**INFLUENCE OF GROWTH REGULATORS ON THE ORGANOGENESIS AND MULTIPLICATION OF *Ortegocactus macdougallii* ALEXANDER, A THREATENED MEXICAN CACTUS.**

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**Abstract***Ortegocactus macdougalli* A., is an 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 rates of propagation, we performed a series of experiments to establish the scientific basis for a more reliable and efficient method through micropropagation. The response of explants comprising tissues of tubercles and axillary meristems (areoles) to two plant growth regulators were initially studied. Then, the conditions for the shoot proliferation stage were optimized by modifying the best treatment obtained from the initial cultures with two new treatments of 6-Benzylaminopurine (BA) and the same concentration of α-Naphtalenacetic acid (NAA). During the rooting stage, four treatments to induce adventitious root formation were tested. The regenerated plantlets were transplanted, acclimatized, and evaluated for plant performance and survival after six months of greenhouse culture. A successful *in vitro* propagation system in which shoot induction and proliferation are induced by two organogenic phenomena (direct adventitious bud formation and activation of pre-formed axillary meristems of areoles) is published for the first time, in this article. It is estimated that the use of this system may regenerate about 15,625 plantlets after five rounds of subcultures and about 21 months of culture.**Key Words:** *Ortegocactus macdougalli* Alexander, Biznaga Pistache de Chico Ortega, micropropagation, *in vitro* propagation, Cacti, Cactaceae.

Running Title: Micropropagation of *Ortegocactus macdougallii* A*.*

Abbreviations: NAA: α-Naphthaleneacetic acid, BA: 6-Benzylaminopurine, MS: Murashige and Skoog (1962) culture medium, PPFD: photosynthetic photon flux density, ANOVA: Analysis of variance.

**INTRODUCTION**The Cactacea family includes about 130 genera and 2,000 species. These plants are important from an ecological point of view [Arias 1993, Bravo-Hollis 1978, Comisión Nacional para el Conocimiento y Uso de la Biodiversidad (CONABIO) 1997]; as ornamentals (Anderson 2001, Cullmann et al. 1986), and for the products they provide —fruits, seeds, stems, roots, flowers—; which are known to contain several useful chemical compounds with desirable nutritional and medicinal properties (Gentile et al. 2004, Karimi-Narges and Ebrahimi-Morteza 2010, Utkarsha et al. 2010). *Ortegocactus macdougallii* A., in particular, is a highly appreciated ornamental cactus, commonly referred to, in Spanish, as “Biznaga Pistache de Chico Ortega". This plant is endemic in Mexico and is distributed in a small area located in the municipality of San José Lachiguiri of the State of Oaxaca (Alexander 1961, Weightman 2004). It belongs to the monotypic genus *Ortegocactus,* which only includes the *macdougallii* species (Pilbeam and Weightman 2006, Weightman 2004). Unfortunately, native populations of this cactus show a clear tendency to disappear as the result of a combination of different factors, including soil degradation and human over-collection of adult plants, fruits, seeds, and seedlings. Adult plants are coveted by collectors because of their unique morphological traits (yellow flowers, tiny size, and the olive-green color of the stems) (Pilbeam and Weightman 2006). Other factors, which relate to its biology and propagation, make the future of this plant species a critical issue. Some of these factors are: very slow growth rates, reduced sprout production, low germination, incompatibility that results in a poor fruit and seed set, and low survival of seedlings growing under natural habitats. In response to this situation, the Mexican government recently classified *Ortegocactus macdougallii* A., as a threatened plant species (NOM-059-SEMARNAT-2010). It has now become urgent to study its biology and physiology to establish the scientific basis and the strategies to rescue and protect native populations**.**

*Ortegocactus macdougallii* A. can be propagated by seeds or through cuttings, however, the rates of propagation are very low because the plants produce very few propagules per year. In this sense, the use of tissue culture techniques, which have proven to be an excellent tool for propagating many plant species (Hartmann et al. 2010), could assist not only in the rescue and sustainable conservation of this plant but also in the massive propagation to ensure plant availability for reforestation of native communities and, also, to satisfy the commercial demand from the nursery and ornamental industries. Micropropagation, in particular, has been studied extensively to establish the scientific basis for cloning more than 50 different cacti, including at least 35 threatened or endangered species. The success of micropropagation systems, when used for cacti, lies in the fact that they offer additional advantages when compared with conventional propagation systems: 1) they reduce the time for shoot production (Ault and Blackmon 1987); 2) they produce true-to-type or genetically stable individuals through activation of axillary meristems from areoles (Pérez-Molphe-Balch and Dávila-Figueroa 2002, Wyka et al. 2006); 3) they produce healthy shoots that, in addition to regenerating whole plantlets, can be used for micro-grafting different genotypes for ornamental purposes (Estrada-Luna et al. 2002); 4) they are extremely efficient when used for massive propagation (Ali et al. 2001, Escobar et al. 1986, Estrada-Luna et al. 2008, Velázquez-Enciso and Soltero-Quintana 2001); 5) the *in vitro* culture has shown great potential as a method for conservation and rescue of endangered species or to establish germoplasm banks (Cardarelli et al. 2010, Dávila-Figueroa et al. 2005, Giusti et al. 2002, Ramírez-Malagón et al. 2007). Cacti can be tissue-cultured by several means, including areole activation (axillary shoot formation) (Machado and Prioli 1996, Mauseth 1977, Wyka et al. 2006), adventitious shoot formation, in which shoots usually arise through calli differentiation (indirect organogenesis) (Minocha and Mehra 1974), and somatic embryogenesis produced from calli culture (Ali et al. 2001, Angulo-Bejarano and Paredes-López 2011, Infante 1992). Direct organogenesis—adventitious shoot formation—in which shoots are generated from explants without nodes, has not been reported yet. Depending on the availability of biological materials, the cultures for breaking areole dormancy may be started from seedlings (Choreño-Tapia et al. 2002, Gómez-Juárez et al. 2006, Moebius-Goldammer et al. 2002) or tissues derived from adult plants such as isolated areoles, mammillae, tubercles or flower sections (Estrada-Luna et al. 2008, Rubluo et al. 2002, Wyka et al. 2008). The objectives of this research were to: 1) study the particular conditions required to establish an efficient micropropagation system; 2) determine the requirements of hormone type, concentration, and combination, for shoot initiation and optimum proliferation; 3) determine the conditions for adventitious root formation in regenerated shoots; and 4) evaluate plantlet survival after transplantation.**MATERIALS AND METHODS**We divided our study into five experimental stages. The first stage consisted in the culture and preparation of mother plants to initiate the micropropagation. The other four stages included the typical steps of micropropagation: culture initiation, subculture and propagule proliferation, adventitious root formation, and plantlet transfer to greenhouse and acclimatization.Healthy adult plants of *Ortegocactus macdougallii* A*.*, about six to seven years 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/m2/s-1 at plant level, an average of day/night temperature of 27/20 ± 3° C, irrigation supplied as needed, and fertilization provided once a month (100 ppm N) with Peters Professional 20-20-20 (Scotts-Sierra Horticultural Products Co., Marysville, OH, USA). During this period, mother plants were also subjected to a regular program of pesticide regime for cleaning and were cultured until they produced new shoots.To start the process of micropropagation, young shoots (0.5–1.5 cm in length) were carefully excised from donor plants. In a laboratory bench, these shoots were washed with running tap water and cleaned by soaking and mechanic agitation for 15 minutes in a solution containing commercial detergent (Liqui-Nox, Alconox Inc., New York). Under sterile conditions provided by a hood, the shoots were initially immersed in ethanol (70%, v/v) for ten minutes and immediately subjected to a surface disinfecting treatment with commercial bleach [Clorox (6% NaOCl)] plus Tween-20 (SIGMA-ALDRICH Chemical) (0.1%), for 20 minutes. Finally, they were washed three times with de-ionized sterile water. Single or multiple tubercles, including one to three areoles, were dissected with a sterile scalpel and used as initial explants.For the first step of micropropagation, a simple experiment to study the response of explants to two different plant growth regulators was conducted. The regulators used were α-Naphthaleneacetic acid (NAA), (SIGMA-ALDRICH Chemical, Cat. Num. N1145) and 6-Benzylaminopurine (BA), (SIGMA-ALDRICH Chemical), Cat. Num. B3408), which were added alone or in combinations at different concentrations (0, 1.3, 4.4, and 13.3 µM) (total of 12 experimental treatments) to the MS (Murashige and Skoog, 1962) medium [pH 5.7, supplemented with 5% sucrose (SIGMA–ALDRICH Chemical), and 0.7% bacto-agar (Difco Laboratories, Detroit, MI) and autoclaved at 121° C for 20 min]. Each treatment was represented by six explants (n = 6). After 116 days of culture, shoot number per explant, percent of explants producing calli, root number, and root length (mm) were determined. Data of the percentage of explants producing calli were transformed by arcsin before performing the statistical analysis. The conditions of the culture room for this and the rest of the micropropagation experiments were adjusted to 27 ± 3 ºC of temperature and a photoperiod of 16 h light (250 µmol/m2/s-1 of maximum PPFD) at flask level.For the proliferation stage, a simple experiment was set up to compare the best treatment obtained from the induction cultures (NAA 4.4 µM:BA 13.3 µM) against two new BA concentrations (19.9 µM and 26.6 µM) combined with the original NAA (4.4 µM) concentration, giving a total of three experimental treatments. Each combination was added to a MS medium [pH 5.7, supplemented with 5% sucrose and 0.7% bacto-agar, and autoclaved at 121º C for 20 min]. All treatments were represented by eight culture flasks (n = 8) containing six explants each, which were used to calculate the explant average per replication. After 110 days of culture, shoot number per explant, total shoot length (mm), total shoot fresh weight (mg), root number, and root length (mm) were determined. The experiment was repeated at least two times to confirm the data obtained and to calculate mean values used for the statistical analysis.For the rooting stage, four treatments were tested in a simple experiment to determine adventitious root formation in the proliferated shoots. The treatments resulted from the reduction of the original component formulation of the MS medium (25 and 50%) plus the addition of Indole-3-butyric acid (IBA), (SIGMA-ALDRICH Chemical, Cat. Num. I7512) (1.6 and 4.9 µM) (Total of four experimental treatments: MS25, MS50, MS50+IBA 1.6 µM, MS50 + IBA 4.9 µM). The MS medium was prepared by using standard protocols with a pH adjusted to 5.7, supplemented with 5% sucrose, 0.7% bacto-agar, and autoclaved at 121º C for 20 minutes. Each treatment was represented by eight flasks or replications (n = 8) containing three explants each, which were used to calculate the explant average for data analysis. After 50 days of culture, the percent of explants producing roots, root number per explant, and total root length (mm) were determined. The data of the percent of explants producing roots were transformed by arcsin before performing the statistical analysis. Fifty regenerated plantlets of *Ortegocactus macdougallii* A., were transplanted to 125 ml capacity plastic pots (6.5 cm high by 5.8 cm wide), previously filled with an artificial substratum [42.63% Peat Moss (PREMIER®), 42.63% sand, 13.95% organic fertilizer supplemented with plant growth promoter bacteria (OrganoDel®), and 0.77% of lime to adjust the pH to 6.8-7.2)], and acclimatized for six days in a bench with low light conditions (400 µmol/m-2/s-1 PPFD) prior the transfer to a different greenhouse with 30% UV filtration and 800 µmol/m-2/s-1 PPFD. In this place they were grown for 180 days, after plant performance and survival were evaluated. All data obtained from each experiment were subjected to an analysis of variance and a mean separation test (Tukey, α = 0.05).**RESULTS**The data obtained during stage 0 of micropropagation revealed very low rates of contamination (4%) and minimum tissue oxidation (0%). After 116 days of culture initiation, we observed two morphogenic responses in the explants: differentiation of disorganized tissues (caulogenesis) and differentiation of organs (organogenesis), which produced both roots and shoots. Calli were differentiated in all treatments including auxins (NAA), independently of the concentration and the presence of cytokinin. These structures were originated from cells of both epidermis and parenchyma tissues located at the edge of the cut area (Fig. 1b, c). During the first stages of differentiation and growth, calli were soft and friable with crystalline appearance and anthocyanin accumulation (Fig. 1a, 1c). Over time, they became hard and compact, showing very slow growth rates. The ANOVA detected statistical significance among treatments, with a clear tendency of higher percentage of explants producing calli with increased auxin concentrations (Table 1). We also observed that treatments with low to medium auxin concentrations (4.4 and 13.3 µM) produced adventitious roots, in addition to the caulogenic structures.The organogenic responses of explants showed that the interaction between BA and NAA in an adequate balance (NAA 1.3 µM:BA 4.4 µM and NAA 4.4 µM: BA 13.3 µM) produced both calli and shoot primordia, which were visible after 56 days of culture. Fortunately, detailed stereoscopic observations of the cultures gave us the opportunity to detect that the origin of the regenerated shoots was the result of two morphogenic events: direct *de novo* differentiation (adventitious bud formation) and axillary meristem activation of the areoles (breaking areole dormancy) located on each tubercle. Based on these observations, we identified the tiny adventitious shoot primordia, which were apparently differentiated from cells of the epidermis in areas delimiting tubercles or at the basal zone of explants, where no areoles were present (Fig. 1d-f). We were not able to perform an anatomical study to confirm these observations. After induction and early stages of cell differentiation, the primordia looked like a disorganized group of round-shaped cells with opaque white color (Fig. 2a). However, several weeks later, these groups of cells grew-up to became organized structures with tiny tubercles colored with the typical olive green observed in young and adult plants (Figs. 1d, e, f, g, h). The shoots that regenerated from axillary meristem activation of areoles were also evident after 56 days of the *in vitro* culture. The sequence of events producing both types of shoots is shown in Fig. 1g and h (adventitious buds) and Figs. 1i and j (areole activation). In this stage, the explant response for shoot production was considered to be very poor, because only two of the twelve treatments evaluated induced shoot differentiation and, from these treatments, four explants produced a total of 17 shoots (treatment NAA 4.4 µM:BA 13.3 µM produced 15 shoots). The treatments supplemented only with BA (1.3, 4.4, 13.3 µM) were not able to break the dormancy of axillary buds, as we expected. In the second organogenic response (root production), we observed that three treatments (NAA 0 µM:BA 1.3 µM, NAA 0 µM:BA 4.4 µM, and NAA 0 µM:BA 13.3 µM), were able to differentiate adventitious roots and that treatments with NAA 1.3 µM:BA 4.4 µM, NAA 4.4 µM:BA 1.3 µM, and NAA 13.3 µM: BA 1.3 µM also induced calli formation in addition to root formation. It became clear then that in this stage we were able to regenerate whole plants with six treatments. The ANOVA obtained for root number and root length showed significant differences among treatments. The superior treatments for root number were NAA 0 µM:BA 4.4 µM (9), NAA 13.3 µM:BA 1.3 µM (8.5), and NAA 4.4 µM:BA 1.3 µM (8.25). It was clear that the treatment including NAA 0 µM:BA 4.4 µM could be considered the best, since it produced more roots. For the root length variable, the mean separation test (Tukey α = 0.05) revealed four statistically different groups in which the treatments with NAA 0 µM:BA 1.3 µM and NAA 0 µM:BA 4.4 µM resulted in higher averages (11.33 and 10.28 mm in length, respectively), as compared to the other treatments (Table 1). During Stage II of micropropagation, we studied and established the optimum conditions for propagules proliferation in order to increase the propagation potential of our protocol. After performing two rounds of subcultures, we calculated the shoot proliferation rates for each of the three treatments tested. Our data showed that shoot proliferation seems to be controlled by an adequate balance of auxins:cytokinins and that the optimum concentration of NAA is 4.4 µM. In combination with BA in concentrations of 13.3, 19.9 and 26.6 µM, the optimum concentration of NAA produced between five to six shoots on average. From the ANOVAs and the mean separation tests applied to the data obtained (Tukey α = 0.05), we were able to see that the shoot number and shoot fresh weight variables did not show significance among treatments. For the shoot length, the treatment NAA 4.4 µM:BA 13.3 µM produced significantly higher shoots (4.78 mm) than the other two treatments: NAA 4.4 µM:BA 19.9 µM and NAA 4.4 µM:BA 26.6 µM, which produced shoots of 3.93 and 3.67 mm in length, respectively (Table 2). It was interesting to notice that increased concentrations of BA (19.9 µM and 26.6 µM) produced crested or monstrous shoots. Given that *Ortegocactus macdougallii* A., is a slow growing plant species, the adequate time between subcultures was considered to be 110 days. As observed with the induction cultures, the origin of shoots during subcultures occurred through areole activation and direct *de novo* differentiation (Fig. 1d-j). In addition to shoot proliferation, these treatments also induced adventitious root formation in some explants. The statistical analysis of data obtained for root number and root length showed high significance among treatments in which treatment NAA 4.4 µM:BA 13.3 µM resulted in better outcomes than NAA 4.4:BA 19.9 µM and NAA 4.4:BA 26.6 µM in both variables. This treatment was the only one that regenerated roots (Table 3); however, the number of roots produced can be considered to be a poor response: an average of 1.25 roots per explant were recorded.For the rooting stage, we recorded the number of roots produced, root length, and percentage of explants with adventitious roots formation. After 50 days of culture, we observed that the four treatments evaluated induced adventitious roots in the explants. The ANOVA showed differences among treatments in the root number and percentage of explants with adventitious roots formation. As a general trend, we could see that the presence of auxins in the MS medium, in particular IBA when supplied in low (1.6 µM) and medium (4.9 µM) concentrations, significantly improved the explant responses for both root number and percentage of explants with roots; which resulted statistically similar. The highest concentration of IBA (4.9 µM) produced better results, enhanced the root number (9.9 in average) and resulted in a higher percentage of rooted shoots (75%), compared with the other treatments (Table 4). Contrary to what was observed in the other experiments, in which adventitious roots were also produced in long periods of cultures, in this case the root initials and primordia were visible on explants only 30 days after the subculture was initiated (Fig. 2a) and 20 days later (50 days in total) highly differentiated and elongated roots were observed (Fig. 2b, c). Detailed observations of the origin of adventitious roots suggest that they possibly differentiated from parenchymatic cells and pith ground tissues in the neighborhood of vascular tissues (Fig. 2d); however, an anatomical analysis to corroborate this observation was not performed. Fifty *Ortegocactus macdogallii* A., plantlets regenerated through this micropropagation scheme were carefully transferred from the laboratory to *in vivo* conditions. After 180 days of transplantation we observed an active plantlet growth and recorded 98% of survival under the culture conditions imposed (Fig. 2f).**DISCUSSION**The methods for propagation of cacti include seed germination, apomixis, and the rooting of sprouts, cuttings, and suckers. Grafts, micrografts, and micropropagation are also common (Anderson 2001, Cullmann et al. 1986, Estrada-Luna et al. 2002). We report here a new way to propagate *Ortegocactus macdougallii* A., through tissue culture. Our system is reliable and very efficient and the complete cycle of micropropagation requires about 13 months of culture to produce 625 plantlets, after three rounds of subcultures. The proliferation rate might be significantly increased with five rounds of subcultures and a culture period extended to 21 months, yielding 15,625 plantlets on average. The protocol is based on the induction of two morphogenic responses: differentiation of shoots and adventitious roots; however, calli may also be produced. During the initial cultures, fungi and bacteria contamination and tissue oxidation are common constrains and adversely affect the physiology of the explants, limit morphogenetic responses, and reduce the potential rate of propagation (Kyte and Klein 1999, Villalobos and Thorpe 1985). However, we were successful in obtaining low rates of contamination (4%) and minimum tissue oxidation (0%), as the result of adequate plant management and culture conditions imposed in the greenhouse and of the cleaning and disinfection treatment prior explant dissection.In this study we demonstrated the role and importance of cytokinins and auxins during the micropropagation of *Ortegocactus macdougallii* A*.* In general, we observed that BA, in combination with NAA in an adequate balance, was able to control and promote three morphogenic responses: caulogenesis, adventitious root formation, and bud production. These responses were produced during initial cultures and subsequent proliferation sub-cultures. When caulogenesis was produced, all calli were originated from cells of both epidermis and parenchyma tissues located at the edge of the cut area (Fig. 1a, b). This response was induced by auxins, independently of the concentration and the presence of cytokinin. Particularly, the auxin concentration requirements for *Ortegocactus macdougallii* A., were relatively low and the optimum responses were in agreement and within the range reported for most cacti (Choreño-Tapia et al. 2003, Karimi et al. 2010, Ruvalcaba-Ruiz et al. 2010, Vyskot 1984, Wyka 2006), which lies between 2.23 and 22.29 μM NAA. Other species such as *Cephalocereus senilis* respond better with higher concentrations (Nava-Esparza and Yañez 1984). Natural auxins are produced in the meristems of higher plants and are involved in many functions such as the control of cell elongation, tissue expansion, and cell division (callus formation) (Davies 1995, Fay and Gratton 1992, Taiz and Zeiger 2010); however, its commercial use and application in plant tissue culture and conventional propagation systems is related to the control of adventitious root formation, inhibition of axillary and adventitious shoot formation, somatic embryogenesis, establishing cell suspension cultures, and as herbicides (Hartman et al. 2010, Pierik 1990, Salisbury and Ross 1994, Taiz and Zeiger 2010).Shoot development in *Ortegocactus macdougallii* A., was produced only by the auxin-cytokinin interaction. However, the regenerated shoots were differentiated through two processes: direct *de novo* adventitious differentiation (adventitious shoots) and activation of axillary pre-formed meristems present in dormant areoles (axillary shoots). According to Hartmann et al. (2010) adventitious bud formation is produced 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 calli production. The process involves the capability of previously developed and highly differentiated cells to initiate cell divisions and form a new meristematic growing point. In our study, adventitious shoot primordia emerged from epidermic cells, which after differentiation and initial growth, looked like a group of poorly organized structures that later became 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. 1d-h). We took these observations as evidence that they did 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 (Hartmann et al. 2010, Machado and Prioli 1996). Interestingly, during the shoot proliferation we observed subcultures in which the two treatments evaluated with higher concentrations of BA (19.9 and 26.6 μM) induced the production of crested forms with more robust constitution. This result is possibly related to the adventitious origin of some buds.In all plant species, including cacti cultured under propagation *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 with the mere presence of cytokinins (Estrada-Luna 1988, Estrada-Luna et al. 2002, 2008, Dabekaussen et al. 1991, Perez et al. 1999, Ruvalcava-Ruiz et al. 2010).Cytokinins are growth regulators that participate in several developmental and physiological activities at cellular and whole plant level; including the cell cycle, cell differentiation (shoot formation and apical meristem identity), control of chloroplast development, control of apical dominance, retarding leaf senescence, etc. (Salisbury and Ross 1994, Davies 1995, Taiz and Zeiger 2010). In tissue culture, cytokinins promote the formation of shoots in different types of explants such as calli, leaf, cotyledons, and stem sections (Hartmann et al. 2010, Taiz and Zeiger 2010). In particular, BA is able to induce both axillary and *de novo* bud differentiation in many species subjected to *in vitro* propagation and it can be used alone or in combination with other growth regulators (Dávila-Figueroa et al. 2005, Giusti et al. 2002, Mata-Rosas et al. 2001, Moebius-Goldammer et al. 2003). During micropropagation, the cacti responses are genotype dependent on different types and different concentrations of cytokinin. Some species have relatively high requirements, such as *Hylocereus undatus* and *Leuchtembergia principis*, which produce 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, but most of the studied species have optimal responses in 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 Molphe-Figueroa (2002) for *Mammillaria formosa, Nyctocereus serpentinus, Pelecyphora aselliformis,* and *P. strobiliformis*. The present study shows that low-level concentrations of cytokonin yielded the best results for bud induction and proliferation. The proliferation rate (number of shoots regenerated per explant) of *Ortegocactus macdougallii* A*.*, is within the range obtained for most tissue culture cacti (four to 10 shoots). As for optimum subculture time, it is much longer (110 days) than the subculture period of other cacti. Escobar et al. (1986) and Estrada-Luna (1988, 2008) reported an optimal subculture frequency of 45 days for several *Opuntia* species, while Ramírez-Malagón et al. (2007) and Ruvalcaba-Ruiz et al. (2010) mentioned that 60 days were better for *Mammillaria* and *Coryphantha*. In contrast, *Obregonia denegrii*, which is also a slow growing plant species, requires subcultures every 120 days of culture (Cardelli et al. 2010). *Ortegocactus macdougallii* A., is an easy to root plant species. To achieve whole plant regeneration we found that the shoots can be rooted through various treatments: MS medium supplemented with 1.3, 4.4, and 13.3 μM of BA or with the combination of NAA 1.3 μM:BA 4.4 μM, NAA 4.4 μM:BA 1.3 μM, and NAA 13.3 μM:BA 1.3 μM as we reported in stage I (Tables 1 and 3). This response may be due to the endogenous auxin concentration of these explants being adequate to promote the generation of adventitious roots, as it occurs when the explants are subjected to cutting propagation in nursery conditions (Jackson 1986). Adventitious roots were also produced in a MS medium with reduced concentrations of the original formulation (25 or 50%) or in a MS medium at 50% plus IBA at 1.6 or 4.9 µM. Notwithstanding these results, it was clear that the addition of IBA (4.9 µM) enhanced the rhizogenic process; which resulted in plantlets with higher quality, because more root number and higher percentage of rooted shoots were produced (Table 4). In horticulture, the stimulatory effect of auxins on adventitious root formation has been successfully used for cutting propagation (Hartmann et al. 2010, Taiz and Zeiger 2010). Many plant species differentiate roots when cultured *in vitro* or *in vivo* without the presence of auxins, including several cacti such as *Opuntia* (Estrada-Luna 2008), *Pilocereus robinii* (Quiala et al. 2009), *Hylocereus undatus* (Mohamed-Yasseen 2002), *Turbinicarpus* *laui* (Mata et al. 2001); however, most species require the addition of auxins, in particular IBA, to achieve rooting or to improve the root number and length (Escobar et al. 1986, Pérez-Molphe-Balch and Dávila-Figueroa 2002). The process of root differentiation seems to be direct and does not include the initial differentiation of calli. Detailed observations of the origin of these adventitious roots revealed that they were differentiated from parenchymatic cells and pith ground tissues in the neighborhood of vascular tissues (Fig. 3d). These cells could be companion cells of the xylem and phloem, parenchyma cells in the periphery of vascular bundles, or pericycle, as described by Jackson (1986). The dividing cells became root apical meristems and later elongated roots following an analogous process to the formation of lateral roots (Jackson 1986, Hartmann et al. 2010).The acclimatization refers to the transition period in which micropropagated materials are transferred from the *in vitro* to greenhouse or growth chamber conditions before transplantation to the field (Kozai 1991, Preece and Sutter 1991). At this time, most species experience a transplantation shock that negatively affects growth rates and increases plantlet mortality ⎯40 to 100% (Varma and Schuepp 1995). The factors responsible for this situation are related to the environmental conditions imposed during acclimation [drastic changes in relative humidity, temperature, light conditions, and water and nutrient availability (Varma and Schuepp 1995, Hartmann et al. 2010)] and to the physiology of the plantlets, which may develop non-functional roots or deficient vascular systems (Grout and Aston 1978, Pospisilova et al. 1999). In some species the readiness in inoculating beneficial microorganisms to re-establish mycorrhizal associations and the interaction with plant growth promoting rhizobacteria populations (Estrada-Luna and Davies 2003) are key factors that dictate the success or failure after micropropagation; because most plant species are mycorrhiza dependent and the interaction with these micro-organisms provides water, nutrients, and other important metabolites to the young plantlets (Estrada-Luna et al. 2000, Estrada-Luna and Davies 2003, Hata et al. 2010). The mixotrophic condition of tissue culture plantlets allows only low rates of photosynthesis and reduced chlorophyll concentrations, which limit overall plant growth (Grout and Millan 1985). The difference in moisture content of the culture flasks (nearly 100% relative humidity) and the natural environmental conditions or greenhouse (20-80% relative humidity) during the first days after transplantation challenge the functionality of stomata to control transpiration (Brainerd and Fuchigami 1982, Díaz-Pérez et al. 1995, Pospisilova et al. 1999). Micropropagated plants will only be able to survive and grow normally if they quickly control the activity of their stomata and photosynthesis (Kozai 1991). The results obtained in this study suggest that fortunately *Ortegocactus macdougalli* A., is a plant species that does not suffer from this type of abnormalities and deficiencies and because of this, the acclimatization problems were minimal and produce the high rates of survival (98%). A possible explanation of these results is that micropropagated plants of *Ortegocactus macdougalli* A., developed functional roots and stomata and a cuticle thick enough to allow appropriate control of transpiration and normal rates of photosynthesis and growth (Fig. 3f). Our high survival rate is consistent with the observations for other cacti such as *Pilosocereus robinii*, *Pelecyphora aselliformis* and *P*. *strobiliformis*, *Astrophytum* spp., *Cephalocereus senilis*, *Coryphantha* spp., *Echinocactus* spp., *Echinocereus* spp., *Echinofossulocactus* spp., *Ferocactus* spp., *Mammillaria* spp*., Nyctocereus* spp*., Stenocactus* spp. These cacti are reported to have survival values ranging from 70 to 100% (Escobar et al. 1986, Estrada-Luna 1988, Estrada-Luna et al. 2008, Martínez-Cárdenas et al. 2007, Pérez-Molphe-Balch et al. 1998, Pérez-Molphe-Balch and Dávila-Figueroa 2002, Quiala et al. 2009).

**ACKNOWLEDGEMENTS:** Authors want to thank the Universidad De La Salle Bajío for the economical support provided through the Office of Research Council, CINVESTAV-IPN Unidad Irapuato for allowing the use of facilities and equipment, and Miriam Zachs for the copy editing of this paper.**REFERENCES**Alexander E.J. (1961). *Ortegocactus*, a unique new genus. Cactus of Succulent Journal of the United States, 33: 39-40.Ali A., Naz S., Ahmad Siddiqui F., Iqbal, J. (2001). Callogenesis, embryogenesis and organogenesis in christmas cactus (*Schlumbegera bridesi*). Pakistan Journal of Botany, 33: 569-574.

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**Table 1.** Effect of cytokinin and auxin on *in vitro* cultured explants of *Ortegocactus macdougallii* A.116 days after the establishment of initial cultures.

|  |  |  |  |
| --- | --- | --- | --- |
| Treatment (*μ*M) | Calli Production  (%) | Root Number | Root Length (mm) |
| BA 1.3 + NAA 0.0 | 0.00 ± 0.00 b | 5.00 ± 0.71 b | 11.33 ± 2.60 a |
| BA 4.4 + NAA 0.0 | 0.00 ± 0.00 b | 9.00 ± 1.47 a | 10.28 ± 1.25 ab |
| BA 13.3 + NAA 0.0 | 0.00 ± 0.00 b | 4.25 ± 0.95 b | 4.48 ± 1.11 cd |
| BA 1.3 + NAA 1.3 | 50.00 ± 28.87 a | 0.00 ± 0.00 c | 0.00 ± 0.00 d |
| BA 4.4 + NAA 1.3 | 75.00 ± 25.00 a | 0.00 ± 0.00 c | 0.00 ± 0.00 d |
| BA 13.3 + NAA 1.3 | 100.00 ± 0.00 a | 0.00 ± 0.00 c | 0.00 ± 0.00 d |
| BA 1.3 + NAA 4.4 | 100.00 ± 0.00 a | 8.25 ± 0.63 a | 4.10 ± 0.90 cd |
| BA 4.4 + NAA 4.4 | 100.00 ± 0.00 a | 0.00 ± 0.00 c | 0.00 ± 0.00 d |
| BA 13.3 + NAA 4.4 | 100.00 ± 0.00 a | 0.00 ± 0.00 c | 0.00 ± 0.00 d |
| BA 1.3 + NAA 13.3 | 50.00 ± 28.87 a | 8.50 ± 0.96 a | 6.38 ± 0.90 bc |
| BA 4.4 + NAA 13.3 | 100.00 ± 0.00 a | 0.00 ± 0.00 c | 0.00 ± 0.00 d |
| BA 13.3 + NAA 13.3 | 100.00 ± 0.00 a | 0.00 ± 0.00 c | 0.00 ± 0.00 d |
| MSD\* | 23.7203 | 3.1334 | 3.1582 |

\*Minimum significant difference. Means with the same letter are not significantly different according to the Tukey mean separation test (∝= 0.05). Mean ± SE.

n = 6.

**Table 2.** Effect of cytokinin and auxin on shoot proliferation subcultures of *Ortegocactus macdougallii* A., 110 days after *in vitro* culture.

|  |  |  |  |
| --- | --- | --- | --- |
| Treatment (*μ*M) | 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 | 138.17 |

\*Minimum significant difference. Means with the same letter are not significantly different according to the Tukey mean separation test (∝= 0.05). Mean ± SE. n = 6.

**Table 3.** Effect of cytokinin and auxin during proliferation subcultures on adventitious root formation of regenerated shoots of *Ortegocactus macdougallii* A.*,* 110 days after *in vitro* of culture.

|  |  |  |
| --- | --- | --- |
| Treatment (*μ*M) | Root Number | Root Length (mm) |
| BA 13.3 + NAA 4.4 | 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 |
| BA 26.6 + NAA 4.4 | 0.00 ± 0.00 b | 0.00 ± 0.00 b |
| MSD\* | 0.5125 | 2.4408 |

\*Minimum significant difference. Means with the same letter are not significantly different according to the Tukey mean separation test (∝ = 0.05). Mean ± SE. n = 8 ± SE.

**Table 4.** Effect of culture media and auxin concentration on adventitious root formation of regenerated shoots of *Ortegocactus macdougallii* A.*,* 50 days after *in vitro* of culture.

|  |  |  |  |
| --- | --- | --- | --- |
| Treatment | Root Formation  (%) | Root Number | Root Length (mm) |
| MS25+ | 58.34 ± 8.34 ab | 4.17 ± 0.48 b | 11.32 ± 1.95 a |
| MS50• | 37.49 ± 11.68 b | 5.00 ± 1.37 b | 9.96 ± 3.05 a |
| MS50-IBA 1.6⊕ | 45.83 ± 6.11 ab | 6.33 ± 0.77 ab | 8.99 ± 0.91 a |
| MS50-IBA 4.9º | 75.03 ± 5.45 a | 9.92 ± 1.91 a | 8.37 ± 1.56 a |
| MSD\* | 3.9990 | 4.9854 | 7.9985 |

**+**MS culture medium at 25% of original concentration; **•**MS culture medium at 50% of original concentration; ⊕MS culture medium at 50% of original concentration plus 1.6*μ*M IBA; º MS culture medium at 50% of original concentration plus 4.9*μ*M IBA. \*Minimum significant difference. Means with the same letter are not significantly different according to the Tukey mean separation test (∝ = 0.05). Means ± SE. n = 8.

**Figure Legends:**

**Fig. 1.** Effect of growth regulators on explants of *Ortegocactus macdougallii* A during stage I of micropropagation. Calli development: (a) callus differentiated from epidermis, (b) calli grown from the edge of cut areas, (c) calli grown from the epidermis. Adventitious shoot development: (d) adventitious buds differentiated from the epidermis, (e, f) and the edge and base of the explant. Sequence of events producing adventitious shoots: (g) bud initials after 54 days of culture, (h) young emerging shoots differentiated from epidermis Sequence of events producing axillary shoots: (i) bud initials from areole activation after 54 days of culture, (j) young emerging shoot.

**Fig. 2.** Rooting and acclimatization of regenerated shoots of *Ortegocactus macdougallii* A. Adventitious root formation stage: (a) emergence of root initials after 30 days of culture (arrows), (b) elongation of root primordial (arrows), (c) adventitious roots differentiated after 50 days of culture, (d) adventitious roots differentiated in the neighborhood of vascular tissues (arrows), (e) regenerated plantlet, (f) acclimatized plantlet 180 days after transplantation.