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In vitro propagation of *Hylocereus purpusii* Britton & Rose, a mexican species in danger of extinction

Manuel de Feria[1]*, Daniel Rojas[2], Maité Chávez[1], Mireya Reyna[2], Elisa Quiala[1], Juan Solís[2], Florentina Zurita[2]. *Author for correspondence.

[1]Instituto de Biotecnología de las Plantas, Universidad Central Marta Abreu de Las Villas, Carretera a Camajuaní km 5.5, Santa Clara, CP 54 830 Cuba. e-mail: mdeferia@ibp.co.cu

[2]Centro Universitario de la Ciénega, Universidad de Guadalajara, Av. Universidad 1115, Ocotlán, CP 47 820, Jalisco, México. e-mail: drojas@cuci.udg.mx

ABSTRACT
The purpose of this work was to evaluate different conditions and culture parameters for the *in vitro* establishment and multiplication of *Hylocereus purpusii*. Seeds were used as plant material and a workflow was developed as an alternative for the propagation and recovery of this species in danger of extinction. In the establishment phase, the best result was obtained in the treatment with 1% NaOCl for 15 minutes achieving a 90% of germinated seeds and a final 77.7% of *in vitro* established plants. In the multiplication phase when combining 8.88 µM 6-BAP with different concentrations of IAA, a significant increment in the length of the shoots was observed with respect to the control (no addition of IAA). A combination of 8.88 µM 6-BAP with 3.42 µM IAA was selected as the best multiplication condition reaching a multiplication rate of 8.8. In the acclimatization phase, after two months of culture *in ex vitro* conditions, the survival was higher than 98% and after six months of growth, the plants were transplanted to field.

Key words: cactus, disinfection, growth regulators, seed germination.

Propagación *in vitro* de *Hylocereus purpusii* Britton & Rose, una especie mexicana en peligro de extinción

RESUMEN
El propósito de este trabajo fue evaluar diferentes condiciones y parámetros de cultivo para el establecimiento *in vitro* y multiplicación de *Hylocereus purpusii*. El protocolo de trabajo se desarrolló como una alternativa para la propagación y la recuperación de esta especie en peligro de extinción. Se utilizaron semillas como material vegetal inicial. En la fase de establecimiento, el mejor resultado se obtuvo en el tratamiento con hipoclorito de sodio al 1% durante 15 minutos. Se logró un 90% de semillas germinadas y finalmente un 77.7% de plantas establecidas *in vitro*. En la fase de multiplicación cuando se combinaron 8.88 µM de 6-BAP con diferentes concentraciones de AIA, se observó un incremento significativo en la longitud de los brotes con respecto al control (sin adición de AIA). La combinación de 8.88 µM de 6-BAP con 3.42 µM de AIA fue seleccionada como la mejor para la fase de multiplicación y para alcanzar un coeficiente de 8.8. En la fase de aclimatización, tras dos meses de cultivo en condiciones *ex vitro*, la supervivencia fue superior al 98%. Después de seis meses de crecimiento, las plantas fueron transplantadas a campo.

Palabras clave: cactus, desinfección, reguladores del crecimiento.

INTRODUCTION

Cacti are very common plants in Mexican landscapes. The country has the privilege to have the biggest quantity of species of this family around the world. Great quantities of cactus species are present in the daily diet of several human communities, either as fresh fruit or vegetable.

Among the broad range of cacti species, cultivation of members of the *Hylocereus* genus has recently become amenable in different countries. *Hylocereus* plants constitute an alternative in regions where climatic factors are obstacles for fresh fruits production (Mohamed-Yasseen, 2002). Due to their proper adaptation to wild conditions, their productive and economic potential (Legaria *et al.*, 2005) offers the possibility of cultivation in semi-arid regions (Chávez, 2002). Moreover, *Hylocereus* fruits contain a high nutritional value (Castillo *et al.*, 2005).

Potentially, the propagation of *Hylocereus* plants can be achieved by two strategies: 1) from steam sections and 2) from seeds. The first

has as prerequisite having a large number of elite donor plants which is extremely difficult considering the small number of individuals available in natural conditions. In the case of propagation from seeds, very few studies have been carried out about it. Propagation from seeds has, as main drawback, the large lapse of time that mediates from germination until harvest (3 to 4 years) (Mizrahi *et al.*, 2004). Other limitation of the use of seeds is related to the environmental conditions of arid and semi-arid regions, where the water is a restrictive factor for germination (Osorio *et al.*, 2001).

In the particular case of *H. purpusii* (Pitahaya purple), the main problems for its propagation, using traditional methods (see 1 and 2 above), are related with the low availability of plant material and its sexual autoincompatibility (Tel *et al.*, 2004; Castillo *et al.*, 2005). In this sense, *in vitro* propagation can be an alternative method to multiply selected genotypes of *H. purpusii*.

In vitro propagation in the *Hylocereus* genus has not been well described. Authors such as Johnson and Emino (1979), mentioned the potential of tissue culture for the propagation of *H. calcaratus.* Mohamed-Yasseen (2002) described his results in *H. undatus*. Pelah *et al.* (2002) worked with *Selinecereus megalanthus*, while Cuellar *et al.* (2006) were able to establish *H. undatus in vitro*, always using seeds as starting material. However, there is no report of *in vitro* studies with *H. purpusii* so far.

The objective of this work was to evaluate different conditions and culture parameters for the *in vitro* propagation of *H. purpusii* from seeds. The *in vitro* culture of *H. purpussi* is a possible alternative for the propagation and recovery of this species in danger of extinction.

MATERIALS AND METHODS

In vitro establishment

Seeds were obtained from fresh fruits of wild Pitahaya purple plants at the semitropical zone of the southern part of Puebla, México. Seeds extraction was done by removing the flesh from the fruits carefully. These were further placed in distilled water with constant agitation. Washing was carried out five times, until the seeds were free of mucilaginous material and acquired a brilliant black color.

A basal culture medium of inorganic salts proposed by Murashige and Skoog (1962) was used for *in vitro* seeds germination and plant establishment. This medium was supplemented with 20 g l^{-1} sucrose and 2 g l^{-1} Phytagel. Finally, pH was adjusted to 5.8.

Fifteen milliliters of culture media were dispensed per glass tube and sterilized during 15 minutes in an autoclave at 1.2 kg cm^{-1} of pressure and 121°C.

One seed was placed per glass tube with a total of 30 seeds per treatment. Glass tubes were kept at 28 ± 2°C in a room with a photosynthetic photon flux density (PPFD) between 38 and 47.5 µmol m^{-2} s^{-1}.

For the statistical processing of the data, the SPSS package for Windows version 16.0 was used. Significance levels were determined by a simple varince analysis (ANOVA) and the differences among mean values were statistically assesed by a Duncan multiple ranges test.For the variables expressed in percentages, differences among values were determined using a two samples proportion test ($p \leq 0.05$) by the statistical package STATISTIX version 1.0.

In all the experiments (see below), visual evaluations were carried out daily until 35 days to determine the germination time of the seeds under *in vitro* conditions. The number of germinated seeds and established plants was determined as well.

Effect of sodium hypochlorite concentration and immersion time on seeds germination

The effects of the immersion time on seeds germination in different sodium hypochlorite concentrations were evaluated. Firstly, an immersion time was fixed to 15 min and three sodium hypochlorite concentrations were assayed: 1, 1.5 and 2%. In a second part of the experiment, the sodium hypochlorite concentration with the highest germination percentage was assayed at 5, 10 and 15 minutes. All the combinations were cultured in the basal medium mentioned before with a 100% MS salts.

Effect of MS salts concentration on seeds germination

Afterwards the interest was focused on knowing whether the reduction of MS salt had an effect on seeds germination. Basal media containing a 25, 50, 75 and 100% of MS salts were evaluated taking into account the number of germinated seeds after 35 days of culture.

In vitro multiplication

Effect of different concentrations of 6-BAP and the type of explant

For the *in vitro* multiplicación the basal culture medium was composed of 100% MS salts, 30 g l^{-1} sucrose, pH 5.8 and 2 g l^{-1} Phytagel. The plant material used in the multiplication phase was apical shoots with two subcultures and an approximate length of 5 cm. In this phase, different concentrations of 6-BAP (0, 2.22, 4.44, 6.66, 8.88, 11.1 and 13.32 µM) were evaluated. The multiplication rate was used as evaluation criteria in all the treatments.

From the results of the previous analysis, three concentration of 6-BAP with the best results splited into selected to determine the influence of the explant type on the multiplication rate. Shoots were halves obtaining two new explants called apex and base. Twenty explants of each type were used per treatment and after 40 days of culture the number of shoots per explant was counted.

Effect of the combination between different concentrations of 6-BAP and IAA

Finally, in this phase, the effect of different concentrations and combinations of 6-BAP (6.66; 8.88 and 11.1 µM) and indolacetic acid (IAA) (1.14; 2.28 and 3.42 µM) was also studied. After 40 days of culture, number of shoots per explant, shoot length, (cm) fresh and dry weight of the shoots per treatment and the relationship dry weigh-fresh weigh were determined.

The plants obtained from the previous experiment were placed in a culture medium without growth regulators, with 100% MS salts, 30 g l^{-1} sucrose, pH 5.8 and 2 g l^{-1} Phytagel to induce roots formation. After 30 days of culture in these conditions, plants were transferred to *ex vitro* conditions.

RESULTS AND DISCUSSION

In vitro establishment

Effect of sodium hypochlorite concentration and immersion time on seeds germination

Seeds germination took place between the 5th and 12th day of culture, but a great percentage (76.6%) germinated between the 8th and 9th days. The increase in NaOCl concentration affected germination, although between the treatments with 1.5 and 2% differences were no significant (Table 1).

The best results were obtained in the treatment with 1% NaOCl and an immersion time of 15 minutes where the 90% seeds germinated. However, when the concentration of NaOCl increased to 1.5 and 2%, some plants died (21.7 and 20.1%, respectively) as a consequence of seeds damage.

When combining different immersion times (5, 10, 15 minutes) in a solution of 1.0% NaOCl, there were no significative differences in the germination percentage and *in vitro* plants establishment.

Table 1. *In vitro* establishment of *Hylocereus purpusii* plants, from germinated seeds after disinfection with NaOCl during 15 minutes.

NaOCl concentration (%)	Germination (%)	*In vitro* establishment (%)
1	90.0 a	77.7 a
1.5	80.0 b	58.3 b
2	76.6 b	56.5 b

Percentages with different letters in the same column differs significativelly for $p<0.05$ according to the proportion test for two samples

However, the treatment with 5 minutes, presented 13.3% of microbial contamination, and hence, it was decided to use the treatment with 1.0% NaOCl for 10 minutes.

Effect of MS salts concentration on seeds germination

Differences were not on seeds germination among the treatments with 25, 50, 75 and 100% MS salts in the culture medium. In Figure 1, plants with 8, 16 and 25 days of culture are shown.

Different results have been described in other cactus species regarding *in vitro* seeds germination. Quiala *et al.* (2004) germinated seeds of *Pilosocereus robinii* and the best results (91.4%) were obtained with 50% MS salts. In the species *Melocactus actinacanthus* same authors obtained the best germination percentage (81.4%) with 25% MS salts. These results confirm the influence of the genotype in the response to similar *in vitro* culture conditions.

In vitro multiplication

Effect of different concentrations of 6-BAP and the type of explant

The results demostrated that 6-BAP had effect on *in vitro* multiplication of *Hylocereus porpusii*.

The best multiplication rate (6.7) and new shoots length (1.75 cm) were obtained (Table 2 and Fig. 2e) in the treatment with 11.1 µM 6-BAP, the largest number of shoots (8.3) was reached in the treatment with 8.88 µM 6-BAP but they showed an average in length of 0.67 cm which limited their multiplication.

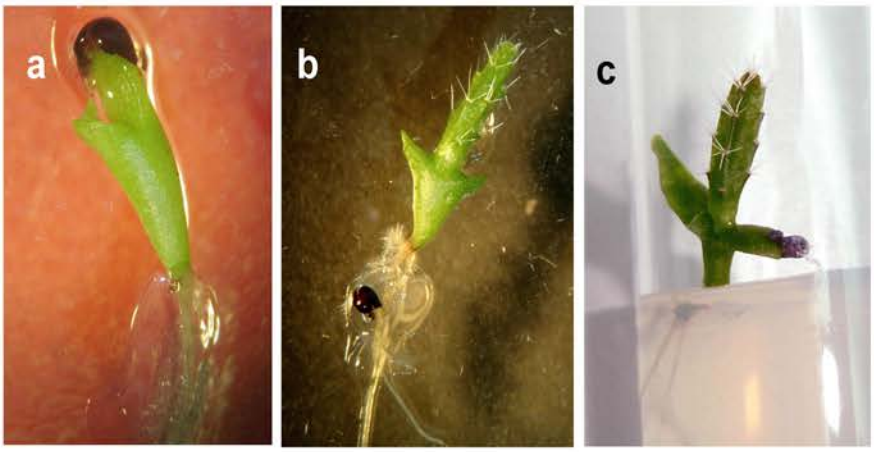

Figura 1. *In vitro* plant of *Hylocereus purpusii* germinated with 100% MS salts at different times after seed germination: a) 8 days, b) 16 day c) 25 days.

Table 2. Responses of *Hylocereus purpusii in vitro* plants after during shoots development 40 days of culture using different 6-BAP concentrations.

6-BAP concentration (µM)	Number of new shoots	Multiplication rate	Length of the new shoots (cm)	Length of the principal shoot (cm)
0	1.2 f	2.1 d	0.70 d	5.5 a
2.22	1.6 f	3.8 b	1.48 b	4.7 b
4.44	3.5 e	1.9 d	0.50 e	3.8 c
6.66	6.1 d	2.6 c	0.93 c	3.8 c
8.88	6.9 c	6.7 a	1.75 a	3.7 c
11.1	8.3 a	2.1 d	0.67d	3.7 c
13.32	7.6 b	2.0 d	0.60 de	2.8 d

Means with different letters in the same column differs for p <0.05 according to Duncan test

When the cactus species *Ariocarpus kotschoubeyanus* was multiplied by Moebius *et al.* (2003), shoots development depended on the presence of 6-BAP in the culture medium.The largest number of shoots was obtained when the concentration of this growth regulator oscillated between 4.44 and 13.32 µM. Similar results were obtained in our study, with *H. purpusii*.

Many species cultivated *in vitro* (including cacti) form roots in the absence of growth regulators. This response was observed in the species *Ariocarpus kotschoubeyanus* by Moebius *et al.* (2003) and Burdyn *et al.* (2006) in *Aloysia polystachya*. However, in our study *H. purpusii* formed as an average up to 2.5 roots per plant when cultivated in auxins depletion and with a concetration of up to 4.44 µM 6-BAP (Fig. 2 a, b, c).

It is well known that the explant type influences the *in vitro* multiplication of many species (Dhar and Joshi, 2005). This study included two explants types called apex and base (see Materials and Methods). In all the evaluated treatments, the first shoots were observed after three weeks of culture starting from both, apex and base explants. In the treatment with 0 µM of 6-BAP the presence of roots in the explants (apex and base) coincided with the formation of new shoots. On the contrary, in the treatments with 6-BAP no roots were observed.

New shoots formation increased proportionally with the adition of 6-BAP. In the treatment with 11.1 µM 6-BAP the greatest number of shoots per explant was obtained on average. Similarly to previous experiments, shoots were poorly developed which limited their use in the *in vitro* propagation.

Similar responses were described by Kumar *et al.* (2005) when working with the species *Holarrhena antidysenterica* and by Liu *et al.* (2006) with the species *Rhodiola fastigiata*.

In the control treatment (0 µM 6-BAP), apex explants hardly emitted new shoots, while base explants activated some areoles located where the cut was done and formed shoots.

According to the multiplication rate obtained and the length of the shoots emmited by both type of explants, the treatment with 8.88 µM 6-BAP was selected as the best one.

Effect of the combination between different concentrations of 6-BAP and IAA

When combining 8.88 µM 6-BAP with different concentrations of IAA, a significant increase in the length of the emmited shoots was observed respect to the control treatment (no addition of IAA) (Table 3). A combination of 8.88 µM 6-BAP with 3.42 µM IAA was selected as the best multiplication condition giving a multiplication rate of 8.8.

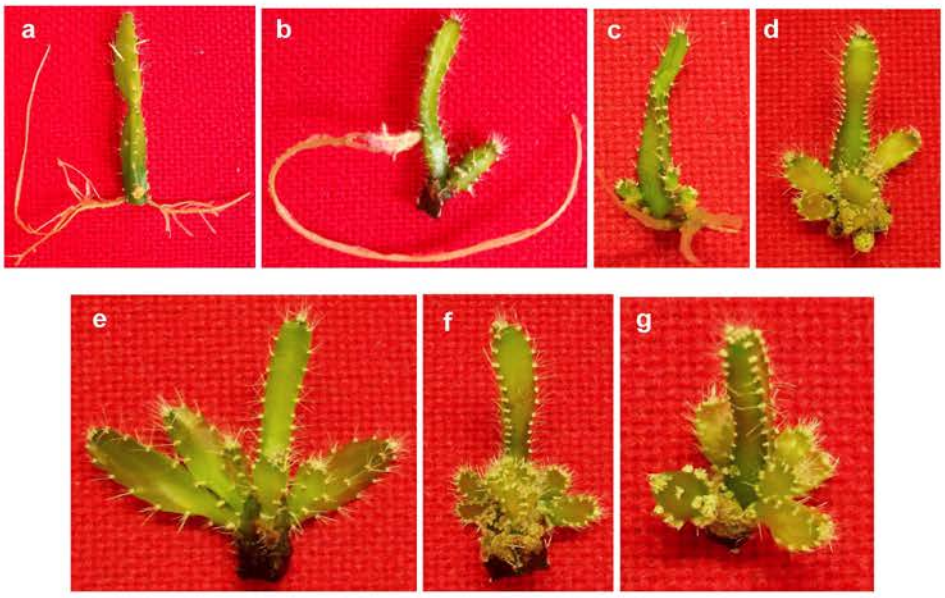

Figura 2. *Hylocereus purpusii in vitro* plants multiplied with different 6-BAP concentrations , a) 0 µM, b) 2.22 µM, c) 4.44 µM, d) 6.66 µM, e) 8.88 µM, f) 11.1 µM, g) 13.32 µM.

Table 3. Responses of *Hylocereus purpusii in vitro* during shoots development plants in presence of different combinations of 6-BAP and IAA.

Concentrations (µM)		Number of new shoots	Multiplication rate	Length of the new shoots (cm)	Length of the principal shoot (cm)
6-BAP	IAA				
8.88	0.0	7.0 e	4.7	1.49 e	3.4 d
6.66	1.14	6.2 f	4.2	1.09 g	3.8 c
6.66	2.28	5.1 g	5.7	1.79 d	4.4 b
6.66	3.42	7.4 de	7.8	2.43 b	4.7 ab
8.88	1.14	6.3 f	5.9	1.85 d	3.8 c
8.88	2.28	7.6 d	6.5	2.04 c	4.1 bc
8.88	3.42	7.1 e	8.8	2.75 a	4.9 a
11.1	1.14	8.7 b	2.6	0.81 h	3.8 c
11.1	2.28	9.4 a	3.7	1.15 f	4.2 b
11.1	3.42	8.1 c	5.9	1.84 d	4.3 b

Means with different letters in the same column differs for p <0.05 according to Duncan test

Figura 3. *Hylocereus purpusii* plants obtained via organogenesis in acclimatization conditions after six months of culture.

Liu *et al.* (2006) reported in *R. fastigiata* the best results in the multiplication rate when combining concentrations of cytokinins and auxins, maintaining a favorable proportion to the cytokinins.

After two months of culture under *ex vitro* conditions, survival was higher than 98% and after six months of culture (Fig. 3) plants were transplanted to the field.

This results were the first reports worldwide on the *in vitro* propagation of *Hylocereus purpusii*. The studies in the multiplication phase demonstrated that it is possible to propagate this species with good multiplication rates. However, it is important to assist the *in vitro* response of this species with different concentrations of 6-BAP and combinations of this growth regulator with IAA.

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