In Vitro Regeneration from Longitudinal Sections of Seedlings of *Beaucarnea purpusii* Rose, an Endemic and Endangered Species

María del Carmen Vadillo-Pro, Luis Hernández-Sandoval, and Guadalupe Malda-Barrera

Faculty of Natural Sciences, Autonomous University of Queretaro, Boulevard de las Ciencias S/N, Juriquilla Querétaro 76230, México

María Luisa Osorio-Rosales

Environment and Sustainability Network, Institute of Ecology A.C., Carretera Antigua a Coatepec 351, El Haya 91070, Xalapa, Veracruz, Mexico

Martín Mata-Rosas¹

Biotechnology Resources Management Network, Instituto de Ecología A.C., Carretera Antigua a Coatepec 351, El Haya 91070, Xalapa, Veracruz, Mexico

Additional index words. micropropagation, cytokinins, pulses, direct organogenesis

Abstract. The present study establishes an efficient protocol for in vitro propagation from longitudinal sections of seedlings of *Beaucarnea purpusii*, a threatened and highly appreciated ornamental species. The effect of three cytokinins: N⁶-benzyladenine (BA), kinetin (Kin), and thidiazuron, 1-phenyl-3-(1,2,3-thiadiazol-5-yl)urea (TDZ), in semisolid media and three different concentrations, as well as the effect of BA and TDZ pulses at higher concentrations in liquid culture medium, were investigated. Adventitious shoot formation by direct organogenesis was observed from all treatments. Additionally, adventitious shoot formation was recorded from the leaves of the new shoots; this particular response was exclusive to treatments supplemented with TDZ. In the experiment using semisolid culture media, the highest means of shoots per explant were obtained from treatments containing TDZ, particularly at a concentration of 0.45 µM (25.8 shoots per explant). For the pulses experiment, the liquid culture media supplemented with TDZ at 22.35 µM for 24 hours and 136.21 µM for 96 hours, induced a mean of 3.9 and 3.3 shoots per explant, respectively. Subculturing individual shoots on MS and half-strength MS (1/2MS) media, both supplemented with activated charcoal at 1 g·L⁻¹, induced rooting in 85% to 95% of shoots. A survival rate of 100% under greenhouse conditions was achieved. The results of this study provide an efficient alternative for mass propagation of *B. purpusii* and may also contribute to the conservation and sustainable use of this valuable natural resource.

The genus *Beaucarnea* belongs to the family Asparagaceae, subfamily Nolinoideae, and occurs from northeastern Mexico to northern Central America (Bogler, 1998). Mexico harbors 10 of the 11 recognized species, of which eight are catalogued as threatened, one as endangered (*B. purpusii*), and one under study to determinate its status (Hernández et al., 2012).

The critical situation of most of *Beaucarnea* species is due to habitat alteration and over-collection of seeds, seedlings, and adult

individuals, which are highly appreciated as ornamental plants. These circumstances affect the population size and the sex ratio, causing a low fertilization rate and consequently a reduced seed production (Contreras et al., 2008; Golubov et al., 2007).

B. purpusii occurs in small areas of the Tehuacán-Cuicatlán Biosphere Reserve, Mexico, which encompasses the states of Puebla and Oaxaca in Mexico (Fig. 1A), and the main reasons for this species being endangered are the small size of its populations, its restricted geographic distribution, the lowest growth ratio of the 11 species in the genus, and the local use of the inflorescences and infrutescences as small Christmas trees, leading to a decreasing number of seeds available for natural germination (Hernández et al., 2009). Because of this situation, it is critical to take action to guarantee its continued survival and sustainable use.

For species like *B. purpusii*, the traditional conservation approach is in situ conservation

(Engelmann, 2011); however, it is now recognized that these can be efficiently complemented by ex situ techniques. Actually the latter may represent the only feasible option for conserving certain highly endangered and rare species (Sarasan et al., 2006).

Plant tissue culture has proven to be a valuable technique for propagation, conservation, and sustainable use of many species, specially of those with sexual and asexual reproduction limitations, slow growth rate, low seeds availability, and endangered conservation status (Pence, 2011; Sarasan et al., 2006), all of which is true for most *Beaucarnea* species (Contreras et al., 2008).

To initiate in vitro propagation, plant growth regulators (PGRs) can be applied to explants following two different methods: 1) traditionally the explants are placed on nutrient medium containing PGRs for 3–4 weeks, transferring them afterward to medium lacking PGRs. 2) Explants can be pulse treated with a concentrated liquid solution of cytokinin and auxin solution for several hours, after which they are placed on medium free of these PGRs, and some studies report improved organogenesis when using the liquid pulse method (Goldfarb et al., 1991; Madhulatha et al., 2004; Ramírez-Malagón et al., 2008).

In spite of the botanical and ornamental interest in *Beaucarnea* species, studies concerning their in vitro propagation are scarce. There are some reports for *Beaucarnea recurvata* (Bettaieb et al., 2008; Osorio-Rosales and Mata-Rosas, 2005; Reyes et al., 2013; Sajeve et al., 1994; Samyn, 1997), *Beaucarnea gracilis* (Osorio-Rosales and Mata-Rosas, 2005), and *Beaucarnea inermis* (Guillén et al., 2015), but to the best of our knowledge, there are no reports on in vitro propagation of *B. purpusii*.

In the pursuit of determining an efficient protocol for in vitro propagation of endangered species with low growth rates such as *B. purpusii*, it is necessary to study the effects of several PGRs at different concentrations on various explant types, with special interest to variables that have not been evaluated previously. Therefore, the present study attempts to provide an efficient protocol for in vitro propagation of *B. purpusii* from longitudinal sections of seedling using different concentrations of BA, Kin, and TDZ on semisolid media, as well as to evaluate the effects on the shoot formation of explants exposed to pulse treatments of higher concentrations of BA and TDZ.

Materials and Methods

Mature seeds were collected from a natural population of *B. purpusii* growing in Tehuacán-Cuicatlán Biosphere Reserve. The seeds were stored at 4 °C until use. They were successively washed with liquid detergent for 30 min, immersed in a 0.3% fungicide solution (Captan® 50, Bayer de México S.A. de C.V., Mexico City) for 24 h, rinsed once with sterile distilled water, surface sterilized with a 3% hydrogen peroxide solution for 10 min and with

Received for publication 3 Sept. 2015. Accepted for publication 25 Jan. 2016.

María del Carmen Vadillo-Pro thanks CONACyT for the grant offered to conduct her studies. We thank Félix Kregel for constructive comments and language corrections and Edgar Couttolenc for his assistance in statistical analysis.

¹Corresponding author. E-mail: martin.mata@inecol.mx.

Fig. 1. (A) Adult individual of *Beaucarnea purpusii* on wild; (B) initial growth of adventitious shoots from longitudinal section of seedlings cultured in MS medium + TDZ at 0.45 μM ; (C) multiple shoot formation from longitudinal section of seedlings cultured in MS medium + TDZ at 0.45 μM ; (D) hyperhydrated shoots observed from explants cultured in MS medium + BA at 13.3 μM ; (E) development of adventitious shoots from leaves of shoots previously formed on medium supplemented with TDZ; (F) cross section through the foliar blade (FB) showing the development of an adventitious shoot (AS), it is possible to observe connection of vascular bundles (VB), epidermis (EP), and parenchyma cells connection between leaf and adventitious shoot; (G) rooting of individual shoot on MS medium after 45 d of culture; and (H) plants of *B. purpusii* after successfully established in soil for 2 months. Bar = 1 cm.

70% ethanol for 1 min, immersed in a 30% (v/v) commercial chlorine solution (sodium hypochlorite, 6% of active chlorine) containing Tween 80® (Sigma, St. Louis, MO) at 0.1% for 30 min, and finally rinsed three times for 5 min each in sterile distilled water. All steps were carried out under continuous stirring.

Germination. Seeds were sown on a semi-solid MS medium (Murashige and Skoog, 1962) (M519; PhytoTech Laboratories, Shawnee Mission, KS), supplemented with sucrose at 30 g·L⁻¹. The pH of media was adjusted to 5.7 ± 0.1 with 0.5 N NaOH and 0.5 N HCl before adding 0.8% of agar (A296; Phyto-Tech Laboratories) and autoclaving at 120 °C for 20 min.

Five seeds per 125 mL baby food jar containing 30 mL of media were cultured, and there were 20 replicates. All cultures were incubated in a growth chamber at 25 ± 1 °C, under a 16-h photoperiod provided by cool-white fluorescent lamps (50 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$).

Induction stage on semisolid culture media. Two-month-old seedlings, 6 cm height, were dissected in two longitudinal sections, trying to divide the meristem to

avoid the effect of apical dominance, and the leaves and roots were trimmed away, each longitudinal section was considered as an explant. The sections were cultured on semisolid MS media, as described above, and supplemented with either BA or Kin at four concentrations (0, 4.4, 13.3, or 22.2 μM) or TDZ (0, 0.45, 2.3, or 4.5 μM). Each treatment consisted of 10 replicates containing two explants, which were placed with the cut surface directly onto the medium. The induction period lasted 30 d.

Induction stage by pulses. Explants were cultured in groups of 16 in 500 mL Erlenmeyer flasks containing each 200 mL of liquid MS media supplemented with different concentrations of BA (0, 110, 220, or 440 μM) or TDZ (0, 22.35, 45.40, or 136.21 μM). The flasks were placed on an orbital shaker at 120 rpm and incubated as described above for 6, 24, 48, or 96 h. Each treatment consisted of eight replicates.

Growth stage. After the induction period, the explants of either semisolid or pulse treatments, were subcultured every 30 d on semisolid MS medium without PGRs but with activated charcoal at 1 g·L⁻¹. After

3 months, survival rate and mean of shoots per explant were recorded.

Rooting stage. At the end of the growth stage, shoots were individualized, transferring 180 of a uniform size of $\approx 0.5 \times 5.0$ cm to test tubes (25 × 200 mm) containing 20 mL of either MS or 1/2MS (salts and vitamins) semisolid medium, both supplemented with sucrose at 30 g·L⁻¹ and activated charcoal at 0, 0.5, or 1 g·L⁻¹. The overall rooting rates of each treatment were registered after 45 d.

Ex vitro culture. Rooted shoots were extracted from the test tubes and washed thoroughly under running tap water. The plantlets were then transferred to propagation trays (Hummert International, Earth City, MO), containing a mixture of worm compost, loam, and pumice (1:1:1) and kept under greenhouse conditions (average temperature 30 °C, relative humidity 80% to 90%). To prevent fungal growth, the plantlets were sprayed with a 0.3% fungicide solution (Cap-tan). The trays were covered with plastic translucent lids the first 15 d, after which, the relative humidity was gradually decreased to between 50% and 60% by removing the lids during increasing amounts of time. Survival rates were recorded after 2 months.

Statistical analysis. Shoot formation was analyzed by a Friedman two-way non-parametric analysis of variance, and different treatments were classified using Duncan's test ($P \leq 0.05$) (SAS 9.1; SAS Institute, Cary, NC). We tested rooting data for differences between treatments using Kruskal–Wallis test using the NPAR1WAY procedure in SAS ver. 9.2. Multiple comparisons were made with least squares means and Bonferoni adjustment procedures, $P \leq 0.05$.

Results and Discussion

First signs of seed germination were observed on the 6th day, and the whole process continued until day 40. It was considered as a germinated seed at the moment the radicle started to emerge (Ranal and García de Santana, 2006). By the 15th day, 80% of seeds had germinated, and after 40 d, a germination rate of 98% was registered. Similar high in vitro seeds germination rates have been reported for *B. gracilis* (90% to 96%), *B. recurvata* (95%) (Osorio-Rosales and Mata-Rosas, 2005; Reyes et al., 2013), and *B. inermis* (92%) (Guillén et al., 2015).

Responses on semisolid medium. The dissection of the seedlings to obtain the longitudinal sections used as explants affected the survival rates of the latter, as in all treatments a given percentage of explants suffered oxidation and subsequent necrosis (Table 1). Such necrosis could be due to the autocatalytic production of phenolic compounds in response to tissue damage caused at the moment of the dissection (Azofeifa, 2009). Osorio-Rosales and Mata-Rosas (2005) reported up to 35% oxidation and necrosis in explants of *B. gracilis*, but none in the case of *B. recurvata*, which may

Table 1. Effect of different cytokinin concentrations on shoot formation from longitudinal sections of seedlings of *Beaucarnea purpusii* cultured on semisolid MS medium.

Treatment (μM)	Survival rate (%)	Mean of normal shoots per responding explant $\pm$ SE	Mean of hyperhydrated shoots per responding explant $\pm$ SE	Mean of total shoots per responding explant $\pm$ SE
Control	61.3	1.75 $\pm$ 0.31 b	0.28 $\pm$ 0.18 d	2.03 $\pm$ 0.37 b
BA				
4.4	56.7	3.53 $\pm$ 0.93 b	0.67 $\pm$ 0.36 cd	4.2 $\pm$ 0.84 b
13.3	61.3	6.74 $\pm$ 1.99 b	2.05 $\pm$ 0.72 bcd	8.79 $\pm$ 1.17 b
22.2	72.4	5.67 $\pm$ 1.34 b	3.62 $\pm$ 0.69 bc	9.28 $\pm$ 1.40 b
Kin				
4.4	51.7	1.6 $\pm$ 0.48 b	0.47 $\pm$ 0.27 d	2.44 $\pm$ 0.45 b
13.3	61.5	3.25 $\pm$ 0.67 b	1.13 $\pm$ 0.34 cd	4.37 $\pm$ 0.58 b
22.2	75.0	3.37 $\pm$ 0.74 b	0.96 $\pm$ 0.41 cd	4.33 $\pm$ 0.93 b
TDZ				
0.45	90.3	25.82 $\pm$ 4.60 a	0.78 $\pm$ 0.54 cd	26.61 $\pm$ 4.60 a
2.3	72.4	23.38 $\pm$ 4.23 a	4.38 $\pm$ 1.98 ab	27.76 $\pm$ 4.33 a
4.5	87.5	22.57 $\pm$ 3.94 a	7.21 $\pm$ 1.96 a	29.78 $\pm$ 4.50 a

Results after 4 months.

Different letters within columns indicate significant difference (Duncan's test, $P \leq 0.05$).

BA = N⁶-benzyladenine; Kin = kinetin; TDZ = 1-phenyl-3-(1,2,3-thiadiazol-5-yl)urea (thidiazuron).

indicate that phenolic production is species dependent.

The survival rates of *B. purpusii* explants were greater with the treatments supplemented with TDZ, mainly at 0.45 μM , and from those supplemented with BA and KIN at 22.2 μM , lower concentrations of these latter PGRs failed to prevent browning, since the survival of the explants from these treatments was at or below the control (Table 1). A study by Sajeve et al. (1994), who sowed cortex explants of *B. recurvata* in MS media, reported a reduction of browning in the explants cultured with different concentrations of BA. Similar studies show that the addition of cytokinins to the culture medium has a positive effect in preventing browning in different species, resulting therefore in higher survival (Bairu et al., 2009; George et al., 2008).

Shoot formation occurred via direct organogenesis from longitudinal sections of *B. purpusii* seedlings cultured with different concentrations of cytokinins. Similar responses have been described for *B. recurvata* (Bettaieb et al., 2008; Osorio-Rosales and Mata-Rosas, 2005; Reyes et al., 2013; Sajeve et al., 1994; Samyn, 1997), *B. gracilis* (Osorio-Rosales and Mata-Rosas, 2005; Reyes et al., 2013), *Beaucarnea goldmanii* (Reyes et al., 2013), and *B. inermis* (Guillén et al., 2015).

Within the first 15 d of culture, explants began to swell, and by the 30th day it was possible to observe small yellowish nodules with a rosette arrangement throughout the surface of the explant (Fig. 1B). The explants were subcultured to the basal MS medium, and 30 d later, the nodules were consolidated into adventitious shoots (Fig. 1C).

Since seedlings of *B. purpusii* as other monocotyledons species only have one apical meristem and primary thickening meristem, but no axillary or lateral buds, all the resulting shoots obtained from the surface of the explant were adventitious shoots. Three different types of adventitious shoots were obtained from the treatments: 1) normal shoots, i.e., those with an appearance comparable to the seedlings (Fig. 1C); 2) not viable hyperhydrated shoots (Fig. 1D); and

3) adventitious shoots arising from leaves (Fig. 1E). These structures were only observed in treatments containing TDZ and were subjected to histological examination to exclude that they were somatic embryos. Development of adventitious shoots was confirmed by the fact that no independently formed epidermis was found between the leaves and the shoots. In fact, both types of structures were connected by vascular bundles, as well as epidermal and parenchyma cells of the leaves, which continued into the shoots. There were no signs of radicle meristem formation (Fig. 1F). These adventitious shoots were considered as normal shoots.

The technique of dissecting or causing damage to apical meristems of explants has been used in combination with the addition of PGRs to the culture medium to overcome apical dominance. By modifying the endogenous auxin/cytokinin ratio in this way, different morphological responses can be expected (George et al., 2008). In addition, exposing the cut surface to the medium allows the PGRs to penetrate easily into the cells (Norizaku et al., 1985). The longitudinal dissection of seedlings has also been used successfully for in vitro propagation of *B. gracilis* and *B. recurvata*, and the shoot formation proved to be greater than in the case of complete seedlings (Osorio-Rosales and Mata-Rosas, 2005).

The positive effect of the cytokinins on shoot formation was evident since the treatments containing these PGRs at any concentration but 4.4 μM of Kin, induced higher shoot formation than the control. Regarding the number of shoots per explant, a statistically significant difference between treatments was registered ($P < 0.0001$). The three treatments containing TDZ induced the highest means of shoot formation with 22 to 25 shoots per explant (Table 1), whereas the lowest number of shoots was obtained from the control. In the case of the treatments supplemented with BA, 6.74 shoots per explant were induced at a concentration of 13.3 μM (Table 1).

Hyperhydration of shoots was observed in all treatments, being particularly high in the

treatments enriched with 0.45 and 2.3 μM of TDZ, showing 4.38 and 7.21 hyperhydrated shoots per explant, respectively, as well as at 22.2 μM of BA, showing 3.62 hyperhydrated shoots per explant. In most of the other treatments, the means of hyperhydrated shoots were low (Table 1). These kinds of shoots proved nonviable and eventually caused the loss of entire explants through browning. However, this response has not been reported in other studies of different *Beaucarnea* species (Bettaieb et al., 2008; Osorio-Rosales and Mata-Rosas, 2005; Samyn, 1997). Hyperhydration has been linked to the species, the genetics of the donor plants, the endogenous and exogenous levels of PGRs, and the type and concentration of gelling agents (Cassells and Curry, 2001; Kevers et al., 2004; Scholten and Pierik, 1998). Some reports indicate that this morphological and physiologic disorder can be controlled or reduced by increasing the agar concentration, which reduces the relative humidity and osmotic potential of the culture medium, but may also decrease shoot formation (George et al., 2008; Ivanova and Van Staden, 2011; Kevers et al., 2004).

According to our results, a concentration of 0.45 μM of TDZ induced the highest normal shoot production per explant (25.8), while causing a low average of hyperhydrated shoots (0.78) and the highest explant survival rate (90.3%) (Table 1). These results are superior to those reported in previous studies of other species of *Beaucarnea*: for *B. recurvata*, there are some studies that reported the formation of 6 to 11 shoots per explant using different concentration of BA (Bettaieb et al., 2008; Osorio-Rosales and Mata-Rosas, 2005; Reyes et al., 2013; Sajeve et al., 1994; Samyn, 1997); for *B. gracilis*, Osorio-Rosales and Mata-Rosas (2005) and Reyes et al. (2013) achieved the induction of 8.2 and 9.4 shoots per explant, respectively; regarding *B. goldmanii*, 3.9 shoots per explant were obtained (Reyes et al., 2013); and for *B. inermis*, 15.7 shoot per explant were induced (Guillén et al., 2015). Moreover, the effect of the addition of different concentrations of TDZ in this process had not been previously studied for any

of the *Beaucarnea* species. In the case of other succulent plants, some reports indicate that TDZ is the most effective cytokinin for shoot proliferation (Atta-Alla and Van Staden, 1997; Pelah et al., 2002).

Treatments by pulses. Since both BA and TDZ induced morphogenetic responses in our previous experiment, pulse treatments to stimulate shoot induction. In general, shoot proliferation was lower than in the case of explants cultured in semisolid MS medium. The survival rates of explants were also affected by the longitudinal dissection of the plantlets (Table 2), and browning was evident just a few days after subculturing the explants on semisolid MS medium. The control treatments and particularly the explants incubated in liquid media for 24 h showed the lowest survival rates (12.5%), in average all the BA treatments showed an intermediate value (59.4%) and TDZ treatments the highest rate (68.2%).

The induction period had no significant influence on shoot formation ($P \geq 0.05$) but did affect significantly the interaction between PGR concentration and induction period ($P < 0.0001$). The highest means of normal shoots per explant was observed in the treatments supplemented with TDZ at 22.35 μM for 24 h and TDZ at 136.21 μM for 96 h, forming 3.9 and 3.3 shoots per explant, respectively. In the remaining pulse treatments two or less shoots per explant were

recorded (Table 2). Further studies are necessary to elucidate how duration and concentration of PGR pulse treatments influence morphogenetic responses in *B. purpusii* explants. Besides, TDZ promoted the formation of a high number of hyperhydrated shoots, which in some cases were more prolific than normal shoots (Table 2).

Contrary to our findings, other studies reported a higher mean of shoots per explant in pulse treatments than in the semisolid medium, as was reported for *Agave tequilana* (Ramírez-Malagón et al., 2008), *Pseudotsuga menziesii* cotyledons (Goldfarb et al., 1991), and *Musa* spp. (Madhulatha et al., 2004). In the present study, high concentrations of cytokinins and the tested incubation times were not suitable for inducing high shoot production per explants. Moreover, in some treatments shoot formation was inhibited. A similar response was obtained for five species of *Agave*, with 2,4-dichlorophenoxyacetic acid (2,4-D) pulse treatments, where no shoot was obtained (Ramírez-Malagón et al., 2008).

Rooting. After 3 months, most of the shoots in semisolid MS media reached a height of 5 cm. They were thus separated into individual shoots and subcultured using different rooting media (Fig. 1G). Rooting occurred in all media tested (Fig. 2). Kruskal–Wallis tests showed significant differences among rooting treatments ($P < 0.0001$), the highest rooting rates were achieved on semisolid MS and 1/2MS

medium, both supplemented with activated charcoal at 1 g·L⁻¹, showing rooting rates of 90% and 85%, respectively. In the case of shoots obtained from pulse treatments, a rooting rate of 90% was achieved on MS media containing 0.5% of activated charcoal. The other treatments produced lower rooting rates, ranging from 65% to 80%.

Osorio-Rosales and Mata-Rosas (2005) reported rooting of *B. gracilis* and *B. recurvata* shoots on MS medium supplemented with activated charcoal; whereas Sajeva et al. (1994) and Samyn (1997) observed a fast development of roots from shoots cultured on MS medium. They also reported an increase rooting when the shoots were subcultured on medium supplemented with α -naphthaleneacetic acid. On the contrary, Bettaieb et al. (2008) stated that *B. recurvata* shoots did not develop roots on a 1/2MS medium, but this limitation was overcome by using media including a range of β -cyclodextrin concentrations.

Ex vitro culture. After 2 months of ex vitro culture, the plants showed a survival rate of 100% (Fig. 1H). Shoots that had not developed roots were treated by dipping them in commercial rooting powder (Radix® 1500, Intercontinental import export, S.A. de C.V., Gto, Mexico) and cultured ex vitro under the same conditions, resulting in rooting and survival rates of 75%. High ex vitro survival rates of micropropagated plants have also been reported for other *Beaucarnea*

Table 2. Effect of the exposure of longitudinal sections of seedlings of *Beaucarnea purpusii*, in liquid MS medium for short periods (pulses) with different concentrations of two cytokinins, on shoot numbers.

Treatment (μM)	Incubation period (h)	Survival rate (%)	Mean of hyperhydrated shoots per responding explant $\pm$ SE	Mean of normal shoots per responding explant $\pm$ SE	Mean of total shoots per responding explant $\pm$ SE
Control					
0	6	31.3	0.12 $\pm$ 0.12 e	0.19 $\pm$ 0.13 ed	0.31 $\pm$ 0.16 hi
0	24	12.5	0.06 $\pm$ 0.06 e	0.13 $\pm$ 0.12 e	0.19 $\pm$ 0.13 i
0	48	37.5	0.06 $\pm$ 0.06 e	0.44 $\pm$ 0.15 de	0.50 $\pm$ 0.16 hi
0	96	43.7	0.0 $\pm$ 0.0 e	0.50 $\pm$ 0.85 de	0.50 $\pm$ 0.13 hi
BA					
110	6	56.2	0.19 $\pm$ 0.13 e	0.69 $\pm$ 0.35 cde	0.87 $\pm$ 0.40 fghi
220	6	50.0	0.44 $\pm$ 0.22 e	0.81 $\pm$ 0.50 cde	1.25 $\pm$ 0.48 fghi
440	6	68.7	0.94 $\pm$ 0.20 cde	1.25 $\pm$ 0.46 bcde	2.19 $\pm$ 0.46 efghi
110	24	56.2	0.44 $\pm$ 0.26 e	0.44 $\pm$ 0.11 de	0.87 $\pm$ 0.35 fghi
220	24	43.7	0.56 $\pm$ 0.27 de	1.13 $\pm$ 0.74 bcde	1.69 $\pm$ 0.94 fghi
440	24	81.2	1.56 $\pm$ 0.64 cde	1.25 $\pm$ 0.50 bcde	2.81 $\pm$ 0.63 bcdefghi
110	48	93.7	0.37 $\pm$ 0.24 e	2.44 $\pm$ 0.42 abcd	2.81 $\pm$ 0.49 bcdefghi
220	48	56.2	0.56 $\pm$ 0.20 de	1.69 $\pm$ 0.87 bcde	2.25 $\pm$ 0.94 efghi
440	48	75.0	0.37 $\pm$ 0.31 e	2.82 $\pm$ 0.71 abc	3.19 $\pm$ 0.81 bcdefg
110	96	31.2	0.37 $\pm$ 0.12 e	0.0 $\pm$ 0.0 e	0.37 $\pm$ 0.12 hi
220	96	50.0	0.75 $\pm$ 0.34 de	0.19 $\pm$ 0.13 de	0.94 $\pm$ 0.35 fghi
440	96	50.0	0.06 $\pm$ 0.06 e	0.56 $\pm$ 0.36 de	0.62 $\pm$ 0.42 ghi
TDZ					
22.35	6	68.7	1.50 $\pm$ 0.80 cde	1.37 $\pm$ 0.98 bcde	2.87 $\pm$ 1.10 bcdefgh
45.40	6	43.7	1.31 $\pm$ 0.58 cde	1.31 $\pm$ 0.72 bcde	2.62 $\pm$ 0.97 cdefghi
136.21	6	50.0	1.50 $\pm$ 0.79 cde	2.9 $\pm$ 1.66 abc	4.42 $\pm$ 1.74 abcde
22.35	24	87.5	2.44 $\pm$ 0.50 bc	3.88 $\pm$ 1.30 a	6.31 $\pm$ 1.40 a
45.40	24	81.2	3.44 $\pm$ 0.72 ab	1.44 $\pm$ 0.62 bcde	4.87 $\pm$ 0.57 abcd
136.21	24	87.5	3.62 $\pm$ 0.77 ab	1.56 $\pm$ 0.83 bcde	5.19 $\pm$ 1.06 abc
22.35	48	56.2	1.33 $\pm$ 0.42 cde	2.17 $\pm$ 0.93 abcde	3.50 $\pm$ 1.00 bcdef
45.40	48	75.0	0.50 $\pm$ 0.43 de	2.13 $\pm$ 0.71 abcde	2.62 $\pm$ 1.04 cdefghi
136.21	48	87.5	4.37 $\pm$ 0.62 a	1.0 $\pm$ 0.38 cde	5.37 $\pm$ 0.64 ab
22.35	96	50.0	1.25 $\pm$ 0.40 cde	1.13 $\pm$ 0.66 bcde	2.37 $\pm$ 0.87 defghi
45.40	96	68.7	1.56 $\pm$ 0.84 cde	1.87 $\pm$ 0.73 abcde	0.18 $\pm$ 1.19 bcdef
136.21	96	62.5	2.00 $\pm$ 0.71 cd	3.31 $\pm$ 0.84 ab	5.31 $\pm$ 1.23 ab

Results after 4 months.

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

BA = N⁶-benzyladenine; TDZ = 1-phenyl-3-(1,2,3-thiadiazol-5-yl)urea (thidiazuron).

Fig. 2. Effect of different culture medium on rooting of individual shoots of *Beaucarnea purpusii*, results after 45 d of culture.

species (Osorio-Rosales and Mata-Rosas, 2005; Samyn, 1997).

Comparing the two culture systems tested, the one using semisolid media, particularly when supplemented with TDZ at 0.45 μ M, clearly induced greater shoot formation, showing a mean of 25.8 shoots per explant. Consequently, a seedling divided in two longitudinal sections has the potential to produce up to 50 new plants through direct organogenesis.

Additionally to in situ protection measures, conservation of endangered species such as *B. purpusii* should be enhanced by ex situ alternatives. For this reason the Mexican Government, through its Ministry of Environment and Natural Resources, has established the policy to stimulate the establishment of legally registered Wildlife Management Units (UMAs, for its name in Spanish), as areas set aside for the sustainable use and protection of fauna and nontimber forest products; they are designed to allow communities to diversify the production of goods and services from wildlife, while minimizing impact on ecosystems and biological resources (Avila-Foucat and Pérez-Campuzano, 2015; García-Marmolejo et al., 2008). Hence, the in vitro propagation protocols established in the present study, in which complete plants were obtained through direct organogenesis, are an important contribution for both propagation and conservation efforts, as they may be legally marketed through UMAs, and this could reduce pressure on wild populations of *B. purpusii* by satisfying the horticultural demand and would generate income for farmers communities, usually low-income population, from conservation activities on private and community lands. In vitro cultured plants could prove useful, in conjunction with genetic and ecological studies, in designing

programs for reintroduction of these plants into the wild.

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