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Hyperhydricity control of *in vitro* shoots of *Turbinicarpus valdezianus* (Möller) GL & F

Control de la hiperhidricidad en brotes *in vitro* de *Turbinicarpus valdezianus* (Möller) GL & F

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Abstract. *Turbinicarpus valdezianus* is a species under special protection, according to the current law NOM-059-ECOL-2010. It spreads preferably through shoot proliferation *in vitro*. A common problem associated with the propagation of this species by tissue culture is the hyperhydricity or excess of water accumulation in the tissues of shoots explants. The literature on this topic indicates that such response is related with oxidative stress. Because of this, the effects of inhibitors of the gibberellins [paclobutrazol (PBZ) and calcium prohexadione (PCa)] and salicylic (SA) and benzoic acids (BA) were tested to diminish the hiperhydrycity of the sprouts, which was measured after twelve weeks of study initiation. The treatment with PBZ (3.4 x 10^{-4}M) completely eliminated hyperhydricity in the sprouts, and significantly increased the organogenesis, with 18 sprouts per explant, in comparison to the 3.55 sprouts per explant observed on the control. In addition to this, PBZ induced several morphological changes in the regenerated material, including a drastic reduction in height and an increase on the thickness of stem and roots. In contrast with these observations, including PCa (10^{-4}M), SA (10^{-4}M) and BA (10^{-4}M) resulted in higher hyperhydricity of shoots when compared to the control. However, they modified the number and length of roots in the sprouts, increasing the survival of the regenerated plantlets when transferred to *ex vitro* conditions. The histological analysis of the hyperhydric plants showed an increase in the number, diameter, and area of xylem vessels.

Keywords: *Turbinicarpus valdezianus*; Hyperhydricity; Paclobutrazol; Calcium prohexadione; Salicylic acid; Benzoic acid.

Resumen. *Turbinicarpus valdezianus* es una especie en protección especial, conforme a la norma vigente NOM-059-ECOL-2001, que se propaga preferemente a través de la proliferación de brotes *in vitro*. Un problema común asociado con la propagación de esta especie es la hiperhidricidad o exceso de acumulación de agua en los tejidos. La literatura sobre el tema indica que la respuesta está relacionada con el estrés oxidativo. Se verificó el efecto de una serie de compuestos con el objetivo de disminuir la respuesta de hiperhidricidad de los brotes, la cual fue evaluada a las doce semanas. Estos compuestos incluyeron a inhibidores de las giberelinas [paclobutrazol (PBZ) y prohexadiona de calcio (PCa)], y los ácidos salicílico (AS) y benzoico (AB). El tratamiento con PBZ (3,4 x 10^{-4}M) eliminó totalmente la hiperhidricidad en los brotes y aumentó significativamente la organogénesis mostrando 18 brotes por explante en comparación con los 3,55 brotes por explante observado en el testigo. Los cambios morfológicos inducidos por el PBZ fueron una disminución en la altura y engrosamiento del tallo y raíces. Por otra parte los tratamientos con PCa (10^{-4}M), AS (10^{-4}M) y AB (10^{-4}M) generaron una respuesta hiperhídrica mayor al testigo. Sin embargo, éstos dieron lugar a modificaciones en el número y longitud de raíces en los brotes los que favorecieron la supervivencia de las plántulas a las condiciones *ex vitro* luego del trasplante. El análisis histológico de las plantas hiperhídricas mostró un incremento en el número de vasos del xilema, con mayor diámetro y área.

Plabras clave: *Turbinicarpus valdezianus*; Hiperhidricidad; Paclobutrazol; Prohexadiona de calcio; Ácido salicílico; Ácido benzoico.

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INTRODUCTION

Turbinicarpus valdezianus (Möller) GL & F (Cactaceae) is an endemic species restricted to a small area of Mexico. It has been included within a special protection programme by Mexican government (NOM-ECOL-059-2010) due to habitat destruction and the collection of wild plants.

These are slow growing plants that sometimes have limited reproductive capacity. Cacti are usually propagated by seeds and rooted offshoots. However, conventional propagation methods are too slow. One alternative is *in vitro* tissue culture with faster growth rates and production of many individuals from a single explant. Nevertheless, there are different problems in plant regeneration, such as hyperhydricity and poor rooting.

The hyperhydricity is characterized by an excessive accumulation of water which is apparently associated to cellular oxidative stress (Chakrabarty et al., 2006). It gives place to a number of morphological, physiological and anatomical abnormalities. This condition is most likely to develop in vegetative materials grown *in vitro* (Debergh et al., 1992). Hyperhydricity limits the propagation and acclimation *ex vitro* of some species, including *T. valdezianus* with losses of up to 65%.

The succulence of cacti favors the hyperhydricity. This is because of the ability of cells to absorb large amounts of water, and the presence of a set of cells with thin walls and little lignification in the inner part of the rind which allows large volume changes (Mauseth et al., 1998). The hyperhydricity of *in vitro* conditions is encouraged by the high humidity and direct contact of the explants with water. Hyperhydrated plants have a translucent appearance with shiny surface and fragile stems (Majada & Sanchez-Tamés, 2003). Various aspects and conditions through tissue culture have been evaluated in cacti in order to diminish this condition; these include changes of the original concentration of the culture medium, medium type, and the use of chemicals as the polyethylene glycol (Santos-Díaz et al., 2003).

The micropropagation of the genus *Turbinicarpus* has been described previously (Rosas et al., 2001; Dávila-Figueroa et al., 2005) but there are no reports which mention decreases of hyperhydricity. After years of work with this plant species in our laboratory, it has been notorious that the propagation of *T. valdezianus* diminishes seriously due to the presence of the state of hyperhydricity, since the 65% of the explants can be lost during cultivation.

Paclobutrazol (PBZ) is an inhibitor of the gibberellic acid synthesis. Its presence reduces levels of endogenous gibberellin, due to its interaction with cytochrome P450, diminishing the monooxygenase reaction and blocking the oxidation of *ent*-kaurene to *ent*-kaurenoic acid inside of the cycle of the mevalonate pathway. This results in a reduction of the gibberellin synthesis and consequent cell elongation. The application of paclobutrazol on *ex vitro* potato cultivation (Tsegaw et al., 2005) showed the following results: increment of the thickness of the cuticle, larger epidermal cells, elongated palisade mesophyll cells, thicker spongy mesophyll, and thickness of the stem. Other studies have shown that the exogenous supplementation with PBZ positively affects aspects related to the growth of plants, besides those already mentioned on the hyperhydricity. For example, PBZ added to the soil at concentrations of 0.125, 1.0, 5.0 and 10 mg per plant has increased the number of roots in *Chrysanthemum* (Burrows et al., 1992) and the proliferation of sprouts in microplants of Araceae (Werbrouck & Debergh, 1996).

Prohexadione calcium (PCa) is also an inhibitor of the synthesis of gibberellins (Rademacher, 2000). Its structure is similar to the carboxilic acid 2-oxoglutaric acid. It is cosubstratum of the dioxygenases that catalyze the final stages gibberellins' formation by blocking the 3-ß hydroxylase, which gives rise to the active gibberellin. It has been reported that the application of PCa in *ex vitro* conditions in a concentration of 250 ppm favors the synthesis of carbohydrates, increases non-structural carbohydrates, favors the accumulation of nitrogen, affects the metabolism of phenolic compounds, and enhances of net photosynthesis in apple plants (Sabatini et al., 2003). These features are useful to reduce the hyperhydric response.

The salicylates are a group of phenolic compounds synthesized under conditions of oxidative stress. Implementation of salicylic acid (SA) delays the senescence of leaves and flowers; this effect is apparently related to ethylene synthesis inhibition. It also affects the photosynthetic process, stomatal conductivity, and transpiration with foliar applications (concentration of 10^{-3}, 10^{-5} M) in corn and soybeans (Khan et al., 2003).

Benzoic acid (BA) is a precursor of salicylic acid. It is synthesized from phenylalanine by combining several metabolic pathways (Wildermuth, 2006). The BA is an organic acid with positive effects when applied to soil (Benavides-Mendoza et al., 2007) or plant leaves (Ramirez et al., 2006).

The aim of this work was to assess the effects of paclobutrazol, prohexadione calcium, and salicylates in the reduction of the hyperhydricity in shoots of *T. valdezianus* under conditions of tissue culture.

MATERIALS AND METHODS

The study was conducted in the Universidad Autónoma Agraria Antonio Narro in 2006. *Turbinicarpus valdezianus* seedlings were germinated *in vitro* using the following protocol: seed disinfection with ethanol 70% for 1 minute and sodium hypochlorite 20% for 15 minutes, followed by rinsing three times with distilled and sterilized water. Seeds were placed in glass jars of 60 mL capacity, with 20 mL of culture medium (MS: Murashige & Skoog, 1962) supplemented with 100 mg/L of myo-inositol (SIGMA®, I-3011); 1 mg/L thiamine-HCL (SIGMA®, T-3906); 1 mg/L Pyridoxine-HCL

(SIGMA®, P-8666); 30 g/L sucrose (SIGMA®, K-0753); 8g/L agar (SIGMA®, A-1296). The pH was adjusted to 5.7, and the medium was sterilized for 15 minutes. The obtained seedlings were sub-cultivated in the same medium MS every four weeks during four consecutive times to increase the amount of material. There was a selection of shoots of 0.5 cm diameter, 0.8 cm tall, and without hyperhydricity symptoms. The shoots were divided transversely into cylindrical pieces and placed in glass jars of 60 mL capacity with 20 mL of the same culture used during the seed germination medium; they were supplemented with 1 mg/L kinetin. The bottles were placed at a temperature of 25 ± 1 °C, with 8 hours of darkness and 16 hours at 2500 lux light. The medium was renewed every four weeks. Five repetitions were established for each treatment. Each repetition consisted of four explants per glass. Treatments were: (1) control (without growth promoters); (2) paclobutrazol (PBZ); (3) calcium prohexadione (PCa); (4) salicylic acid (SA), and (5) benzoic acid (BA). The concentration of PBZ was 3.4×10^{-4} M while those of PCa (BASF®), SA (SIGMA®), and BA (SIGMA®) were of 10^{-4} M. The experiment was repeated three times, and the average results were reported. We carried out the evaluations in two forms. The first, registered 12 week after the start of tissue culture, corresponded to the sprouting response, expressed as the number of shoots produced by each explant. The second in the 13th week, after effecting a necessary transplant to a new culture medium, was determined as the growth response, measured by the length of shoots. Other growth variables verified after 17 weeks of cultivation were: fresh weight (g), dry weight (g), number of roots, and root length (cm). The absence of hyperhydricity was assessed qualitatively based on morphological characteristics. The data were subjected to analysis of variance under a completely randomized experimental design, and a Tukey ($p \leq 0.05$) test for mean separation was conducted with the statistical software SAS (SAS, 1989).

In order to characterize the potential effects produced by the hyperhydricity in the shoots, as well as the effect on the shoots differentiated *in vitro*, a histological analysis of the differentiated representative materials of each treatment was carried out. Three individuals were taken for each treatment, and set with formalin-acetic-acid-alcohol (F.A.A.). They were then dehydrated in a series of alcohol solutions at 50, 60, 70, 85 and 98% for two hours using a modification of the Johansen technique (1940), with tertiary butyl alcohol rather than ethanol 96%. Next, a sequence of changes in tertiary butyl alcohol (100%), tertiary butyl alcohol solutions plus xylene in different proportions (3:1, 1:1, and 1:3, respectively), and finally pure xylene for 2 hours in each solution were made. When finished, the samples were included in aluminum molds with paraffin at 55 °C, assembled, prepared for the realization of cuts, cut into 12 cross-sections at the middle part of the shoots with a microtome (MOD.820 Spencer, American Optical) handheld to 18 micrometers thick, cut stuck on a slide with Kaupt adhesive applying heat with a lighter. Staining of the sample was carried out with safranin (1%) for 15 minutes, previous hydration in a series of alcohol (absolute alcohol, alcohol at 96, 85, and 70%) and in distilled water, later dehydration with a series of alcohol (alcohol 70, 85, 96% and absolute alcohol), coloration with fast green 0.5% (30 seconds), and rinsing with absolute alcohol. After setting with carbol-xylene (Sass, 1958), mounting was made with balsam of fir.

Histological evaluation was based on the 6 best preparations out of the 12 originally obtained. Measurements were made at a magnification of X40 objective, through Axion Vision software, of the anatomical elements located in 15 areas of 100 x 100 micrometers of a vascular bundle. In each area, the number of xylem vessels was determined. The cross-sectional area and the thickness of the wall of two vessels from each vascular bundle systematically chosen (the positions 12 and 3 of the area were selected) were measured. An analysis of variance under a completely randomized experimental design, and a Tukey ($p \leq 0.05$) test for mean separation were conducted with the software SAS (SAS, 1989).

RESULTS AND DISCUSSION

Analysis of variance showed significant differences in number of shoots in the paclobutrazol treatment. Treatment with paclobutrazol (PBZ) completely eliminated the hyperhydricity, reduced the number of roots, but increased their length (Table 1). Paclobutrazol response on shoots was positive, confirming the results obtained by Escalona et al. (1999) in *Ananas comosus*. In a medium with cytokinins to induce sprouting in *T. valdezianus*, 7.8 shoots per explant were obtained (Dávila-Figueroa et al., 2005). In our study, 18.0 shoots per explant were obtained when adding PBZ. The observed difference may arise from a synergistic effect of PBZ with kinetin, a response previously observed by Werbrouck & Debergh (1996) in the proliferation of Araceae.

Treatments of prohexadione calcium (PCa), salicylic acid (SA), and benzoic acid (BA) increased the number of hiperhydrated shoots in comparison with the control. It is possible that the hyperhydricity resulting from SA and BA in the indicated concentrations resulted from an oxidative response in the cells (Janda et al., 2000), biochemical condition which could induce greater over-hydration. The observed increase of hyperhydric shoots in PCa was unexpected; a possible explanation consists in associating the response with the presence of a series of factors that promote vegetative growth (Evans et al., 1996).

The shoots produced in the SA rich culture showed stems with lower longitudinal growth, but thicker; decrease in the number of shoots, and significant increases in the length of roots. In other species such as *Solanum tuberosum* (Mora-Herrera & Lopez-Delgado, 2006) and *Sechium edule* (Alvarenga-Venutolo et al., 2007) an inhibition of stem and

root growth was observed in the presence of SA. An increase in thickness and length of roots was observed in this study; this difference could be interpreted as the synergistic effect of the SA concentration with the endogenous auxin; a similar result was reported by Bojarczuk & Jankiewicz (1975).

The BA decreased the organogenesis and inhibited rhizogenesis (Table 1). Santos-Díaz et al. (2003) reported that the low percentage of rooting was related to the hyperhydric status of the cultured explants.

Table 1. Effect of inhibitors of gibberellins and salicylates on shoot growth and root differentiation on explants of *T. valdezianus* at 12 weeks after planting.
Tabla 1. Efecto de compuestos inhibidores de giberelinas y salicilatos sobre el crecimiento de brotes y diferenciación de raíces en los explantes de *T. valdezianus* a las doce semanas después de la siembra.

Treatment	# of shoots / explant	% with explants hyperhydricity	Fresh weight (mg)	# of roots/ explant	Root length (cm)
Control	3.55b	25	0.19ab	4.6a	0.30c
Paclobutrazol ($3.4x10^{-4}$M)	18.00a	0	0.21ab	1.4b	0.67b
Calcium Prohexadione (10^{-4}M)	3.25b	50	0.26a	1.6b	0.34c
Salicylic acid (10^{-4}M)	1.15b	75	0.15b	2.2b	0.81a
Benzoic acid (10^{-4}M)	1.20b	75	0.17ab	0.4b	0.02d

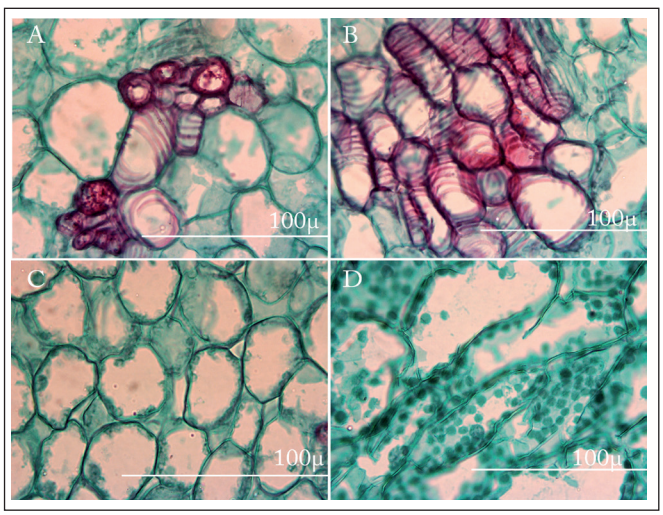
Means with equal letters are not statistically different (Tukey, $p < 0.05$).
Promedios con la misma letra no son estadísticamente diferentes (Tukey, $p < 0,05$).

Table 2. Histological observations of the xylem in *T. valdezianus* plants propagated *in vitro*, and treated with different inhibitors of gibberellins and salycilates.
Tabla 2. Observaciones histológicas del xilema en plantas de *T. valdezianus* propagadas *in vitro* y tratadas con diferentes inhibidores de giberelinas y con salicilatos.

Treatment	Diameter of lumen of xylem vessels (µm)	Average area of xylem vessels (µm²)	Thickness of the wall of xylem vessels (µm)	Average number of vessels per mm²
Control	7.08b	44.48b	2.00bc	9.72bc
Paclobutrazol (3.4×10^{-4}M)	6.37b	33.89b	2.90ab	19.86ba
Calcium Prohexadione (10^{-4}M)	8.54b	50.32b	3.62a	7.60c
Salicylic acid (10^{-4}M)	10.59ab	75.77b	1.54c	29.66a
Benzoic acid (10^{-4}M)	17.86a	234.82a	2.14bc	21.21a

Means with equal letters are not statistically different (Tukey, $p< 0.05$).
Promedios con la misma letra no son estadísticamente diferentes (Tukey, $p< 0,05$).

Fig. 1. Histological cross-section of stalks of *T.valdezianus* plants *in vitro*, observed with the X40 objective. On the left or on the right, cross-sections of plants without or with hyperhydricity, respectively. Differences in cellular volume are clearly shown. A and B: xylem cells; C and D: parenchyma cells.
Fig.1. Corte histológico de plantas de *T. valdezianus* de cultivo *in vitro*, observadas con el objetivo de X40. A la izquierda los cortes de plantas sin hiperhidricidad, a la derecha los correspondientes a plantas con hiperhidricidad. Se aprecia claramente la diferencia en el volumen celular. A y B: células del xilema; C y D: células parenquimáticas.



Morphological characterization of *T. valdezianus* developed *in vitro* without tested compounds presented vessels with an average diameter of 7.08 µm (4 - 11.43 µm), an average area of vessels of 44.48 µm² (10.3 - 77.2 µm²), and a frequency of vessels of 9.72 per mm². The application of PCa, salicylic and benzoic acid modified some of these characters (Table 2). Moreover, anatomical study results demonstrated that seedlings with hyperhydricity had larger xylem vessels, higher variation of size in all directions between plants, and a great number of vessels (Fig. 1).

Treatment with PCa showed reduced root number and length, and increasing stem length with regard to the control. On the other hand, results indicated that PBZ totally declined the formation of hyperhydric shoots in *T. valdezianus*. PCa, SA and BA increased hyperhydricity. PCa and SA treatments led to rhizogenesis in shoots, favoring plant survival in *ex vitro* conditions.

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