Micropropagation of Endemic and Endangered Mexican Species of Ponytail Palms

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Abstract. Experiments were conducted to establish an efficient protocol of micropropagation of *Beaucarnea gracilis* and *B. recurvata* two endemic and endangered Mexican species. Multiple shoots were induced by direct organogenesis from in vitro seedlings and longitudinal sections of seedlings in both species. The highest formation of shoots per explant, both *B. gracilis* and *B. recurvata*, was obtained from longitudinal sections of seedlings on Murashige and Skoog (MS) medium supplemented with 22.2 μM 6-benzylaminopurine, induced 8.2 and 11.1 shoots per explant respectively. In vitro rooting was readily achieved on MS medium with 1 g/l activated charcoal without growth regulators. According to initial treatment and depending on where the shoots come from, the rooting rates were 61% to 100% for *B. gracilis*, and 83% to 100% for *B. recurvata*. Survival rates in greenhouse conditions for both species were 80% to 100% after 3 months. These results indicate that the micropropagation of these species of *Beaucarnea* is technically feasible, and that in vitro culture is a useful option for the conservation and propagation of these important endangered species.

The *Beaucarnea* genus is part of the Nolinaceae family, endemic to North America and part of Central America (Eguarte et al., 1994; Hernández, 1993b). *Beaucarnea* distribution extends from southern Tamaulipas, Mexico (near the Tropic of Cancer) to Honduras and probably to northern Nicaragua in Central America. All species of *Beaucarnea* (about 10) are considered threatened or endangered. Of these, nine are endemic to Mexico with *B. gracilis* and *B. recurvata* catalogued as threatened species. (Hernández, 1993a; Semarnat, 2002)

Beaucarnea gracilis Lem. is a tree measuring 6 to 12 m in height, distributed throughout Mexico's Oaxaca and the Puebla states (Fig. 1a). *B. recurvata* Lem. reaches 4 to 15 m, and is found in the states of Oaxaca and Veracruz (Fig. 1b) (Hernández, 1993b). The wild populations of these two species are threatened as a result of their habitat alteration caused by the opening of areas for agriculture and livestock, as well as by urban expansion and the illegal collection of individuals (Cardel et al., 1997; Franco, 1995; Hernández, 1993a). Both species have great commercial demand as ornamentals, especially *B. recurvata*. Despite the fact that some nurseries are legally propagating *Beaucarneas* (ponytails), the production and size of the specimens available do not satisfy the national and international market demands, and thus the seeds are constantly and illegally over-collected;

juvenile and adult plantlets are extracted selectively (Hernández, 1993a). This has caused the reduction and even the disappearance of wild populations, which are affected mainly by the reduction of seed production, a phenomenon that influences the size, the population structure, and natural regeneration (Franco, 1995).

In view of the difficulty in achieving efficient in situ conservation for species in this genus, plant tissue culture represents an excellent option for the study and conservation of threatened species, as well as a tool for efficient and fast propagation (Carneiro, et al., 1999; Fay, 1994; González-Benito, et al., 1999; Martínez-Vázquez and Rubluo, 1989; Mata, et al., 2001a, 2001b; Mauseth, 1979; Rodríguez-Garay and Rubluo, 1992; Stuppy and Nagl, 1992).

Despite the great worldwide interest for ponytails as ornamentals (in the U.S. mature plants may be sold for \$600 to \$700) (Cardel et al., 1997), there are few reports on propagation by tissue culture for *B. recurvata* (Mekers, 1988; Samyn, 1993, 1997) and, as far as we know, there are no reports for *B. gracilis*. The development of viable methods for the propagation of ponytails would thus be beneficial for both commercial and conservation propose. The present study describes the micropropagation of *B. gracilis* and *B. recurvata* from seedlings and longitudinal sections of seedlings germinated in vitro, and their establishment under greenhouse conditions.

Materials and Methods

Seed germination. Seeds of *B. gracilis* and *B. recurvata* were collected in Puebla and Veracruz (Mexico), respectively. Seeds were washed with a Dawn detergent (Colgate Palmolive, S.A. de C.V., Mexico) for 30 min, then sterilized with 70% ethanol for 1 min, followed by 30% v/v commercial chlorine (sodium hypochlorite, 6% of active chlorine), plus Tween 80 (2 drops/100 mL) for 30 min. Seeds were then rinsed three

times with sterilize, distilled water under aseptic conditions. Finally, the seeds were kept in a solution of Benlate (3 g·L⁻¹) for 20 h, and subsequently rinsed with sterilize distilled water. Seeds were sown in 125 mL baby food jars with 25 mL of Murashige and Skoog MS medium (Murashige and Skoog, 1962), with 2 mg·L⁻¹ glycine; 100 mg·L⁻¹, myo-inositol, and 30 g·L⁻¹ sucrose (basal MS medium). The pH was adjusted to 5.7 with NaOH and HCl 0.1 N before adding 5.5 g·L⁻¹ agar high gel strength (Sigma Chemical Co., St. Louis, Mo.) and autoclaving at 120 °C for 15 min. Five seeds were sown per container with 60 repetitions (300 seeds). Cultures were incubated at 25 ± 1 °C, under a 16-h photoperiod provided by cool-white fluorescent lamps (50 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). Germination percentages were recorded weekly.

Induction of multiple shoots. Two types of explants were excised from 7 cm of in vitro-germinated seedlings for both species: a) seedlings with roots and tips of leaves trimmed and placed into culture vertically (Fig. 1c); b) seedlings as above, but dissected in two longitudinal sections and placed into culture with the cut surface in contact with the medium (Fig. 1d). The explants were placed on basal MS medium supplemented with different concentrations of 6-benzylaminopurine (BA) (0, 4.4, 13.2, or 22.2 μM). Forty explants were used per treatment, with two explants placed in each jar for 20 repetitions. After the induction period (30 d), monthly subcultures were carried out in basal MS medium, containing 1 g·L⁻¹ activated charcoal and without growth regulators. The number of shoots per explant was recorded after 3 months.

Rooting of shoots. Regenerated shoots were individualized and subcultured to MS basal medium with 1 g·L⁻¹ activated charcoal and without plant growth regulators and in order to induce the formation and development of roots. Two months later, rooting was observed and rooting percentage recorded.

Ex vitro survival. The regenerated plants were removed from the jars, washed thoroughly under tap water, and finally with distilled water. The plants were then transferred to propagation trays (Hummert International) with a soil mixture containing of leaf litter, loam and perlite (1:1:1), and placed under greenhouse conditions that averaged 30 °C and with a high relative humidity (80% to 90%) keeping the trays covered with plastic translucent lids, and perforated weekly to decrease the relative humidity until 50% was reached. Plants then were individually repotted.

Statistical analysis. Shoot production was recorded and analyzed using a one-way analysis of variance (ANOVA) followed by a multiple comparison of means using Tukey's HSD criterion ($p \leq 0.05$).

Results and discussion

Seed germination. Seed germination for both species started on the fifth day after being placed in culture and reached 89.8% (*B. gracilis*) and 95.3% (*B. recurvata*) after 30 d in culture. The development of in vitro-seedlings was similar to that observed for seed germination in soil (data not shown). Cardel et al. (1997) and Flores and

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Fig. 1 (a) Adult plant of *Beaucarnea gracilis*. (b) Adult plant of *B. recurvata*. (c) In vitro germinated seedlings of ponytail used as explants. (d) In vitro germinated seedlings of ponytail dissected longitudinally and used as explants. (e) Initial phase of development of adventitious shoots, showing nodular structures (arrow). (f) Primordial buds showing the meristematic dome and primordial leaf arrangement (arrow). (g) Adventitious shoots from *in vitro* grown seedlings of *B. gracilis*. (h) Adventitious shoots from in vitro grown seedlings of *B. recurvata*. (i) Adventitious shoots from longitudinal section of *B. gracilis* after subculture to basal MS medium. (j) Shoots before division. (k) Rooting in *B. gracilis* (left) and *B. recurvata* (right). (l) Regenerated plantlets of *B. gracilis* (left) and *B. recurvata* (right) after 7 months in soil. Bars = 0.3 cm.

Briones (2001) obtained a germination rate of 95% to 100% for *B. gracilis* seeds germinated on agar and filter papers. This high germination level under controlled conditions contrasts with the low establishment of plantlets observed in the field, which can be attributed to several factors, such as soil quality, the hydric potential during the establishment season, depredation and pasturing (Cardel et al., 1997; José and Valiente-Baunet, 2001).

Induction of multiple shoots. Seedlings and longitudinal sections of seedlings of both species formed shoots in all treatments after 30 days. Most treatments produced a generalized response of adventitious shoot development mainly at the base of the explants (Fig. 1e). Regeneration was not observed from the cut areas of the seedling's longitudinal sections.

Around the tenth day of cultivation, the thickness at the explant base and the development of nodular structures was observed. On the twentieth day, the formation of small nodular protuberances was evident. Observations under the stereoscopic microscope revealed the presence of tiny buds with meristematic activity and several leaf primordia (primordial buds), which after a few days elongated and differentiated into adventitious shoots (Fig. 1f). These shoots arose by direct organogenesis without callus formation. Generally, propagation from

callus is considered unsuitable because of the risk of undesirable genetic aberrations (Feijoo and Iglesias, 1998; George, 1993).

***Beaucarnea gracilis*: Responding explants.** The highest average number of shoots per explant was on longitudinal sections, even though the number of responding explants was lower than with seedlings (Table 1). This could have happened as a consequence of the damage caused at the moment the seedling were divided in two parts, because the 75% of explants began to oxidize during the first 2 weeks, and the 35% of them died by necrosis. There is evidence that when a cell is injured, some phenolic compound could be released into the culture medium and new compounds could be produced (Bonga and von Aderkas, 1992).

As the concentration of BA was increased the number of adventitious shoots increased. At the highest concentration of BA (22.2 μM), an average of 8.2 shoots per explant were obtained from longitudinal sections of seedlings and 5.4 shoots per explant from in vitro seedlings (Table 1) (Fig. 1g). However, considerable variation among explants was observed. For example, a few explants formed as many as 20 shoots, mainly from longitudinal sections of seedlings, and two explants with only BA (4.4 μM) formed one shoot at the tip of the leaf and another from the root.

In general, the development of the shoots was similar, using both kinds of explants, and the growth of its structures was very similar to that obtained during seed germination. In most cases, it was possible to note the swollen stem base and the growth of leaves in an alternate way. Shoots with greater size, produced an incipient arrangement of a small rosette, typical of adult individuals. In many cultures, the development of the first shoots inhibited or limited the development of subsequent shoots. This could explain the heterogeneity observed for shoot height, ranging from 0.3 to 10 cm.

***Beaucarnea recurvata*.** Unlike *B. gracilis*, survival was not affected by the dissection of the explants; 100% of the explants from the longitudinal sections, responded, and no oxidation was observed.

Shoots formation per explant was highest on longitudinal sections of seedlings (11.1), when 22.2 μM BA was used (Fig. 1h). The use of cytokinins is common, mainly BA, to induce multiple shooting in diverse species (Hubstenberger et al., 1992; Pérez et al., 1998; Mata et al., 2001b; Moebius-Goldammer et al., 2003). Shoot formation in *B. recurvata* was heterogeneous, forming on some longitudinal sections up to 47 shoots. In contrast, formation of shoots was limited when using in vitro seedlings, with only 1.9 shoots per explant with 13.2 μM of BA (Table 2).

As in the case of *B. gracilis*, a greater number of shoots were obtained when using longitudinal sections of seedlings as explants in compared to shoots obtained from in vitro seedlings. The size of the shoots was larger and more homogenous and vigorous in growth (Fig. 1i). This could be the result of dividing the plant and releasing the apical dominance, and the surface of the explants was in more contact with the induction medium, allowing an increase in the meristematic activity. This kind of response has been recorded in several species (Hubstenberger et al., 1992).

In both species of *Beaucarnea* there is a statistically significant difference between the shoot formation from longitudinal sections of seedlings and in vitro seedlings ($p \leq 0.0001$), i.e., from all treatments using longitudinal section of *B. recurvata*'s seedlings, an average of 7.8 shoots per explant were formed. In contrast, 1.4 shoots were formed per explant from in vitro seedlings. In the case of *B. gracilis*, from all treatments using longitudinal section, 4.9 shoots per explant were formed and for in vitro seedlings 3.0 shoots per explant were formed. In both cases the highest formation of shoots was obtained by longitudinal section of seedlings in basal MS medium with 22.2 μM BA, being greater in the case of *B. recurvata*. The development and growth of adventitious shoots were faster, since the shoots were evident from the first month in culture, and in the case of *B. gracilis*, the shoots were not evident until the second month in culture.

Another statistically significant difference found between the morphogenetic response of these two species was that when using seedlings as explants ($p \leq 0.0001$), the shoot formation obtained in *B. gracilis* was more numerous (3.0), whereas in *B. recurvata* the shoot formation was

Table 1. Effect of 6-benzylaminopurine concentration on shoot number, root formation and ex vitro survival from two different explants of *Beaucarnea gracilis*.

BA (μM)	Explants with responses ^z (no.)	Total shoots (no.)	Avg no. shoots/ responding explant $\pm$ SD	Rooting (%)	Ex vitro survival ^y (%)
Longitudinal sections ^x					
0	25	27	1.1 $\pm$ 0.3 a ^w	100	100
4.4	31	129	4.2 $\pm$ 4.3 b	88	91
13.2	35	195	5.6 $\pm$ 4.2 bc	70	83
22.2	30	247	8.2 $\pm$ 5.6 c	61	86
In vitro seedling ^v					
0	40	40	1.0 $\pm$ 0.0 a	76	100
4.4	36	72	2.0 $\pm$ 2.2 ab	77	90
13.2	34	120	3.5 $\pm$ 5.3 bc	64	87
22.2	40	214	5.4 $\pm$ 4.2 c	51	80

^xN = 40, for ANOVA only the explants with response were considered.

^yResults after 3 months.

^zIn vitro longitudinal sections of seedling.

^wDifferent letters within columns indicate significant difference, $P \leq 0.05$.

^vIn vitro seedlings with roots and tips of leaves trimmed.

Table 2. Effect of 6-benzylaminopurine concentration on shoot number, root formation and ex vitro survival from two different explants of *Beaucarnea recurvata*.

BA (μM)	Explants with responses ^z (no.)	Total shoots (no.)	Avg no. shoots/ responding explant $\pm$ SD	Rooting (%)	Ex vitro survival ^y (%)
Longitudinal sections ^x					
0	40	56	1.4 $\pm$ 0.8 a ^w	100	96
4.4	40	245	6.1 $\pm$ 4.2 b	96	100
13.2	40	383	9.6 $\pm$ 6.2 c	95	99
22.2	40	441	11.1 $\pm$ 9.2 c	91	97
In vitro seedling ^v					
0	40	40	1.0 $\pm$ 0.0 a	97	100
0	40	58	1.5 $\pm$ 1.7 a	94	100
4.4	40	74	1.9 $\pm$ 2.4 a	97	96
13.2	40	59	1.5 $\pm$ 2.0 a	88	92

^xN = 40, for ANOVA only the explants with response were considered.

^yResults after 3 months.

^zIn vitro longitudinal sections of seedling.

^wDifferent letters within columns indicate significant difference, $P \leq 0.05$.

^vIn vitro seedlings with roots and tips of leaves trimmed.

lower (1.4), sometimes limited to the growth of the original explant.

For both species and from longitudinal sections of in vitro seedling, the highest formation of shoots was induced, for *B. gracilis* 8.2 shoots per explant were obtained (Table 1a), and for *B. recurvata* 11.1 shoots per explant were obtained (Table 2a). This data come from a half of a seedling. If we add the two parts, we could state that 16 and 22 plants can be induced from a whole seedling respectively after 6 months in culture. This report surpasses previous reports about *B. recurvata* where a 1 to 2 mm shoot apex (from young seedlings germinated in peat), was cultured in medium Quoirin et al. with 4.4 μM BA, where three to four plants per explant can be produced after 9 months in culture (Mekers, 1988; Samyn, 1993).

Rooting. When shoots were 5 to 10 cm in height they were individualized to induce root formation. In a few cases, the explants started to develop roots, even before being individualized (Fig. 1j). Some shoots, from both species, continued shoot proliferation, possibly as a result of buds that remained inactive from the apical dominance exerted by the taller shoots. This result was observed mainly from 15% to 30% shoots that originated from longitudinal sections of seedlings, initially cultured on high concentrations of BA (13.2 and 22.2 μM). In those cases, the majority of explants continued forming from one to three new shoots. It has been found that the exogenous levels of cytokinin influence the endogenous auxin to cytokinin ratio and therefore some species maintain a continued capacity to form new shoots even in cultured medium without plant growth regulators (George, 1993).

Rooting of *B. gracilis* shoots from both kinds of explants was influenced by the induction medium, i.e., the rooting percentage diminished in inverse proportion to BA concentration (Table 1).

In shoots of root formation was obtained between the first and the fourth week when shoots were cultured in MS medium with activated charcoal (1 g L⁻¹). One to four vigorous roots were formed (Fig. 1k), similar to those obtained from in vitro seed germination. At the end of the rooting period (2 months), most of the roots had formed secondary roots. Samyn (1993) reported that *B. recurvata* rooting occurred easily on one-third-strength MS medium without growth regulators, but the number of roots was increased by NAA (0.67 and 1.35 μM) and the in vitro root initiation occurred in 2 weeks.

In general, the rooting percentage of *B. gracilis* was 80% to 100%. For *B. recurvata*, the rooting percentage was higher than 90% except for shoots induced on 22.2 μM BA where the percentage was 88% (Table 1 and 2). Hubstenberger et al. (1992) found that MS medium was appropriate for rooting cactus and other CAM species. Others also report spontaneous rooting in a medium free of exogenous auxins (Ault and Blackmon, 1987; Escobar et al., 1986; Li et al., 2002; Martín and Pérez, 1995).

Given the aforementioned results, there is a correlation between rooting in vivo and the treatments required in vitro; the species which root freely in vivo, as *Beaucarnea* species, also

root spontaneously in a medium without growth regulators (Hubstenberger et al., 1992).

Ex vitro survival. Most of the plants that formed roots in vitro and potted in soil survived and continued their development (Fig. 1l). The survival percentage for *B. gracilis* was 80% to 100%, and for *B. recurvata* 92% to 100% (Tables 1 and 2). Plantlets that did not root in vitro survived with a percentage higher than 50% (data not shown). The plantlets developed a swollen stem base and one shoot apex from the base after seven months, as in the case of the seedlings obtained from seed germination in soil.

The over-collection of young and adult plants of these species affects not only the population size but also the sex proportion, because they are dioecious species. The fertility possibly decreases and hence the seed production, which can lead to the risk of extinction through the reduction of the minimal viable size of the population. The micropropagation of plants by means of direct organogenesis will not only contribute to satisfy the demand in the horticultural market, but also to design reintroduction programs in the wild, contributing to the conservation of these endangered species.

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