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Article

Micropropagation and *ex-vitro* rooting of *Beaucarnea hookeri* (LEM.) Baker

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Abstract

Beaucarnea hookeri is a species endemic to Mexico and is listed as threatened in NOM-059-SEMARNAT-2010. In order to micropropagate this species, an experiment was established at the Montecillo *Campus*, College of Postgraduates in 2022, where the response of the shoots during *in vitro* multiplication with different concentrations of benzylaminopurine (BAP) and their capacity for *ex-vitro* rooting during acclimatization were evaluated. An experiment was established with three subsequent subcultures (SC3, SC4, SC4-1); in SC3, shoots generated with 0.5 mg L^{-1} BAP (SC2) were used; in SC4, healthy shoots generated from SC3; and SC4-1 with shoots from SC3 but with initial hyperhydration (IHH). In SC3, 0.5, 1, 1.5, 2, and 2.5 mg L^{-1} BAP were evaluated. In SC4, 0 and 0.5 mg L^{-1} BAP and in SC4-1, concentrations of 0, 0.5, and 1 mg L^{-1} BAP. During acclimatization, four treatments of Radix® 10 000 (0, 1 000, 2 000, and 4 000 mg kg^{-1}) were evaluated, and shoots from SC2 were used. During multiplication, the number of shoots (NS) was recorded, and SC3 generated 6.35 shoots with the concentration of 1.5 mg L^{-1} BAP, with the presence of IHH. On average, 9.7 shoots were obtained in SC4, and 5.3 shoots in SC4-1, both subcultures with 0.5 mg L^{-1} BAP. In addition, there was a recovery of IHH in SC4-1. In rooting, there were no significant differences between treatments, and the control generated 73.3% of shoots with roots (SSR) 80 days after transplantation. Only the shoots of the control treatment in multiplication presented roots, which allowed complete plants to be obtained in 30 days.

Keywords:

Calibanus hookeri, endangered, in vitro culture, Nolinaceae, organogenesis.

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Introduction

Beaucarnea hookeri, synonymy of *Calibanus hookeri*, is endemic to Mexico, it is recorded in Tamaulipas, San Luis Potosí, Guanajuato, Querétaro, and Hidalgo (De-Nova *et al*., 2018; Hernández-Sandoval, 2020), grows on igneous rock outcrops and rarely on limestone rocks. It is a distinctive member of the Nolinaceae family, the morphology that distinguishes it is its almost spherical caudex of 0.4 to 1 m in diameter, fissured bark of grayish brown color, leaves arranged in almost sessile rosettes distributed in the caudex giving a cespitous appearance, the leaves with concave blades, 60 to 95 cm long and denticulate margins.

It has paniculate inflorescences, 20-40 cm long, staminate flowers on pedicels 2.5 mm long, pistillate flowers of 1-1.5 mm in diameter, almost spherical to pyriform fruits of 5-8 mm in diameter, thickened pericarp with three ribs along it, seeds 3-4 mm long and 3 mm wide, brown (Martínez *et al*., 2014; Hernandez-Sandoval, 2020). *Calibanus hookeri* was formally included within *Beaucarnea* derived from the study carried out by Rojas-Piña *et al*. (2014). Hernández-Sandoval (2019) noted that it is one of the genera that were registered as a synonym of *Beaucarnea* in the Nolinaceae family. In its normative annex III, the DOF (2019) establishes that the species *Beaucarnea hookeri* (Lem.) Baker is what is commonly known as 'inaja' and 'sacamecate' (Hernández-Sandoval, 2020).

The recognized synonyms are *Calibanus caespitosus*, *Calibanus hookeri*, *Dasylirion caespitosum*, *Dasylirion hartwegianum*, *Dasylirion hookeri* (DOF, 2019). According to NOM-059-SEMARNAT-2010, it is a species in the 'threatened' risk category (SEMARNAT, 2010). In this regard, Hernández-Sandoval (2020) reported that *Calibanus hookeri* is a species found in very localized places and in populations with few individuals, so it is considered threatened and endangered.

For the conservation of this species, recently Núñez *et al*. (2021) developed an *in vitro* establishment protocol in MS medium supplemented with cytokinins at high concentrations (2.5, 5, and 7 mg L^{-1}), such as benzylaminopurine (BA), kinetin (K), isopentyl adenine (2ip) and thidiazuron (TDZ), where they determined that the most efficient treatment was with 5 mg L^{-1} BA, this concentration induced 26 shoots per seedling, although they also mention that it presents susceptibility to hyperhydration.

In studies conducted in the multiplication stage, Gil *et al*. (2019) reported that in *Cattleya trianae*, treatments with cytokinins (6-BAP) increased the response in shoots and decreased root formation. Waly *et al*. (2018) evaluated the effect of the type of medium and the concentration of BAP in the multiplication stage of *Beaucarnea recurvata* and concluded that for the production of large quantities of plants, the B5 medium supplemented with 0.4 mg L^{-1} BAP should be used for four weeks. The present study aimed to determine the best treatments with low concentrations of BAP for *in vitro* multiplication, as well as to evaluate the *ex-vitro* rooting during the acclimatization of the plants generated from their culture.

Materials and methods

Seeds

They were collected in October 2019 in the Botanical Garden of the Faculty of Higher Studies of Iztacala, UNAM, Mexico, and donated by MS Marcial Pineda, the person in charge of the aforementioned garden, who made the identification of the plant. The seeds were stored for 14 months in a dry place without any treatment within the facilities of the College of Postgraduates, Montecillo, State of Mexico.

Seed disinfestation

It consisted of qualitative selection after removing the testa (based on the color of the seed), greenish and grayish seeds were removed to avoid the presence of fungi and mechanical

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damage. Once 150 seeds were obtained, they were placed in a beaker containing a solution of 10 g L^{-1} of Roma® biodegradable detergent and tap water, the beaker was placed for 30 min under the fall of the tap water, and at the end of the time, they were rinsed three times. Subsequently, 70% v/v alcohol was added for 1 min, and it was rinsed with tap water (three consecutive times), then a 30% v/v sodium hypochlorite solution with two drops of tween 20® per 100 ml of solution was used for 30 minutes.

Before completing the 30 min, it was taken to the previously sanitized laminar flow hood, where the chlorine solution was removed, and the seeds were rinsed five times with deionized and sterilized water consecutively; finally, a solution of sulfuric acid 4N was used and added to the seeds for a period of 5 min, at the end of the time, five rinses were carried out consecutively with deionized and sterilized water, then the water was drained from the beaker, and the seeds were ready to be sown in the test tubes.

Sowing of seeds and culture medium

The seeding was done in test tubes with 20 ml of MS base medium (Murashige and Skoog, 1962) supplemented with 2 mg L^{-1} glycine, 100 mg L^{-1} myo-inositol, 0.4 mg L^{-1} thiamine-HCl, 0.5 mg L^{-1} nicotinic acid, 0.5 mg L^{-1} pyridoxine HCl, 30 g L^{-1} sucrose and 8 g L^{-1} Merk(agar. The pH of the culture medium was adjusted to 5.7 with NaOH 0.1N or HCl 0.1N, and it was sterilized in an autoclave at 1.5 kg cm^{-2} for 15 min.

The MS medium was used for all subcultures, what changed was the amount of BAP according to the treatments. One seed was sown per test tube of 150 x 22 mm, in a laminar flow hood previously disinfected, and 120 seeds were established for germination. The seeds were exposed to 0, 2.5, and 5 mg L^{-1} BAP for 80 days, the plants with the highest number of shoots showed hyperhydration, so the mother plants were subcultured with the same characteristics described for the medium, and only the concentrations were changed from 2.5 to 0.5 and 5 to 1 mg L^{-1}, respectively. The shoots generated 30 days after the second subculture were the initial material for the experiments.

Multiplication stage (SC3)

To obtain the plant material for the following subcultures, the shoots were extracted from the tubes and separated in Petri dishes. The individualized shoots were established in a fresh culture medium supplemented with different concentrations of BAP, according to the treatments assigned in the subculture (SC3); that is, the shoots generated in SC2 that were during 30 days of culture with 0.5 mg L^{-1} BAP after the separation of the mother shoot (Figure 1B) were established in different concentrations of BAP (0, 0.5, 1, 1.5, 2 and 2.5 mg L^{-1}) with 21 repetitions.

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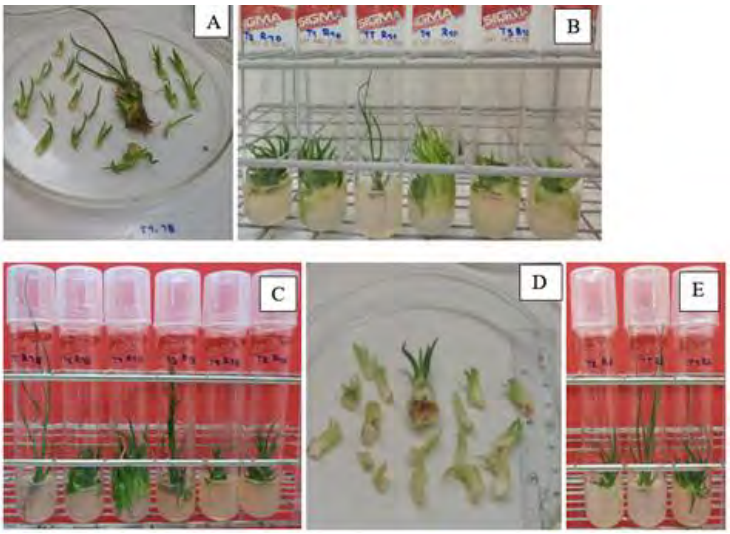
Figure 1. A) separation of shoots to establish SC3; B) repetition 10 from left to right (1, 0.5, 2, 2.5, and 1.5 mg L-1 BAP), SC3; C) repetition 18 of the treatments from left to right (0, 2.5,0.5, 1.5, 2, and 1 mg L^{-1} BAP) SC4; D) shoots with IHH, generated in SC3; and E) SC4-1 treatments R6 treatments from left to right (1, 0, and 0.5 mg L^{-1} BAP) SC4-1.



Subculture (SC4)

Healthy shoots generated at concentrations of 0.5, 1, 1.5, 2, and 2.5 mg L^{-1} BAP, from SC3, were established in 0.5 mg L^{-1} BAP and a control without regulators, 22 repetitions per treatment were used.

Subculture (SC4-1)

Shoots with initial hyperhydration (Figure 1D) were established at random in 0, 0.5, and 1 mg L^{-1} BAP with 22 repetitions. The variables recorded for the experiments were the following: in rooting, number of roots (NR), root length (RL) in cm, percentage of survival of shoots with root (PSSR); and in the subcultures of multiplication, number of shoots (NS), leaf length (LL) in cm, stem base width (SBW) in cm, percentage of shoots with root (PSR) and percentage of propagule shoots hyperhydrated (PSHH).

Ex-vitro rooting

The shoots generated in the second subculture (SC2) after 30 days were used, they were separated from the shoots they generated, and these were returned to their test tube, and after 58 days, 60 shoots were established in a completely randomized experiment with 15 repetitions at 0, 1 000, 2 000 and 4 000 mg kg^{-1} of Radix 10 000®, in cells. The mixture was prepared with Radix® 10 000 and talc without aroma. Cells of 140 ml in volume supported by their bases were used, both were washed with a solution of Roma® biodegradable detergent and rinsed with tap water, and disinfected with a 30% v/v NaOCl solution for 20 min.

The substrate mixture that was used was based on Promix® Peat moss and perlite (1:1 v/v), sterilization was performed in an autoclave at a pressure of 1.7 kg cm^{-2}, 30 min (twice). The shoots were extracted, and the base of the stem was sprinkled with a solution of Timsem® 1 g L^{-1}. The agar was removed from the base of the stem with deionized and sterilized water, excess water was removed from the base of the stem, then it was impregnated with the Radix® mixture according to the assigned treatment, after being transplanted, each shoot was irrigated with 20 ml of sterilized deionized water. An 8 x 26 bag was placed on each cell to create a microclimate,

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holding it with a band to start the rooting process. The cells were transferred to the incubation room, where they were placed in a photoperiod of 16 h light and 8 h dark, with cold-white light lamps, with an intensity of 80 µmol m^{-2} s^{-1}. At day 40, the bag was removed from the cells, and they were kept in the incubation room until 80 days, during which time the evaluation was performed.

Statistical analysis

A completely randomized design was used in all four experiments; data analysis was performed using the one-factor Anova technique, followed by Tukey's multiple comparison test, the honestly significant difference (HSD). In the case of the variables of subculture, PSR and PHH, and that of rooting, PSSR, they were analyzed by logistic regression because they are binomial variables, and the comparison of means was by emmeans Tukey, all analyses were performed in the statistical program RStudio.

Results and discussion

Multiplication in the subculture of shoots (SC3)

Number of shoots (NS)

The formation of adventitious shoots at the base of the stem was significant ($p \leq 0.05$) among the treatments, the highest values (6.35 and 5.71 shoots per propagule) were recorded with 1.5 and 2.5 mg L^{-1} BAP, respectively (Table 1 and Figure 1B). The results of this research are superior to those by (Osorio-Rosales, 2005), when they used seedlings without roots and trimmed leaves as explant, they generated 5.4 shoots per seedling in *B. gracilis* at a concentration of 5 mg L^{-1} BAP, and with 1 mg L^{-1} BAP, they generated 1.9 shoots per explant in *B. recurvata*, but they are lower when they used the explant of longitudinal sections in *B. gracilis* and *B. recurvata*, with 5 mg L^{-1} BAP they induced 8.2 and 11.1 shoots per explant.

Table 1. Response of *B. hookeri* shoots generated in 0.5 mg L^{-1} BAP, exposed to BAP concentrations of SC3 to 30 after the beginning.

Treatments BAP (mg L^{-1})	LL (cm)	NS	NR	RL (cm)	PSR	PHH
0	7.78 a	0.14 b	0.38 a	1.34 a	33 a	10 a
0.5	3.29 b	4.33 a	0 b	0 b	0 a	19 a
1	2.3 b	5.52 a	0 b	0 b	0 a	29 a
1.5	2.09 b	6.35 a	0 b	0 b	0 a	45 a
2	2.56 b	5.66 a	0 b	0 b	0 a	52 a
2.5	1.94 b	5.71 a	0 b	0 b	0 a	67 b
*	$2.2x10^{-16}$	$9.7x10^{-08}$	$4.9x10^{-07}$	$2.1x10^{-07}$	$5.2x10^{-05}$	0.0004

LL= longest leaf length in cm; NS= number of shoots; NR= number of main adventitious roots; RL= Longest adventitious root length; PSR= percentage of shoots with roots; PHH= percentage of hyperhydration of propagule shoots; *= *p*-value. Means with different letters in the same column are statistically different, according to the Tukey test ($p \leq 0.05$).

They also coincide with what was reported by Reyes-Silva *et al*. (2013), by using basal explants of 10 to 15 mm, without leaves or roots in MS medium supplemented with BAP at concentrations of 3 to 4 mg L^{-1} in 10 species of the genera *Beaucarnea*, *Dasylirion,* and *Nolina*, they generated 3.9 to 10.3 shoots per explant. They also coincide with Vadillo-Pro *et al*. (2016), with explants of longitudinal sections of *B. purpusii* and a concentration of 3 mg L^{-1} BA, they induced 6.74 shoots per explant.

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Leaf length (LL) in cm

The longest leaf lengths (7.78 and 3.29 cm) were obtained in 0 and 0.5 mg L^{-1} BAP (Table 1), which were significant ($p \leq 0.05$). The results coincide with Osorio-Rosales (2005), who reported that the development of the first shoot inhibited or limited the development of subsequent shoots, this explains the heterogeneity observed for the height of shoots from 0.3 to 10 cm in *B. recurvata* and *B. gracilis*.

Number of roots (NR), root length (RL) in cm, and percentage of shoots with root (PSR)

The NR, RL, and PSR were significant ($p \leq 0.05$). The NR (0.38 roots) were only present in shoots of the control treatment (0 mg L^{-1} BAP), no roots formed in the other treatments. The root length was 1.34 cm in the control (Table 1). The above results coincide with Gil *et al*. (2019), who found that without the application of regulators, higher root percentages were obtained in *Cattleya trianae*.

The results obtained by Núñez *et al*. (2021) in *C. hookeri* were higher, (100%) of rooting in MS medium without regulators. Regarding the percentage of shoots with roots, 33% was obtained in the control, and the other treatments with BAP did not form roots (Table 1). These results differ from Guillén *et al*. (2015), who registered a higher percentage of *in-vitro* rooting (92.6%) obtained in *B. inermis* with an MS medium without phytohormones. Also, the results they achieved in the genus *Beaucarnea* in MS medium with 2 g L^{-1} of activated carbon were higher, 63 and 75% rooting (Reyes-Silva *et al*., 2013). The result obtained is a lower percentage of rooting in the control compared to *B. gracilis* and *B. recurvata*, where the rooting of more than 60 and 80% of the shoots cultured in MS medium without the application of phytohormones was observed.

Percentage of hyperhydration of propagule shoots

The treatment with the highest hyperhydration was the treatment of 2.5 mg L^{-1} BAP with 67% hyperhydration (Table 1). Similar results were reported by Vargas-Castillo (2010), who found 28.66 to 41% hyperhydration in *Geophila macropoda* at concentrations of 3 and 4 mg L^{-1}. Vadillo-Pro *et al*. (2016) recorded that explant exposure to 0.1 to 0.5 mg L^{-1} TDZ showed 4.38 and 7.31 hyperhydrated shoots per explant, respectively, as well as 5 mg L^{-1} BA showed 3.62 hyperhydrated shoots per explant.

Healthy shoot subculture (SC4)

Number of shoots (NS)

The formation of adventitious shoots at the base of the stem was significant ($p \leq 0.05$) among the treatments, the highest NS was generated with the concentration 0.5 mg L^{-1} BAP, OS (9.7 shoots per propagule), the other two best concentrations that produced the highest number of shoots (7.37 and 7.21 shoots per propagule) were 1 and 1.5 mg L^{-1} BAP of shoot origin (Table 2). The numbers of shoots reported by (Waly *et al*., 2018) in *B. recurvata* in the optimal concentration of 0.4 mg L^{-1} BAP in B5 medium for the multiplication stage are lower. Also, Gil *et al*. (2019) in *Cattleya trianae*, where low doses of BAP (0.05 mg L^{-1}) generated the highest percentage of shoots, treatments with BAP 0.5 and 2 mg L^{-1} increased the shoot response and decreased root formation.

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Table 2. Response of healthy shoots of *C. hookeri* with SC3 origin, subcultured 30 days at concentrations of 0 and 0.5 mg L $^{-1}$ BAP, SC4.

Treatments		LL (cm)	NS	NR30	RL30 (cm)	PSR	PHH
BAP mg L^{-1}	OS						
0	0.5	10.22 a	0.04 c	1.04 a	3.25a	68 a	0 a
0.5	0.5	4.33 b	9.7 a	0 b	0 b	0 a	14 a
0.5	1	3.54 bc	7.37 ab	0 b	0 b	0 a	18 a
0.5	1.5	3.03 bc	7.21 ab	0 b	0 b	0 a	5 a
0.5	2	2.6 c	5.81 b	0 b	0 b	0 a	23 a
0.5	2.5	2.4 c	6.09 b	0 b	0 b	0 a	5 a
	*	$2.2x10^{-16}$	$5.06x10^{-12}$	$2.2x10^{-16}$	$2.2x10^{-16}$	$7.1x10^{-13}$	0.051

OS= origin of SC3 shoot 0.5, 1, 1.5, 2, 2.5 mg L^{-1}; LL= longest leaf length; NS= number of shoots; NR= number of main adventitious roots; RL= Longer adventitious root length; PSR: percentage of shoots with root; PHH= percentage of propagule shoots hyperhydrated; *= *p*-value. Means with different letters in the same column are statistically different, according to the Tukey test (p≤ 0.05).

Leaf length (LL) in cm

The variable LL was significant (p≤ 0.05), where the best treatment for the longest LL (10.22 cm) was the control, and the second-best treatment was 0.5 mg L^{-1} (4.33 cm) (Table 3). Adelberg and Naylor (2012) reported that, when using a liquid medium, the plants were larger and the negative effect of rooting decreased, without affecting the multiplication rate, so the concentration of 6 µM (1.35 mg L^{-1}) BA in a liquid medium was optimal for the multiplication and rooting of *Aloe barbadensis*.

Table 3. Response in the recovery of shoots with IHH, origin of SC3, 1.5, 2, and 2.5 mg L^{-1} BAP and generation of shoots at day 30, SC4-1.

Treatment BAP (mg L^{-1})	LL (cm)	SBW (cm)	NS	NR	RL (cm)	PSR	HH5	HH30
0	6.78 a	0.31 b	0.09 b	0.71 a	1.44 a	43 a	95.2 a	23.8 a
0.5	3.76 b	0.66 a	5.63 a	0 b	0 b	0 a	86.4 a	18.2 a
1	1.89 b	0.74 a	5.4 a	0 b	0 b	0 a	90.9 a	22.7 a
*	$6.4x10^{-6}$	$1.3x10^{-6}$	$5.9x10^{-10}$	$3E^{-04}$	$1E^{-04}$	$7.5x10^{-06}$	0.59	0.89

LL= leaf length in cm; SBW= stem base width in cm; NS= number of shoots; NR= number of adventitious roots; RL= root length; *= *p*-value. Means with different letters in the same column are statistically different, according to the Tukey test (p≤ 0.05).

Number of roots (NR), root length (RL) in cm, and percentage of shoots with root (PSR)

With respect to NR and RL, they were significant at p≤ 0.05; however, the PSR was not significant at the same level of significance. The NR (1.04 roots) were only present in shoots of the control treatment (0 mg L^{-1} BAP) since no roots formed in the other treatments. The root length was 3.25 cm in the control (Table 1). The treatment without phytohormones in the MS medium allowed the formation of roots in *B. inermis*, although to a lesser extent compared to 1 mg L^{-1} IBA (Guillen *et al*., 2015).

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The percentage of shoots with roots was 68% in the control, and the other treatments with BAP did not form roots (Table 1). For their part, Vadillo-Pro *et al*. (2016) reported that in shoots subcultured individually in *B. purpusii* in MS medium and ½ MS, both supplemented with 1 g L^{-1} of activated carbon, they induced 85 to 95% of shoots.

Percentage of hyperhydration of propagule shoots (PHH)

The percentage of hyperhydration ranged from 5% to 23% (Table 2); nevertheless, this variable was not significant ($p \leq 0.05$). The results obtained coincide with Vargas-Castillo (2010), who found hyperhydration in percentages less than 20% in the media that were supplemented with 0 to 2 mg L^{-1} BAP in *G. macropoda*.

Subculture of shoots with IHH (SC4-1)

The variables LL, SBW, NS, NR, and RL were significant, except for PSR and PHH, at *p*# 0.05. At the concentration of 0.5 and 1 mg L^{-1} of the origin of the shoot, no significant differences were found (Table 3). The results obtained by Ramírez-Gottfried *et al*. (2021) were superior when comparing the MS medium with vermicompost in the multiplication stage of *Dasylirion cedrosanum*. The addition of 1 mg L^{-1} BAP plus 0.2 mg L^{-1} IAA in MS medium generated higher average shoots (8.25 shoots/explant). In *Aloe barbadensis*, the concentration of 6 µM (1.35 mg L^{-1}) BA in a liquid medium was optimal for multiplication and rooting.

Leaf length (LL) in cm

The longest leaf lengths (6.78 cm) were obtained in the control (0 mg L^{-1} BAP) (Table 3). The importance of larger leaf lengths was proven by Aureoles-Rodríguez *et al*. (2008), who determined that plant size and culture medium affected plant rooting in *Agave inaequidens*. Plants 4 cm long with more than two roots had a survival of 100%.

Number of roots (NR), root length (RL) in cm, and percentage of shoots with root (PSR)

The NR, RL, and PSR were significant ($p \leq 0.05$). The NR (0.71 roots) were only present in shoots of the control treatment (0 mg L^{-1} BAP) since no roots formed in the other treatments. The root length was 1.44 cm in the control. The results coincide with Zhang *et al*. (2013), who, in an MS medium free of regulators, obtained shoots of *Agave hybrid* regenerated by organogenesis.

The percentage of shoots with roots was 43% in the control, and the other treatments with BAP did not form roots (Table 3). In this regard, Reyes-Silva *et al*. (2013) reported that when evaluating rooting at 45-50 days in shoots generated in multiplication with a base medium at 50% in species *Beaucarnea goldmanii*, *B. recurvata* and *B. gracilis*, the lowest percentages of rooting were obtained (34, 23 and 14% respectively), but not in the genera *Dasylirion* and *Nolina*, which reached 75 to 100% rooting. Likewise Vargas-Castillo (2010) determined hyperhydration in percentages less than 20% in the media that were supplemented with 0 to 2 mg L^{-1} BAP in *G. macropoda*.

Percentage of shoots hyperhydrated

At day 5 after being established in the subculture medium, the shoots did not show significant differences, the percentages of hyperhydration ranged from 86.4 to 95.2% (Table 4). Initial hyperhydration occurred in younger leaves with light green to light yellow coloration in shoots sown at 1 mg L^{-1} BAP. In the case of 1.5, 2, and 2.5, in addition to the above symptoms, they showed wider bases than the mother shoot, as well as slight leaf deformation and a smaller number. At 30 days of subculture, hyperhydration was evaluated, the concentrations in the three treatments helped in the recovery of the initial hyperhydration, and there were no significant differences between treatments (Table 3 and Figure 1E). The above results coincide with (Millán-

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Soto *et al*., 2019), who reported a reduction of hyperhydration by 10.4% in *Asparagus officinalis* when reseeding rhizomes in a culture medium without growth regulators.

Ex-vitro rooting

Percentage of survival of rooted shoots (PSSR)

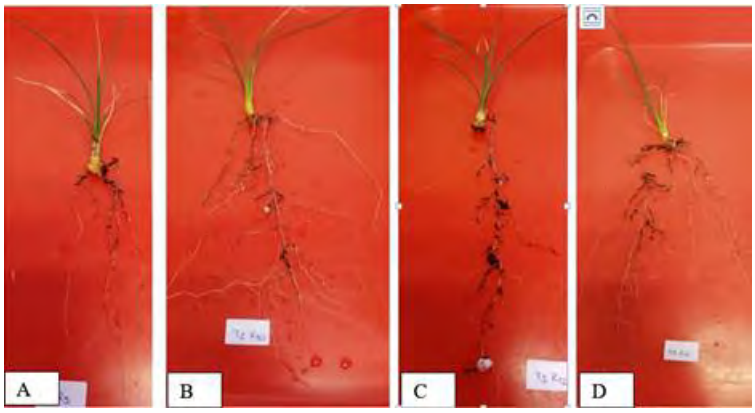
Root formation was observed in all treatments; however, the highest percentage of survival of shoots with roots (73.33%) was obtained in the control (Table 4 and Figure 2). The increased survival of rooted shoots (propagule shoots subjected to rooting after separation from their shoots) can be explained by the ability of some species to regenerate roots. This behavior is similar to what was obtained by Guillen *et al*. (2015) in shoots of *B. inermis*.

Table 4. *Ex-vitro* rooting and acclimatization of shoots after their first shoot production in *B. hookeri* 80 days after transplantation.

Treatments (mg kg^{-1})	NR	RL	PSSR
0	2.41 a	11.43 a	73.3 a
1 000	2.12 a	9.87 a	33.3 a
2 000	1.76 a	5.73 a	46.7 a
4 000	1.2 a	4.89 a	40 a
*	0.41	0.1	0.1272

NR= number of primary roots; RL= primary root length; PSSR= percentage of survival of shoots with roots; *= p≤ 0.05. Means with different letters in the same column are statistically different, according to the Tukey test (p≤ 0.05).

Figure 2. *Ex-vitro* rooting treatments, 80 days after establishment. A) 1 000 mg kg^{-1} ; B) 2 000 mg kg^{-1} ; C) 4 000 mg kg^{-1} ; and D) 0 mg kg^{-1} .



In *Cattleya trianae*, treatment without BAP formed roots in 7.35% (Gil *et al*., 2019). On the other hand, Puente-Garza *et al*. (2015) established the shoots obtained in multiplication in MS medium without regulators, and at 30 days, they generated roots in *Agave salmiana*, which allowed their successful acclimatization. Zhang *et al*. (2013) employed regulator-free MS medium in *Agave hybrid* shoots, regenerated by organogenesis, these generated an average number of 5.39 roots per shoot with a length of 8.44 cm per root within 30 days.

The low rooting of the shoots that were immersed in a mixture with Radix® could possibly be affected by the amount of talc that was impregnated at the base of the stem, being a physical obstacle between the shoot and substrate. In the subcultures of this research, the shoots in the control treatments that did not have regulators generated 33% in SC3, 68% in SC4, and 43% in

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SC4-1 in a period of 30 days of subculture, these were only exposed to BAP for 30 days when they were generated from the propagule shoot, while the shoots in *ex-vitro* rooting were twice as long.

The results are similar to those by Adelberg and Naylor (2012), who reported that exposure of shoots to cytokinins such as BAP inhibited rooting. This may be because a cytokinin remnant inhibited rooting, and plants in agar were more affected than plants in the liquid medium. This remnant reduced rooting from 92% (control) to 68% with 10 µM (2.25 mg L^{-1}) BAP in *Aloe barbadensis* plants.

Conclusions

The dose of 0.5 mg L^{-1} BAP allows the highest shoot production in 30 days, with increased leaf length. In addition, this concentration contributes to *ex-vitro* rooting and decreases the probability of the appearance of hyperhydrated shoots. The control treatment (BAP 0 mg L^{-1}) can be used to root healthy and good-sized shoots in 30 days since, at the same time they grow, they form roots, and in 60 days, there is a complete and acclimatized plant.

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