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In vitro micropropagation of *Nopalea cochenillifera* (Cactaceae)

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Summary

Here we present a protocol for the mass propagation of *Nopalea cochenillifera*, a cactus species widely used in semi-arid regions as a forage crop. Areoles were isolated from young cladodes of field-grown plants and cultivated in Murashige and Skoog (MS) medium supplemented with 6-benzyladenine (BA) at 7.5 µM and indolacetic acid (IAA) at 1.0 µM. Axillary bud proliferation was achieved by transferring explants containing up to 8 areoles to MS medium supplemented with BA at 7.5 µM and IAA at 0.2 µM . Rooting was achieved by transferring *in vitro* grown shoots to hormone-free MS medium. The plantlets successfully survived acclimatization *ex vitro*. By using the protocol presented here, it is possible to reach a propagation rate of up to 20 individuals for each cycle of multiplication.

Introduction

The cactus *Nopalea cochenillifera* (L.), is a succulent shrub with green, flattened stem segments called cladodes. In Mexico, the tender young cladodes are called nopalitos and are consumed as a vegetable (NERD et al., 1997). In the northeast of Brazil it is widely cultivated as a forage crop, where it is known as "palma doce". Its importance is highlighted in the years of drought and in the summer months, where in many occasions its fleshy cladodes are sole source of water and nutrients for livestock (NETO, 2003; BATISTA et al., 2003). In Brazil, the area on which this crop is cultivated is expanding very rapidly and it is estimated to reach 500,000 hectares (SANTOS et al., 2001). As the interest in the crop grows, there is a need to develop reliable protocols for the rapid multiplication and distribution of selected genotypes and of disease-free planting material. Tissue culture techniques are also essential in order to facilitate the application of modern biotechnological methods to this crop. The techniques for *in vitro* culture of Cactaceae are still not well developed and the effect of different growth regulators on morphogenesis is not well understood (FAY et al., 1995; LLAMOCA-ZÁRATE et al., 1999a; GIUSTI et al., 2002). Here we present a detailed protocol for the *in vitro* propagation of this species.

Material and methods

Young cladodes about 10 cm long of *N. cochenillifera* were obtained from plants grown in the experimental field of the Agronomy School, Federal University of Ceará, Fortaleza, Brazil. The cladodes, collected during the dry-season (August do December), were disinfected by immersion in 70% ethanol, followed by immersion in 1.5% sodium hypochlorite for 10 min. Disinfected tissues were rinsed three times with sterile distilled water and sectioned transversely or longitudinally. Explants containing one areole and 2-3 mm of the surrounding tissue were cultivated on MS medium (MURASHIGE and SKOOG, 1962) solidified with 0.7% agar. Different combinations of benzyladenine (BA) (0.0, 0.06, 0.3, 1.5, 7.5 and 37.5 µM) and indolacetic acid (IAA) (0.0, 0.04, 0.2, 1.0 and 5.0 µM) were tested for shoot development and the results obtained were recorded after 5 weeks of culture. To achieve axillary proliferation 2 cm shoots developed *in vitro* were cut in half longitudinally and the halves were sectioned in segments containing up to 6 areoles and cultivated on MS medium solidified with 0.7% agar. Different combinations of BA (0.0, 0.06, 0.3, 1.5, 7.5 and 37.5 µM) and IAA (0.0, 0.04, 0.2, 1.0 and 5.0 µM) were tested for shoot proliferation. The effect of the different treatments were measured after 5 weeks of culture. The induction of roots was tested by cultivating individual shoots in MS medium solidified with 0.7% agar and supplemented with a range of concentrations of IAA (0.0, 0.9, 4.5, 22.5 and 112.5 µM). After reaching at least 4 cm rooted plants were transferred to plastic pots (9.0 x 6.5 cm), filled with washed sand river and watered with MS salts and submitted to a 15 days acclimation period in which they were covered with transparent plastic bags. All media were prepared by standard procedures, pH adjusted to 5.8 and autoclaved at 121°C for 15 min. Explants were incubated at 27±2 °C under a 16 hours photoperiod with an irradiance level of 70 $\mu E \cdot m^{-2} \cdot s^{-2}$ provided by white fluorescent lights. A minimum of 30 explants was used for each treatment and all experiments were performed three times. Percentage of developed and proliferated shoots was calculated from the total number of preexisting buds. The results were evaluated by the analysis of variance and Tukey test to compare the mean (level of significance at 5%) (STEEL and TORRIE, 1980).

Results

The effect of different concentrations of BA and IAA on shoot initiation in explants containing individual areoles isolated from field grown plants was determined after 5 weeks of culture. By examining the results shown in Tab. 1 it is apparent that BA at 7.5 µM either alone or in combination with IAA favored best the initiation of shoot development, even though IAA at very low concentration (0.04 and 0.2 µM) exerted an inhibitory effect on areole activation. Although the frequency of shoot initiation at higher concentrations of BA was equally high, most of the shoots initiated failed to develop or grew very slowly. The frequency of shoot development was very low when the areoles were cultivated in basal medium or in basal medium supplemented with auxin alone. Taking into account the frequency of shoot initiation and the growth and development of the shoots, BA at 7.5 µM in combination with IAA at 1.0 µM was chosen as the ideal cytokinin/auxin combination to achieve the establishment of *in vitro* culture of *N. cochenillifera*.

After 5 weeks in culture, the shoots grown in MS medium supplemented with BA at 7.5 µM and IAA 1.0 µM reached up to 2.0 cm in length, and were used as source of explants for axillary proliferation. This was done by removing the shoot apex and dividing the shoots longitudinally into two halves and sectioning the halves in portions containing up to six areoles. These portions were then transferred to Petri dishes containing MS medium supplemented with the same combinations of BA and IAA as those used in the experiments for inducing shoot initiation from field grown plants. Although BA alone

Tab. 1: Percentage of shoot initiation in explants of *Nopalea cochenillifera* containing individual areoles, cultured in MS medium supplemented with combinations of benzyladenine (BA)and indolacetic acid (IAA).Shoot initiation was determined after five weeks of culture.

IAA (μM)[a]	BA (μM)[b]					
	0.00	0.06	0.30	1.50	7.50	6.25
0.00	2.50 dB	7.10 cdB	6.03 cdC	10.80 cD	49.00 aB	25.53 bC
0.04	3.40 dB	3.67 dB	14.47 cB	26.47 aA	20.03 bD	26.80 aC
0.20	4.27 dB	6.37 dB	9.13 dC	14.27 cC	31.63 bC	45.83 aB
1.00	3.50 cB	12.97 bA	10.90 bBC	14.43 bC	57.70 aA	53.13 aA
5.00	10.30 eA	16.77 dA	24.70 cA	21.77 cB	44.97 bB	54.53 aA

[a] In each column, mean values followed by same capital letters do not differ at p<0,05.
[b] In each line, mean values followed by same lower-case letters do not differ at p<0,05.

Tab. 2: Percentage of axillary proliferation in explants of *Nopalea cochenillifera* containing up to six areoles, submitted to combinations of benzyladenine (BA) and indolacetic acid (IAA).

IAA (μM)[a]	BA (μM)[b]					
	0.00	0.06	0.30	1.50	7.50	6.25
0.00	0.00 dB	0.00 dC	10.00 cB	13.33 cD	73.33 aB	40.00 bB
0.04	6.67 cA	0.00 dC	6.67 cC	33.33 bC	46.67 aC	30.00 bC
0.20	3.33 eA	15.00 dA	3.33 eC	50.00 bA	96.67 aA	30.00 cC
1.00	0.00 eB	10.00 dB	3.33 eC	50.00 bA	76.67 aB	41.33 cB
5.00	0.00 dB	16.67 cA	40.00 bA	43.33 bB	43.33 bC	65.67 aA

[a] In each column, mean values followed by same capital letters do not differ at p<0,05.
[b] In each line, mean values followed by same lower-case letters do not differ at p<0,05.

was able to induce shoot proliferation, the addition of IAA into the medium had a positive effect on the frequency of shoot induction (Tab. 2). The highest proliferation frequencies were achieved when the proliferation medium contained BA at 7.5 μM alone or in combination with IAA at 0.2 or 1.0 μM. Although within these combinations of growth regulators we could not detect a statistically significant difference among the treatments, the shoots proliferated in medium supplemented with BA at 7.5 μM and IAA at 0.2 μM were greener and grew faster. As previously observed with explants obtained from field-grown plants (Tab. 1), IAA at very low concentration caused a decrease in the frequency of shoot induction. The frequency of shoot development was very low when the areoles were cultivated in basal medium or in basal medium supplemented with auxin alone. The frequencies of proliferation in medium supplemented with BA at 37.5 μM alone or in combination with IAA were also high, but the shoots that were initiated grew very slowly or even failed to develop. Therefore the best combination of growth regulators for achieving axillary proliferation in *N. cochenillifera* was chosen as BA at 7.5 μM and IAA at 0.2 μM. By using this medium, it is possible to reach a propagation rate of up to 20 for each cycle of multiplication.

We found that the minimum shoot size for rooting of explanted shoots is 1.0 cm. The results of the experiments in which shoots were cultivated in MS medium supplemented with IAA at various concentrations are shown in Tab. 3. Rooting of the shoots occurred even in the absence of IAA. The roots formed were thick, with an average length of 5 cm. After reaching at least 4 cm rooted plants from all four treatments were transferred to plastic pots (9.0 x 6.5 cm), filled with washed river sand and watered with MS salts and submitted to a 15 days acclimation period in which they were covered with transparent plastic bags. After this period the plants were transferred to a greenhouse. 95-100% of the plants survived. It is interesting to note that even though the number of roots was significantly higher in the medium containing IAA at 22.5 μM, upon transfer to soil rooted plants from all of the treatments grew equally well.

Tab. 3: Rooting of explanted shoots up to 1,0 cm in length, subjected to concentrations of indolacetic acid (IAA).

IAA (μM)	Number of roots*
0.0	4.50 [bc]
0.9	4.47 [bc]
4.5	3.53 [c]
22.5	8.00 [a]
112.5	6.23 [ab]

* Means followed by the same letters do not differ, at p<0,05.

Discussion

This study reveals the feasibility of attaining mass propagation of *N. cochenillifera* through axillary proliferation. Areole activation through breaking of apical dominance is the most efficient way to attain micropropagation in cacti and the activation is generally induced by cytokinins rather than auxins (RUBLUO et al., 2002; ARNHOLDT-SCHMITT et al., 2002). The hormonal requirements for shoot initiation from isolated areoles of *N. cochenillifera* were similar to those found for *Opuntia ficus-indica* (LLAMOCA-ZÁRATE et al., 1999b; GARCIA-SAUCEDO et al., 2005), *Opuntia elisiana* (JUÁREZ and PASSERA, 2002) as well as for several other cacti (MALDA et al., 1999). Shoot development was not observed when the areoles were cultivated in basal medium, suggesting that the endogenous levels of cytokinins and auxins were not conductive to shoot development. Exogenous cytokinin is required for areole activation in *N. cochenillifera*. Although auxin is not strictly necessary, it promotes a more vigorous shoot development as observed in *O. ficus-indica* (LLAMOCA-ZÁRATE et al., 1999b), *Mammilaria san-angelensis* (MÁRTINEZ-VÁZQUEZ and RUBLUO, 1989) as well as for several other cacti (MALDA et al., 1999). The hormonal requirements for areole activation in explants from field grown *N. cochenillifera* plants and from in vitro plants are very similar, except that the frequency of shoot initiation is higher in explants from in vitro grown plants.

Auxin-mediated induction of roots in cacti is well documented (ARNHOLDT-SCHMITT et al., 2002; RUBLUO et al., 1993). In the case of *N. cochenillifera*, rooting of explants was achieved in the absence of IAA, even though the addition of IAA to the culture media led to an increase in the number of roots. However, after transferring the rooted shoots from the control and IAA treatments, no difference in the growth and development of the plants could be observed, thus indicating that the conditions under which the roots developed do not influence the life cycle of the plants. These observations are in agreement with previous observations of ESCOBAR et al. (1986) with *Opuntia amyclaea*.

In conclusion, the culture conditions described here may be utilized for the mass propagation of *N. cochenillifera* and for the mass production of healthy planting material from selected genotypes. The availability of a protocol for the in vitro cultivation of this species will allow for the development of protocols for de novo regeneration of this species either by organogenesis or somatic embryogenesis.

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