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In vitro propagation of *Notocactus magnificus*

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Abstract

Most commercially grown cacti can be easily propagated by seed and/or cuttings. A group of rare and endangered species does not fit into this category and is therefore a good candidate for *in vitro* propagation productions as a tool to overcome habitat and plant-destruction. The number of rare and endangered species of Cacti goes into about 100. Many show a low production and germination of seeds and plantlets are prone to damping-off, making the *in vitro* propagation a feasible alternative for the multiplication and conservation of their germplasm. The aim of the present investigation is to establish a protocol for the *in vitro* culture and plant regeneration of *Notocactus magnificus*, the blue cactus, a highly ornamental species, native to Brazil. The surface sterilization of the explants was achieved with immersion for 10 min in sodium hypochlorite solution for either seeds (0.25% v/v) or ribs segments (1% v/v). Callus formation was observed when explants were cultured on MS medium supplemented with sucrose at 2% (w/v), 2,4-dichlorophenoxyacetic acid 0.5 μM, benzylaminopurine 4.4 μM, thiamine HCl 0.4 mg l^{-1} and i-inositol 100 mg l^{-1}. The regeneration of shoots was carried out on MS medium supplemented with either different concentrations of benzylaminopurine and 1-naphthaleneacetic acid, or kinetin and indole-3-acetic acid. The highest number of shoots occurred when MS medium was supplemented with benzylaminopurine 22.2 μM, sucrose 3% (w/v) and agar 0,6% (w/v). *In vitro* spontaneous rooting of shoots was observed after eight months under culture on MS medium. Only *in vitro* rooted shoots developed into normal plants under glasshouse culture conditions. This *in vitro* protocol should be useful for the conservation as well as mass propagation of *Notocactus magnificus*.

Abbreviations: BAP – benzylaminopurine; 2,4-D – 2,4-dichlorophenoxyacetic acid; IAA – indole-3-acetic acid; Kinetin – 6-furfurylaminopurine; 2iP – N^6-(Δ^2-isopentenyl) adenine; IBA – indole-3-butyric acid; MS – Murashige and Skoog medium; NAA – 1-naphthaleneacetic acid

Introduction

The Cactaceae family thrives from Canada through to the extreme south of Argentina (Rizzini, 1987). Most species are found growing in arid or semi-arid regions, whilst some can be seen in jungles and tropical rainforests. In Brazil there are 32 indigenous genus, comprising 160 species, Bahia State being the dispersion center (Seixas, 2001). According to Moura (1979) every Cactaceae species is at risk of disappearing from its natural habitat.

As cacti seeds are frequently difficult to be obtained (Mauseth, 1977) and plantlets are reported to be susceptible to damping-off (Mauseth, 1979; Ault and Blackmon, 1987), the *in vitro* propagation is a feasible alternative for the rapid multiplication and maintenance of germplasm (Smith et al., 1991;

Johnson and Emino, 1979), therefore lowering the danger of extinction for many Cactaceae.

The surface sterilisation of cacti is extremely important because the spikes and hairs normally found in such plants host a large variety of microorganisms. Several chemicals have been reported for such procedure, such as sodium hypochlorite (Johnson and Emino, 1979), chloramine B (Vyskot and Jara, 1984) and calcium hypochlorite (Escobar et al., 1986).

Johnson and Emino (1979), working with thornless *Mammilaria elongata* tubers found that the largest amount of friable calli was achieved by Murashige and Skoog (1962) high salts supplemented with Murashige et al. (1974) organic mixture adjusted to pH 5.6 using 2–10 mg l^{-1} 2,4-D and 1–2 mg l^{-1} kinetin or 2iP. Shoot induction was optimized with the addition of 10 mg l^{-1} 2iP and 1 mg l^{-1} IAA, while roots formed by culturing on media containing 60 mg l^{-1} NAA or IBA with complementary levels of 1–2 mg l^{-1} kinetin or 2iP.

According to Clayton et al. (1990), many cacti produce an excess of auxin under *in vitro* culture conditions, stimulating the callus production, a negative for micropropagation. *Opuntia amyclaea* was reported to have rooted on medium without the supply of exogenous auxin (Escobar et al., 1986). Ault and Blackmon (1987) also observed spontaneous rooting and 90% of shooting in *Ferocactus acanthodes* on medium lacking auxin. The highest concentrations of cytokinin is the most adverse in subsequent rooting; this adverse effect can be minimized by a passage on hormone-free medium to help to reduce cytokinin carry over, before application of specialized treatments (Hubstenberger et al., 1992).

The aim of the present work was to establish a reliable protocol for the *in vitro* propagation of *Notocactus magnificus*.

Material and methods

Plant material and explant sources

Notocactus magnificus (Ritter) Krainz seeds and 2 years old potted plants were supplied by Cactos Hino, Jundiaí, SP, Brazil. Rib segments of approximately 1 cm^2 from the potted plants (Figure 1e, f) or hypocotyls of *in vitro* germinated seeds were the explant sources. Rib explants were surface sterilised using sodium hypochlorite at 0.25, 0.5 and 1% (v/v) for 10 or 20 min., while seeds were efficiently surface sterilised with sodium hypochlorite at 0.25 (v/v) for 10 min. Both ribs and seeds were subsequently washed six times with sterile distilled water.

Basal media and culture conditions

Murashige and Skoog (1962) basal medium was used in all experiments. Glass cultures vessels with 20 diameter$\times$10 cm high using a plastic cap, were maintained at 25 ± 2 °C with 14-h light/10-h dark photoperiod at 47 μmol m^{-2} s^{-1} DFFF. Plants in the glasshouse were cultured under 80% humidity, 70% shading, 25 ± 5 °C and natural daylength.

Seed germination

Seeds were efficiently surface sterilized with sodium hypochlorite at 0.25 (v/v) for 10 min, followed by six washes in sterile distilled water. After this procedure they were transferred under sterile conditions to test tubes containing MS medium with 2% (w/v) sucrose. As the *in vitro* germination rate was very low (below 13%) a further test was carried out in a germination chamber, using Gerbox with 25 seeds on moist filter paper at alternate temperatures (20–30 °C), under constant illumination (15 μmol m^{-2} s^{-2} DFFF), 4 replicates and 4 assessment times: 5, 10, 15 and 22 days after the beginning of the test.

Callus formation

Hypocotyls of *in vitro* germinated seeds or rib segments were cultured on MS medium supplemented with thiamine HCl 0.4 mg l^{-1}, i-inositol 100 mg l^{-1}, sucrose 2% (w/v), BAP 4.4 μM and 2,4-D 0.5 μM, pH at 5.7 (Smith et al., 1991). Callus formation was carried out in a culture room at 25 ± 2 °C in the dark.

Shoot differentiation

Calli (6–8 mm) were transferred onto MS medium supplemented with three different combinations of growth regulators, sucrose and gelling agent. The first consisted of BAP (0, 2.2, 8.9 or 22.2 μM) with

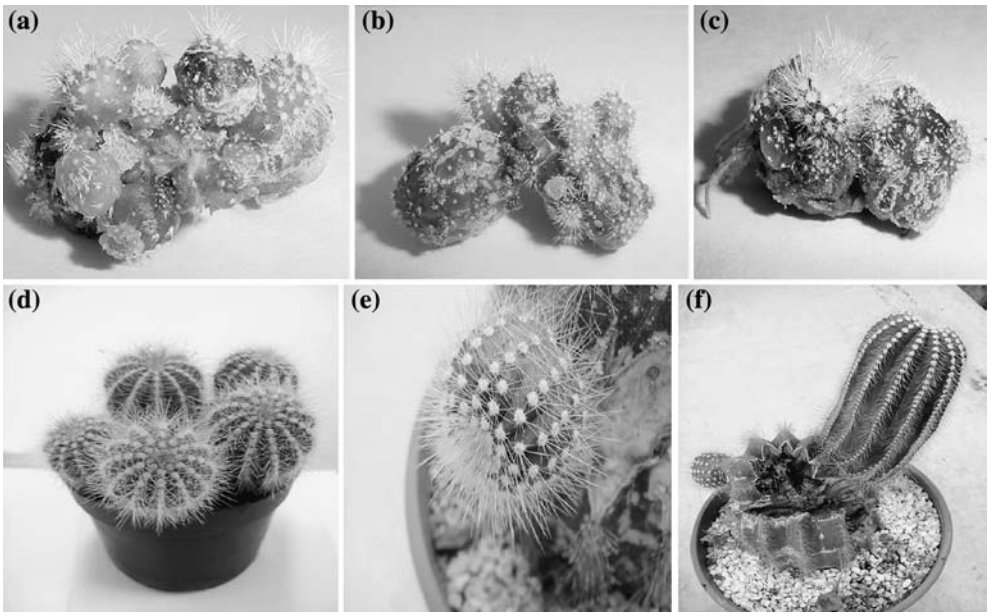

Figure 1. Several phases of *in vitro* micropropagation of *Noctocactus magnificus*. (*a*, *b*) Represents the shoot formation of several shoots/callus after 4 weeks on MS medium supplemented with BAP 22.2 μM, sucrose 3% (w/v), made semi-solid with agar 0.6% (w/v); (*c*) Rooting of callus after 6 months in culture growing on hormone free media. (*d*) Six months old plants acclimated obtained from *in vitro* culture. (*e*, *f*) The original plant from where the explants were obtained.

NAA (0, 2.7, 10.7 or 26.9 μM) as well sucrose (3% w/v) and agar (0.6% w/v). The second one was comprised of kinetin (0, 4.6, 9.3 or 23.2 μM) with IAA (0, 2.9 or 5.7 μM), sucrose (2% w/v) and Phytagel (0.2% w/v). The third medium was supplemented with BAP (0, 4.4, 22.2 and 44.4 μM), sucrose (2% w/v), Phytagel (0.2% w/v) and activated charcoal (500 mg l^{-1}) in order to avoid putative oxidation.

Shoot differentiation and elongation were carried out under 25 ± 2 °C with 14-h light/10-h dark photoperiod at 47 μmol m^{-2} s^{-1} DFFF.

Root formation

The *in vitro* rooting was carried out on MS medium containing (2% w/v) sucrose and Phytagel (0.2% w/v).

Hardening

Ex vitro culture was carried out in a glasshouse under 70% shading and 80% humidity. Rooted shoots were washed to remove culture medium and placed on multicell plastic trays (24 6 × 6 × 6 cm^2 cells/tray), 3 shoots/cell, filled with either 100% Plantmax compost (Eucatex, Jundiai, SP), 100% washed sand or 50% Plantmax and 50% washed sand.

Results and discussion

Seed germination

In vitro germinated seeds showed an average germination of 13%. The germination after 22 days in germination chamber achieved a figure of 26%. The lower percentage of germination under aseptic condition may be due to factors such as low seed viability, inadequate osmotic potential of the medium or high humidity. Seixas (2001) obtained a germination of 2.05% for seeds of *N. magnificus* on seven different composts, well inferior figure to the 70% observed when seeds were cultured for 18 days in a germination chamber under illumination and alternate temperatures (20–30 °C), identical to those used in the present investigation, except for the use of sand instead of filter paper.

Surface sterilisation of explants

Due to the large amount of thorns observed on the stems of *N. magnificus*, the surface sterilization was only efficient when rib explants were submerged in sodium hypochlorite at 1% (v/v) solution for 10 min. This result complies with Smith et al. (1991), working with *Coryphanta macromeris*.

Callus formation

The protocol used by Smith et al. (1991) was also efficient for the formation and proliferation of *N. magnificus* pale (lacking chlorophyll) and compact calli.

Shoot differentiation and ellongation

Some shoot proliferation of *N. magnificus* occurred in the dark after 2–4 months on the same medium used for callus formation. The resulting shoots were subsequently transferred to the light at 47 μmol m^{-2} s^{-1} DFFF onto MS medium supplemented with BAP 4.4 μM and 2% sucrose (w/v), made semi-solid with Phytagel 0.2% (w/v), resulting in several shoots per callus, which after excision gave rise to an average of 6 shoots.

A better regeneration rate, though, was observed when calli were subcultured onto fresh callus inducing medium after 4 weeks and finally transferred onto shoot regeneration medium after a total of 8 weeks in culture. An average of 6 shoots/callus were observed after 4 weeks on MS medium supplemented with BAP 22.2 μM, sucrose 3% (w/v), made semi-solid with agar 0.6% (w/v). Similar results (5 shoots/callus) were observed when calli were transferred onto MS medium supplemented with kinetin 4.6 μM, IAA 2.9 μM, sucrose 2% (w/v), made semi-solid with Phytagel 0.2% (w/v) but the rising of shoots occurred only after 12 weeks in culture (Figure 1 a, b).

In every case, the resulting shoots were transferred onto MS medium lacking growth regulators, but containing sucrose 2% (w/v) and Phytagel 0.2% (w/v) for further development.

Root formation

As also reported by Escobar et al. (1986) for *O. amyclaea* and Ault and Blackmon (1987) for *F. acanthodes*, *in vitro* rooting occurred spontaneously. After 6 months in culture shoots showed a darker shade of green, typical shape and cuticule formation. Efficient rooting occurred after 6 months in culture. On the completion of 8 months in culture 45% of the shoots showed roots (Figure 1c).

Hardening

Non-rooted shoots did not grow under glasshouse conditions, in contrast with the 90% rooting reported by Ault and Blackmon (1987) for *F. acanthodes*. Only 20% of previously rooted shoots survived the glasshouse conditions when cultured on 50% sand and 50% compost. Rooted shoots kept on 100% compost showed a loss of 78% due to diseases stimulated by higher humidity, while those cultured on 100% sand died because of low water retention (Figure 1d).

Conclusion

The protocol herein described is efficient for the *in vitro* multiplication of *N. magnificus* through indirect organogenesis enabling the conservation and propagation of this species. Further studies need to be carried out in order to improve rooting and acclimatization.

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