prioli 1996, Hartmann et al. 2011). During the multiplication stage, the treatments with higher concentrations of BA (19.9 and 26.6 μM) induced both the formation of normal and fasciated shoots with more robust constitution. The fasciation or cristation refers to a phenomenon that produces morphological variation of plant organs (White 1948, Papafotiou et al. 2001, Iliev and Kitin 2011). The variation is the result of changes in the physiology of the plants produced by the environmental conditions or the effect of plant growth regulators, in particular cytokinins (BA), or changes in genetic factors such as the activation and expression of the CLAVATA family genes (Iliev and Kitin 2011).

In all plant species, including cacti cultured *in vitro*, the balance of auxins and cytokinins in the medium is of great importance to induce the process of shoot regeneration. This was described on the first reports on cacti *in vitro* culture (King 1957, Sachar and Iyer 1959) and later confirmed by Johnson and Emino (1979) and Mauseth (1979). However, this observation does not constitute a rule, since the activation and subsequent areolar direct sprouting has also been observed in some species such as *Opuntia* spp. (Estrada-Luna et al. 2002, 2008), *Sulcorebutia alba* Rausch (Dabekaussen et al. 1991), *Schlumbergera truncata* (Haworth) Morán (Perez et al. 1999), *Coryphantha retusa* (Britton & Rose) (Ruvalcava-Ruiz et al. 2010), with the mere presence of cytokinins.

During micropropagation, the cacti responses are dependent on genotype, as well as on different types of cytokinin and different concentrations. Some species such as *Hylocereus undatus* and *Leuchtembergia principis* have relatively high requirements and produced good results with 44.6 μM of BA (Starling 1985, Loeza et al. 2001). Pérez et al. (1999) reported that sprouting production for *Schlumbergera truncata* explants was obtained with even higher concentrations, of 89.16 μM BA; however, most of the studied species have optimal responses at rather low concentrations (4.4 and 8.8 μM BA), as shown in the data obtained by Perez-Molphe-Balch et al. (1998) and Pérez-Molphe-Balch and Dávila-Figueroa (2002) for *Mammillaria formosa, Nyctocereus serpentinus, Pelecyphora aselliformis,* and *P. strobiliformis*. The present study showed that low concentrations of cytokinin yielded the best results for bud induction and proliferation. The proliferation rate of *Ortegocactus macdougallii* was within the range obtained for most tissue culture cacti (4 to 10 shoots).

Ortegocactus macdougallii could produce adventitious roots without the presence of growth regulators (Tables 1 and 3). This may be due to the endogenous auxin concentration in the explants that makes them adequate to promote formation of adventitious roots, as it occurs when the explants are subjected to cutting propagation in nursery conditions (Jackson 1986). Adventitious roots were also produced when the variants of the medium were supplemented with auxins. However, the addition of IBA (4.9 μM) enhanced the rhizogenic process, which resulted in higher percentage of rooted shoots and higher root number (Table 4). In horticulture, the stimulatory effect of auxins on adventitious root formation has been successfully used for cutting propagation (Taiz and Zeiger 2010, Hartmann et al. 2011). Although many cacti such as *Opuntia* (Estrada-Luna et al. 2008), *Pilocereus robinii* (Quiala et al. 2009), *Hylocereus undatus* (Mohamed-Yasseen 2002), *Turbinicarpus laui* (Mata-Rosas et al. 2001), differentiate roots when cultured *in vitro* or *in vivo* without the presence of auxins, most cactus species require addition of auxins, in particular IBA, to achieve rooting or to improve the root quality.

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