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Auxin induced morphogenetic responses in long-term in vitro subcultured *Mammillaria san-angelensis* Sánchez-Mejorada (Cactaceae)

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Abstract

Observations were made as to the influence of auxins, as the sole exogenous growth regulator, on the morphogenesis of long-term in vitro subcultured plantlets of the severely endangered cacti *Mammillaria san-angelensis*. Sections of long-term subcultured shoots were exposed to different auxins at various concentrations, and plant regeneration was recorded as a direct effect of auxin concentration. It was found that morphogenetic potentiality was retained in long-term subcultures, and that the best regeneration was seen in the presence of IAA (34.25 μM). Histological analysis revealed two processes leading to regeneration: de novo production of shoots and axillary meristem activation. Of the two, de novo shoot production was found to occur both in controls and in explants growing in the presence of IAA, while axillary meristem activation was observed only in the presence of IAA.

Keywords: Endangered species; Cactaceae; Auxins action; Areole activation; Growth regulators; Histology development; Tissue culture

1. Introduction

Loss of morphogenetic potential is common in long-term in vitro cultured tissues (Chaturvedi and Jain, 1994), which is a great disadvantage in utilization of such tissue for

Abbreviations: IAA, 3-indolyl-acetic acid; NAA, 1-naphthalene-acetic acid; IBA, indole-butyric acid; 2,4-D, 2,4-dichlorophenoxy-acetic acid; MS, Murashige and Skoog

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in vitro production of plants. In the Cactaceae family, interaction of auxins and cytokinins for in vitro plant regeneration is well documented (Hubstenberger et al., 1992). However, these authors point out that while an exogenous cytokinin is always required to obtain in vitro shoot proliferation, auxins are not strictly necessary. In addition, Dabekaussen et al. (1991) state that auxins are of little importance for cacti micropropagation, and Reyes et al. (1995) reported that the use of antiauxins in in vitro cultures of the cactus *Aztekium ritteri* led to better rates of axillary branching and improved quality of shoot development. This data suggests that elimination of auxins favors micropropagation in cacti.

Mammillaria san-angelensis Sánchez-Mejorada is a nearly extinct cactus which has been successfully micropropagated. In vitro mass propagation of this severely endangered cactus was influenced mainly by two factors: the presence of BAP and the origin of the explant (Martínez-Vázquez and Rubluo, 1989). The aim of the present study was to analyze the influence of auxins, as the sole exogenous source of growth regulators, on the morphogenetic responses of sections of *M. san-angelensis* shoots subcultured and maintained in vitro for several years. Histological analysis of the events was also carried out.

2. Materials and methods

Plantlets of *M. san-angelensis* regenerated on MS (Murashige and Skoog, 1962) basal medium enriched with BAP at 0.1 mg l^{-1} were used as the original source of explants (Martínez-Vázquez and Rubluo, 1989). They were subcultured every 6 months for 7 years in 25 ml MS basal medium with no growth regulators in 5.5 by 7.0 cm baby food jars, and maintained in a growth chamber at $27 \pm 2\ ^{\circ}C$ under cool-white fluorescent lamps with 14.8 μmol m^{-2} s^{-1} light and a 16 h photoperiod. Sections of shoots from this long-term subcultured material were transferred to MS alone (control) as well as to MS supplemented with auxins IAA (11.41, 22.83, 34.25 μM), NAA (10.74, 21.48, 32.22 μM), IBA (9.84, 19.68, 29.52 μM) and 2,4-D (9.05, 18.09, 27.14 μM) and incubated as described above.

Three explants were grown per jar and each experiment was repeated six times, for a total of 18 cultured explants per auxin concentration. Morphogenetic responses were recorded after 3 months in culture. All experiments were conducted using a completely randomized design. Due to high variability in the observations from each treatment, shoots per explant values were transformed to natural logarithms (ln) and subjected to Fisher LSD analysis. Regression analysis was used to test for significant linear and quadratic responses.

After in vitro regeneration, plantlets were individualized and transferred to basal MS devoid of any growth regulator in order to permit strengthening as well as rooting. After 1 month, rooted plantlets were transferred to pots containing soil from the original growing area of this severely endangered species, and were then transferred to a greenhouse.

Histological analysis was performed on control explants as well as those exposed to IAA at (34.25 μM) after 3 months of culture. The samples (callus, callus with buds, and shoots) were fixed in FAA (formalin:acetic acid:ethanol:water, 2:1:10:7). Bud and shoot sections were then embedded in paraplast (Johansen, 1940) and stained with safranin-fast green; afterwards they were sectioned at 10 μm. Other samples were embedded in JB-4 plastic mixture, cut with a glass knife at 2 μm and stained with toluidine blue.

3. Results

3.1. In vitro morphogenetic responses

Table 1 summarizes the in vitro morphogenetic responses of explants from long-term subcultured shoots of *M. san-angelensis* exposed to different auxins at various concentrations. Shoot formation was induced in all tested conditions; however, the number of shoots per explant varied greatly in all treatments (e.g. in IBA 9.84 μM, the range was 5–45, data not shown), resulting in high deviation values. It was also noted that the yield of shoots per explant varied. Regression analysis showed that IAA, NAA and IBA showed a linear positive correlation between auxin concentration and number of shoots per explant. In contrast, 2,4-D showed a negative linear correlation between auxin concentration and number of shoots per explant (Table 1). Fig. 1a shows a typical response of a long-term subcultured explant growing in MS basal medium in the total absence of exogenous growth regulators (control). After 3 months of incubation, buds and shoots were apparent. The highest yield of shoots was achieved by the action of the naturally occurring auxin IAA (Table 1 and Fig. 1b).

Table 1
Effect of four auxins on the morphogenetic response of explants from *M. san-angelensis* subcultured for 7 years in MS at 27 ± 2 °C and 14.88 μmol m^2 s^{-1} 16 h photoperiod[a]

Auxins	Concentration (μM)	Shoot-producing explants (%)	Mean shoots per explant	Mean of ln of shoot per explant	Regression analysis of ln of shoots per explant	Root formation (%) in subcultured shoots in MS hormone-free
IAA	11.41	18/18 (100)	16.38 ± 9.36	2.61 b[b]		83
IAA	22.83	18/18 (100)	20.27 ± 12.50	2.83 b	Linear[**]	77
IAA	34.25	17/18 (94)	26.77 ± 16.71	2.97 b	Quadratic ns[c]	55
Control		18/18 (100)	7.00 ± 5.19	1.60 a		72
NAA	10.74	16/18 (88)	12.50 ± 11.18	2.10 ab		61
NAA	21.48	18/18 (100)	14.44 ± 13.09	2.28 b	Linear[**]	50
NAA	32.22	18/18 (100)	18.72 ± 12.47	2.71 b	Quadratic ns	61
Control		18/18 (100)	7.00 ± 5.19	1.60 a		72
IBA	9.84	17/18 (94)	14.27 ± 12.47	2.31 b		72
IBA	19.68	17/18 (94)	20.33 ± 13.29	2.72 b	Linear[*]	77
IBA	29.52	16/18 (88)	21.33 ± 16.54	2.55 b	Quadratic ns	77
Control		18/18 (100)	7.00 ± 5.19	1.60 a		72
2,4-D	9.05	15/18 (83)	14.61 ± 13.88	2.0 c		16
2,4-D	18.09	6/18 (33)	5.27 ± 10.08	0.79 ab	Linear[*]	22
2,4-D	27.14	5/18 (27)	5.16 ± 10.44	0.71 a	Quadratic ns	0
Control		18/18 (100)	7.00 ± 5.19	1.60 bc		72

[a] Multiple comparison test and regression analysis were performed separately at each auxin group.
[b] Values with the same letter are not significantly different from each other at a 5% level by LSD (Fisher test).
[c] Non-significant.
[*] Significant at $p < 0.042$.
[**] Significant at $p < 0.01$.

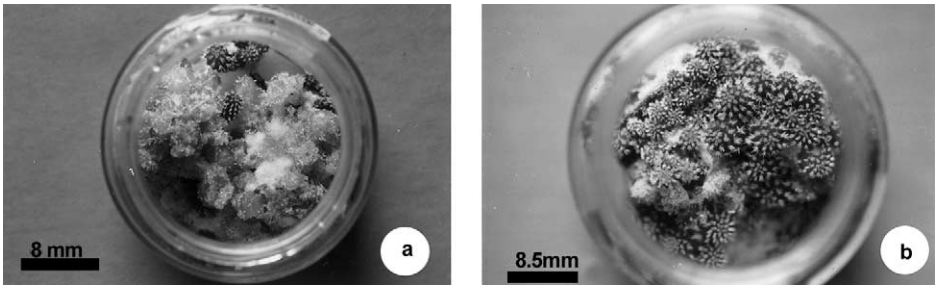

Fig. 1. Morphogenetic responses of *M. san-angelensis* explants after 3 months in culture: (a) without auxins (control); (b) exposed to IAA (34.25 μM).

Most of the isolated shoots rooted to a greater or lesser extent when transferred to MS BM free of growth regulators irrespective of auxin origin, except for those derived from 2,4-D treatments. Shoots derived from the 2,4-D experimental batch showed low values for rooting, and total inhibition of rooting was recorded at the highest concentration of this auxin (Table 1). Root initiation was quantified 3–4 weeks after incubation. There were 3–6 roots per shoot, and the roots were an average of 1.5 cm in length. Rooted plantlets showed 100% survival in greenhouse conditions (Fig. 2).

3.2. Histological observations

Histological analysis of the in vitro derived shoots of *M. san-angelensis* not exposed to auxins (control) as well as those subjected to the action of IAA at 34.25 μM revealed

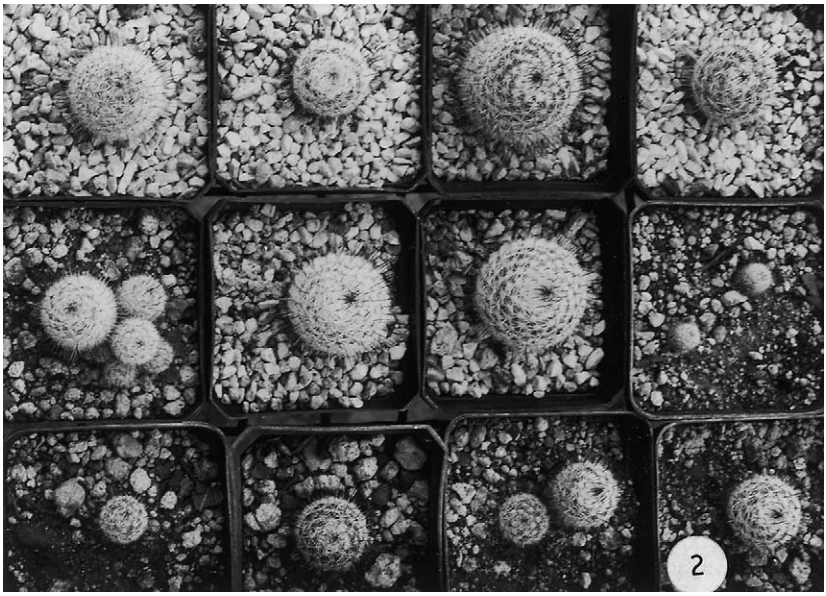

Fig. 2. In vitro derived plants growing at the greenhouse (8 months–1.5 years old).

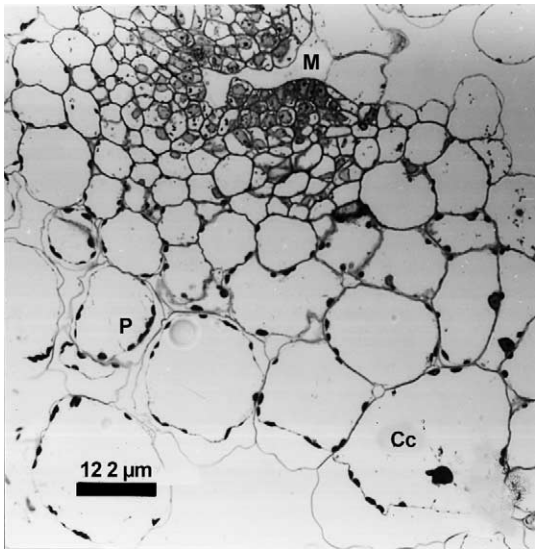

Fig. 3. De novo differentiation of callus cells into meristematic tissue and early stages of bud induction. Cc, callus cells; M, meristem; P, chloroplasts.

that morphogenetic responses were induced in two different ways from the original explant.

3.2.1. De novo meristem formation through callus

Unorganized callus (Fig. 3) showed typical parenchymatous and irregular cells, large and optically empty, with few spaces between them. Callus cells were observed to contain

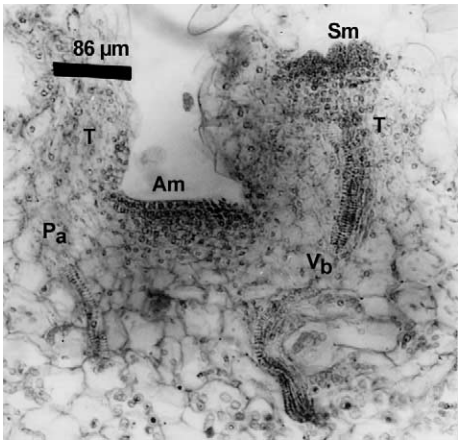

Fig. 4. Longisection of early shoot development showing the typical dimorphic meristem. Am, axillary meristem; Sm, spiniferous meristem; Pa, parenchyma; Vb, vascular bundle; T, tubercles.

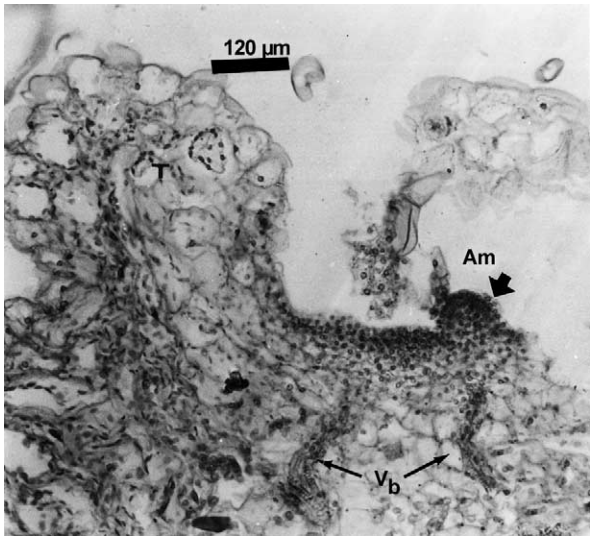

Fig. 5. Activation of axillary meristem (Am) forming a protuberance (arrow); T, tubercles; Vb, vascular bundle.

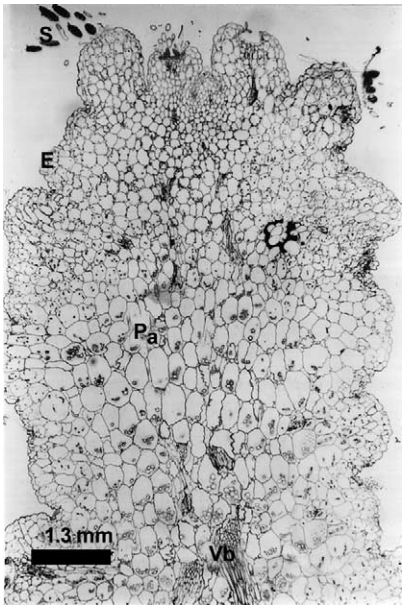

Fig. 6. Longisection of a growing shoot derived from activated axillary meristem showing typical structures of *Mammillaria*. E, epidermis; Pa, parenchyma; S, spines; Vb, vascular bundle.

chloroplasts situated on the cell periphery. Toward the surface of the callus, cells were smaller with a conspicuous nucleus, without vacuoles, and showed meristematic appearance (Fig. 3). After 3–4 weeks, vascular bundles, parenchymatous cells, tubercles, and axillary and spiniferous meristems were apparent (Fig. 4). From these formations, normal shoots grew as in Fig. 1a and b, which were able to be rooted, transplanted to soil and grown to maturity (Fig. 2). These results were observed in controls (no auxins) as well as in explants that had been exposed to the auxins tested in this study; however, in the former only this event was present, whereas in the latter areole activation was also triggered.

3.2.2. Areole activation

Explants exposed to auxins (IAA 34.25 μM) displayed areole activation, whereas control explants did not. Areoles in *Mammillaria* are dimorphic (Boke, 1958; Bravo-Hollis and Sánchez-Mejorada, 1991) with spiniferous and axillary meristems. An activation of axillary meristem was observed in this work to be influenced by auxins. Fig. 5 shows the activation of this axillary meristem. Small and conspicuously organized cells were apparent, active division was observed (Fig. 5) and was followed by the formation of a shoot (Fig. 6). The shoots produced their own tubercules and grew similarly to those in Figs. 1b and 2.

4. Discussion

Areole activation through breaking apical dominance is the most efficient way to attain micropropagation in cacti. However, in practically all reported cases, areole activation is induced by cytokinins rather than auxins (Hubstenberger et al., 1992; Rubluo, 1997). This was also observed in *M. san-angelensis,* in which the best results were achieved in the presence of BAP alone or in combination with NAA, with a reduction in regeneration capacity noted with increased auxin concentration (Martínez-Vázquez and Rubluo, 1989). Moreover, the in vitro morphogenetic responses of many species reported in the literature exhibit a need for an adequate balance between auxins and cytokinins (George, 1993).

In disagreement with most of the current literature, the present study showed an auxin-induced regeneration capacity (Table 1). There is some support for this in the literature Hutabarat (1986) mentioned organogenetic capacities induced by IBA in *Echinocereous* spp. (Cactaceae) and Mauseth and Halperin (1975) found that in *Opuntia polycantha*, auxins inhibit the action of gibberellins and cytokinins, which are expressed only when auxin concentrations are very low. It has been noted that tissue cultures maintained for a long period in vitro often lose their regeneration capacity. However in this study, it was found that totipotency was retained by the tissues in all cases (hormone-free or influenced by auxins) (Table 1). Moreover, our group demonstrated that in in vitro derived shoots similar to those used in this study, there was nuclear genome stability as analyzed through flow cytometry and karyological observations despite the long-term subculture origin and the use of various auxins to attain morphogenetic responses (Palomino et al., 1999). Therefore, the method outlined here provides a large population that clonally retains the genetic make-up of this species and can be used to expand the urgently needed research into this severely endangered species.

In our results, two modes of plant regeneration (i.e. de novo meristem formation and areole activation) were observed (Figs. 3–6) in presence of the auxins tested. However, the mechanisms by which these growth regulators act in this system remains unresolved.

Auxin-mediated induction of roots in cacti is well documented (Hubstenberger et al., 1992; Rubluo et al., 1993), while other studies (e.g. *Aztekium riiteri*) report no root differentiation from auxin-treated shoots (Rodriguez-Garay and Rubluo, 1992). In our results, induction of roots was obtained by simply subculturing shoots to MS basal media devoid of growth regulators, and root induction appeared not to be influenced by the culture origin, except in the case of shoots cultured with 2,4-D, which did not show root induction (Table 1).

In the present study, *M. san-angelensis* responded to the addition of auxins by indirect organogenesis (Figs. 3 and 4) and by activation of the pre-existing axillary meristem. Our results show that in vitro activation of the axillary meristem takes place under the influence of auxins (Figs. 5 and 6). These results disagree with those of Dabekaussen et al. (1991), who found that the addition of NAA is of little importance for areole activation in *Sulcorebutia alba* (Cactaceae). Our results showed that the new calluses were formed de novo (adventitious), and that the axillary meristems were activated.

The ability of auxins to induce in vitro plant regeneration in *M. san-angelensis* is atypical and contrary to most previously published studies. It has been suggested that changes in sensitivity of tissues to auxins may play a fundamental role in developmental responses (Trevawas, 1981), and that a quantitative method is needed to assay these changes (Cline, 1994). The results presented here suggest that auxins may be involved in in vitro morphogenetic responses in Cactaceae, and that further work is needed to understand the delicate balance of growth hormones required for successful regeneration and propagation.

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References

Bravo-Hollis, H., Sánchez-Mejorada, H., 1991. Las Cactáceas de México, Vol. III. UNAM, México, 643 pp.

Boke, N.H., 1958 Areole histogenesis in *Mammillaria lasiacantha*. Am. J. Bot. 45, 473–479.

Chaturvedi, H.C., Jain, M., 1994 Restoration of regeneration potentially in prolonged culture of *Digitalis purpurea*. Plant Cell Tiss. Org. Cult. 38, 73–75.

Cline, N.G., 1994 The role of hormones in apical dominance. New approaches to and old problem in plant development. Physiol. Plant. 90, 230–237.

Dabekaussen, M.A.A., Pierik, R.L.M., van der Laken, J.D., Hoek, S.J., 1991 Factors affecting areole activation in vitro in the cactus *Sulcorebutia alba* Rausch. Hort. Sci. 46, 283–294.

George, E.F., 1993. Plant Propagation by Tissue Culture. Part 1: The Technology. Exegetics Limited, Edigton, 574 pp.

Hubstenberger, J.F., Clayton, P.W., Phillips, G.C., 1992. Micropropagation of Cacti (Cactaceae). In: Y.P.S. Bajaj (Ed.), Biotechnology in Agriculture and Forestry, Vol. 20, High Techniques and Micropropagation, Vol. IV. Springer, Berlin, pp. 49–68.

Hutabarat, D., 1986. Gamma rays induced effects on plant regeneration from callus in *Echinocereus* species (Cactaceae). In: Proceedings of a Symposium on Nuclear Techniques and In Vitro Culture for Plant Improvement, Vienna, IAEA, pp. 187–192.

Johansen, D., 1940. Plant Microtechnique. McGraw-Hill, New York.

Martínez-Vázquez, O., Rubluo, A., 1989 In vitro mass propagation of the near extinct *Mammillaria san-angelensis* Sanchez Mejorada. J. Hort. Sci. 64 (1), 99–105.

Mauseth, J.D., Halperin, W., 1975 Hormonal control of organogenesis in *Opuntia polycantha*. Am. J. Bot. 62, 869–877.

Murashige, T., Skoog, F., 1962 A revised medium for rapid growth and bioassays with tobacco tissues cultures. Physiol. Plant. 15, 473–479.

Palomino, G., Dolezel, J., Cid, R., Brunner, I., Mández, I., Rubluo, A., 1999 Nuclear genome stability of *Mammillaria san-angelensis* (Cactaceae) regenerants induced by auxins in long-term in vitro culture. Plant Sci. 141, 191–200.

Reyes, I., Hubstenberger, J.F., Phillips, G.C., 1995 Antiauxin effects on morphogenesis in cultures of the endangered cactus *Aztekium ritteri*. In Vitro, Part II 31 (3), 52A.

Rodriguez-Garay, B., Rubluo, A., 1992 In vitro morphogenetic responses of the endangered cactus *Aztekium ritteri* (Boedeher). Cactus Succ. J. USA 64, 116–119.

Rubluo, A., 1997. Micropropagation in *Mammillaria*. In: Y.P.S. Bajaj (Ed.), Biotechnology in Agriculture and Forestry. High Techniques and Micropropagation, Vol. VI. Springer, Berlin, pp. 193–205.

Rubluo, A., Chavez, V., Martínez, A.P., Martínez-Vázquez, O., 1993 Strategies for recovery of endangered orchids and cacti through in vitro culture. Biol. Conserv. 63, 163–169.

Trevawas, A., 1981 How do plant growth substances work? Plant Cell Environ. 4, 203–228.