In Vitro propagation of *Pilosocereus robinii* (Lemaire) Byles *et* Rowley, endemic and endangered cactus

Elisa Quiala[1*], Jesús Matos[2], Grecia Montalvo[2], Manuel de Feria[1], Maité Chávez[1], Alina Capote[1], Naivy Pérez[1], Raúl Barbón[1] and Britta Kowalski[3]

[1]Instituto de Biotecnología de Las Plantas, Universidad Central "Marta Abreu" de Las Villas. Carretera a Camajuaní km 5½, Santa Clara, CP 54 830, Cuba.
[*]Author for correspondence; E-mail: elisa@ibp.co.cu

[2]Empresa Nacional para la Protección de la Flora y la Fauna.Unidad Sabana de Santa Clara. Carretera a Placetas km 5½, Santa Clara. Villa Clara. Cuba. Tel. 53 (42) 206285

[3]Institute for Land Use, University of Rostock, Faculty of Agricultural and Environmental Sciences, 18051 Rostock, Germany

Received 2 December, 2008; Accepted 10 January, 2009

Abstract

In vitro propagation systems by areole activation were developed for *Pilosocereus robinii* (Lemaire) Byles *et* Rowley, an endemic cactus of Cuban island in extinction danger. Seeds from mature fruits were collected under field conditions and disinfected with two concentration of NaOCl (1.0 and 2.0%) and two time of disinfection (15 and 20 min). During germination a half- and full-strength MS basal medium were tested. The highest germination rate (92.8%) was reached when half MS basal medium were used. Shoot formation from areoles of in vitro-germinated plantlets was achieved in explants cultured in Murashige and Skoog (MS) basal medium supplemented with 30 g l^{-1} sucrose, 3.0 g l^{-1} Gelrite® and three concentration of 6-Bencilaminopurine (4.44, 6.66 and 13.32 µM). Shoot production in this proliferation medium was evaluated during three culture cycles. Proportionately proliferation rate increase with 6-BAP concentration, on the contrary shoots length decreased. The highest proliferation rate (8.9 shoots per explant) was reached employment 13.32 µM of 6-BAP. On average, rooting efficiency was 100% in MS basal medium free of growth regulators. The frequency of survival of the plants once transferred to substrate composed of cattle manure rotted for eight months was on average 91.6%. Finally, 500 individual plants of *Pilosocereus robinii* were transferred to nursery. We describe for the first time a system for the production of multiple shoots by areole activation, as well as their rooting, acclimatization and nursery establishment of endangered and endemic specie, that are difficult to propagate by conventional methods.

Key words: biodiversity conservation, cactaceae, micropropagation, threatened specie, tissue culture.

Abbreviations: 6-BAP- 6-Bencilaminopurine

Introduction

Pilosocereus robinii (Lemaire) Byles *et* Rowley is an endemic and endangered cactus from Cuban island (Borhidi and Muñiz, 1983). The International Union for the Conservation of the Nature (UCIN) reports areas in Cuba of high conservation priority, mainly the *Pilosocereus robinii* habitat. The area extends over the North coast from Rincón Francés to Camagüey, where natural flora has been adversely affected due to the tourist development.

The seeds germination rate in this specie is low, and their growth is slow. Propagation by stem cuttings is inefficient; the donor plant must be mutilated to obtain a new individual with the additional risk of fungal infection of the cut tissue and subsequent loss through rotting. For these reasons it is difficult to recover endangered populations through conventional propagation methods. Therefore, it is necessary to safeguard these species and to improve, in any possible way, existing propagation techniques.

Tissue culture techniques have been applied successfully in the recovery and *in vitro* propagation of different cacti species such as: *Opuntia* (Escobar *et al*., 1986), *Cereus peruvianus* Mill (Oliveira *et al*., 1995), 21 species of Mexican Cacti (Pérez Molphe-Balch *et al*., 1998), *Opuntia ficus-indica* (Pinheiro da Costa *et al*., 2001), *Pelecyphora aselliformis* Ehrenberg and *P. strobiliformis* Werdermann (Pérez Molphe-Balch and Dávila-Figueroa, 2002), *Ariocarpus kotschoubeyanus* (Lem) K. Schum (Moebius-Goldammer *et al*., 2003), different species of *Turbinocarpus* (Mata-Rosas *et al.* 2001; Dávila-Figueroa *et al*., 2005), *Notocactus magnificus* (Gallo *et al*., 2005), *Mammillaria albicoma* (Wyka *et al*., 2006).

In the present study, we describe for the first time an *in vitro* propagation system for *Pilosocereus robinii,* endangered and endemic Cuban specie of cacti. We refer seeds disinfection and germination, the production of multiple shoots by areole activation, as well as their rooting, acclimatization and nursery establishment. This system could become valuable tools for conservation and rescue of this specie.

Materials and methods

Plant material: Botanical seeds of *Pilosocereus robinii* were obtained from mature fruits collected in their natural habitat from several different plants (Figure 1).

Seeds disinfection and germination: Seeds were washed with water and then disinfected in two concentrations of Sodium Hypochlorite (1.0 and 2.0%), two times of disinfection (15 and 20 min) were tested. Seeds were placed singly in test tubes with 10 ml of incubation MS basal medium Murashige and Skoog (1962), 10 g l^{-1} sucrose, pH 5.6 and solidified with 2.0 g.l^{-1} Gelrite®. After 10 days of culture the number of seeds free of microbial contaminants was evaluated and seeds then placed on germination media. During germination a half- and full-strength MS basal medium supplemented with 1.0 mg l^{-1} thiamine, 30 mg l^{-1} sucrose and 2.0 g l^{-1} Gelrite® were proved. Seed germination was evaluated on alternate days during seven weeks.

The cultures were maintained on a growth chambers with solar light 48.1-62.5 $\mu E.m^{-2}s^{-1}$ at 28±2ºC. These same conditions were used in all subsequent experiments.

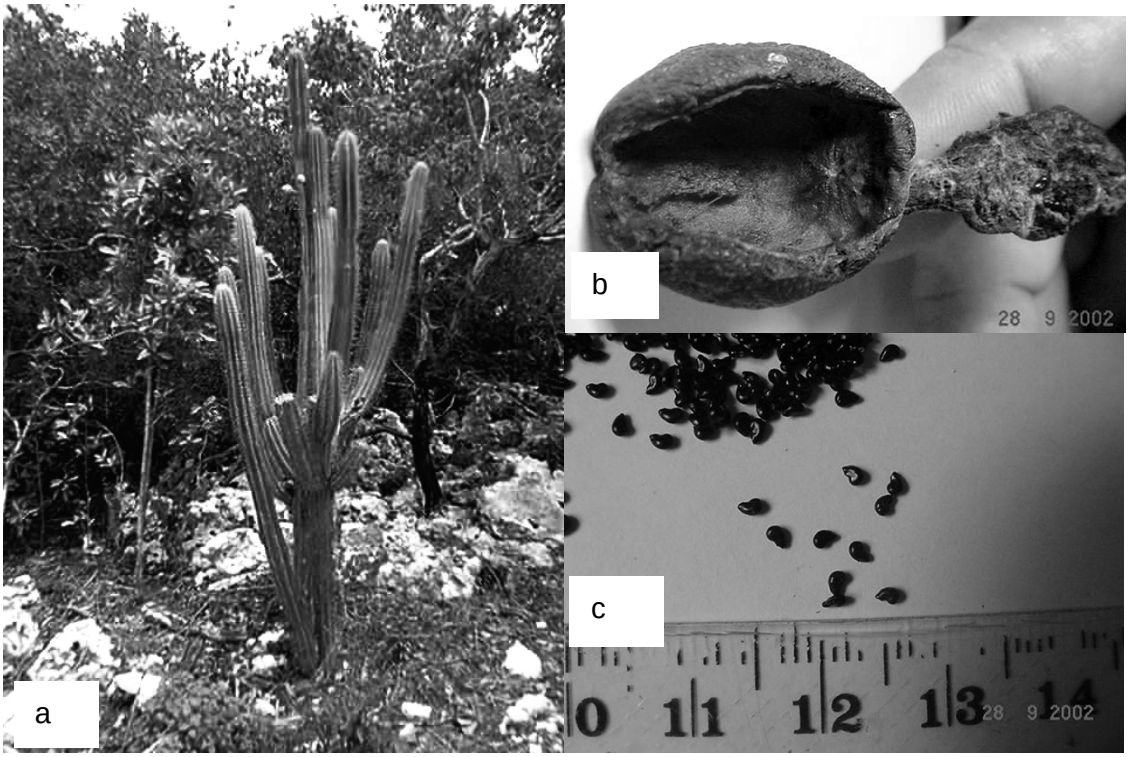

Figure 1 a) Plant of *Pilosocereus robinii* in natural habitat ("Cayo Conuco", north coast of the island), b) mature fruit opened, c) seed collected from mature fruit.

Axillary shoot proliferation and rooting: The proliferation was carried out by means of axillary buds activation. For this, the root systems were excised and each cactus was cut transversely and both the apex and the base were placed into culture flask, containing 25 ml of full-strength MS basal media with 30 g l^{-1} sucrose, 1.0 mg l^{-1} thiamine, 100 mg l^{-1} myo-inositol, 3.0 g l^{-1} Gelrite® and three concentrations (4.44, 6.66 and 13.32 (uM) of 6 BAP. Twenty explants per treatment were used. The number of shoots produced per plant and the length of shoots were recorded after 7 wk of culture during three culture cycle (21 wk).

Shoots were collected from shoot proliferation media and used for rooting. The rooting technique consisted of transferring the shoots to MS basal medium free of growth regulators with 30 g l^{-1} sucrose and 3.0 g l^{-1} Gelrite®. The number of plants with roots was evaluated 8 wk after initiating this phase.

Acclimatization and transfer of plantlets to nursery: Rooted plants were transplanted to pots containing two type of substrate. Substrate 1 was composed by 100% of cattle manure with eight months of decomposition covered with a 2.0 cm layer of zeolite. Substrate 2 was composed of 85% compost mixed with 15% zeolite. Plastic covering were used to reduce 50% of luminous intensity of solar light and to allow acclimatization plants before being transferred to the nursery. Survival percentages were determined 10 wk after transplantation. Finally plants were transferred to nursery and planted onto plastic bags containing a 2:2:1 (v/v) mixture of cattle manure, soil from the natural habitat and ground lime stone.

Experimental design and data analysis: Each experiment was repeated three times. All data collected was compared through analysis of variance. Means values were compared by Duncan multiple range test at the 5% level ($p < 0.05$).

Results and discussion

Seeds disinfection and germination

The sodium hypochlorite was effective for the disinfection of seeds; a 100% of seeds free of microbial contaminants were obtained when 2.0% of sodium hypochlorite for 20 minutes was used. When 2.0% of NaOCl for 15 minutes was applied, an 80% of seeds free of microbial contaminant were reached in this treatment (Figure 2)**.** The seeds of *Pilosocereus robinii* are small (2-3 mm) with a hard and flat texture offering little coverage for the development of microorganisms.

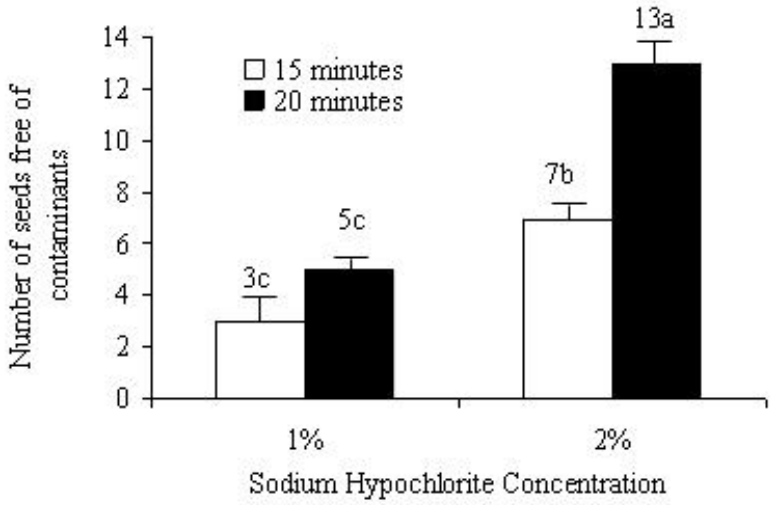

Figure 2. Effects of Sodium Hypochlorite in the seeds disinfestations of *Pilosocereus robinii.* Bars with different letters are significantly different at p=0.05. Data are means ± SE (n =14).

Germination occurred gradually starting 14 d after the inoculation of seeds, achieving a high percentage, in the culture medium with half-strength MS basal medium (Figure 3). In the full-strength MS basal media, germination was inferior and started one week later.

Axillary shoot proliferation and rooting

The cytokinin was indispensable for shoot generation through areole activation. The control treatments without 6-BAP were unable to induce shoot proliferation. On the contrary, when cytokinin was included in the culture media, shoots were obtained in all treatments.

After 4 weeks the explants exhibited shoot production from the areoles in all treatments containing 6-BAP. In the range of concentrations used, proportionately proliferation rate increase with 6-BAP concentration, on the contrary shoots length decreased.

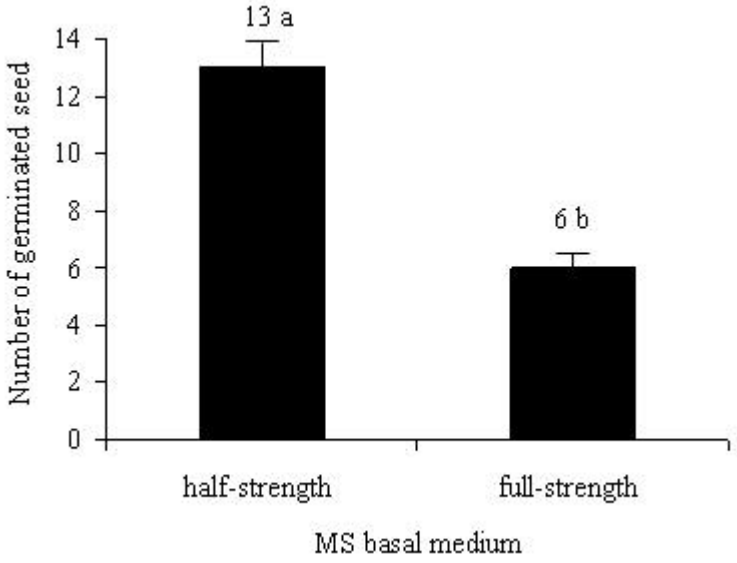

Figure 3. Effects of half-strength and full-strength MS basal medium in the seeds germination of *Pilosocereus robinii.* Bars with different letters are significantly different at p=0.05. Data are means ± SE (n =14).

When apical explants were used, a dominant shoot arises from the elongation of the inoculated apex, and the small shoots appear at its base. While when the base of cactus was used as primary explant, the shoots generated were homogeneous in their size and differentiation stage and appeared in the upper part of the explants, these shoots were easily separated and subculture (Figure 4a). So that in this specie is indispensable to eliminate the apical dominance to produce new shoots with desirable characteristic to subculture. Although the highest proliferation rate (8.9 shoots per explant) (Table 1) was reached with the higher concentration (13.32 µM 6-BAP) we observed in this treatment shoots with hyperhydration symptoms.

Table 1. Effects of 6-BAP concentration on shoot proliferation by areole activation in *Pilosocereus robinii.*

Growth regulators (µM 6-BAP)	Shoots per explant in each culture cycle*		
	First cycle (7 wk)	Second cycle (14 wk)	Third cycle (21 wk)
none	1.00 c	1.30 d	1.80 d
4.44	2.10 b	3.37 c	4.30 c
6.66	2.92 a	4.41 b	5.62 b
13.32	3.30 a	5.53 a	8.93 a
±SE[£]	0.29	0.37	0.41

[£]SE: Standard Error. Data were collected from 20 initiated shoots per treatment in each culture cycle.
*Means followed by the same letter within each column do not differ significantly at p≤ 0.05 according to the Duncan multiple range test.

According to different authors (Elias-Rocha *et al*., 1998; Pérez-Molphe-Balch *et al.,* 1998) hyperhydration of the tissues is a serious problem for *in vitro* culture of cacti. This physiological disorder is due to the physical and chemical conditions of *in vitro* culture; i.e. high humidity, excess of carbohydrates and minerals, high levels of plant growth regulators and low light intensity (Ziv, 1991). In this work, hyperhydration was present, only when higher concentrations of 6-BAP in the proliferation media where used. The 6.66 µM 6-BAP treatment would be more convenient to use for shoot proliferation of *Pilosocereus robinii* to avoid the adverse effect of higher levels of 6-BAP, such as hyperhydricity and somaclonal variation.

The positive effect of 6-BAP on the capacity to induce plant regeneration in cactaceae has been reported previously for species in other cacti genera, also by areole activation for shoot proliferation. Pérez-Molphe-Balch *et al.* (1998) report a range from 2.1 to 17.5 shoots per explant in a study conducted on 21 species in 10 genera of Cactaceae. Elias-Rocha *et al*. (1998) refer seven shoots per explant for *Mammillaria sphacelata*. There are also reports of 13.7 and 12.3 shoots per explant in *Pelecyphora aselliformis* and *P. strobiliformis*, respectively. Pérez-Molphe-Balch and Dávila-Figueroa (2002) refers 5.3, 3.8, and 4.3 shoots per explant in *Carnegiea gigantea, Pachycereus pringlei*, and *Stenocereus thurberi*, respectively.

Although on average, rooting efficiency was 100% and the in vitro-generated roots were vigorous in a basal MS media free of growth regulator (Figure 4b), shoots coming from proliferation media with 6.66 and 13.32 µM 6-BAP, rooted one week later than those coming from treatment without or with 4.44 µM 6-BAP.

Maintaining genetic stability in regenerated plants is essential for endangered species conservation. It is important that shoots proliferation in *Pilosocereus robinii* was from areole activation, because according to Machado and Prioli (1996) as well as Pérez-Molphe-Balch and Dávila-Figueroa (2002) micropropagated cacti regenerated from axillary buds are considered to be genetically stable.

Acclimatization and transfer of plantlets to nursery

The acclimatization of plants was successful. The biggest survival rate (91.6%) was obtained in the substrate with 100% of cattle manure covered with zeolite (Figure 4c), while 66% of survival rate was achieved when the substrate with 85% compost and 15% zeolite was used. In this treatment fungal infection in the base of cactus was observed and subsequent loss through rotting.

In Cuba, according to the experience of the National Botanical Garden, a complex mixture composed by 35% of well washed thick sand, 15% of rotten earth, 35% of humus and 15% of charcoal is recommended for the sowing of cactus. However, according to the experience obtained in this work, with a simple substrate composed by cattle manure with eight months of decomposition it is feasible the culture of this cactus species, with a high percentage of survival in acclimatization. Applying the methodology described in this work, 500 plants of *Pilosocereus robinii* has been transferred to the protected area and planted in nursery conditions with 100% survival rate (Figure 4d).

Conclusions

A protocol for *in vitro* propagation of *Pilosocereus robinii* was established. This protocol is more efficient than traditional propagation methods and can be valuable tool for the in vitro conservation of this specie, and the production of plants for the repopulation of natural areas damaged during construction of tourist facilities.

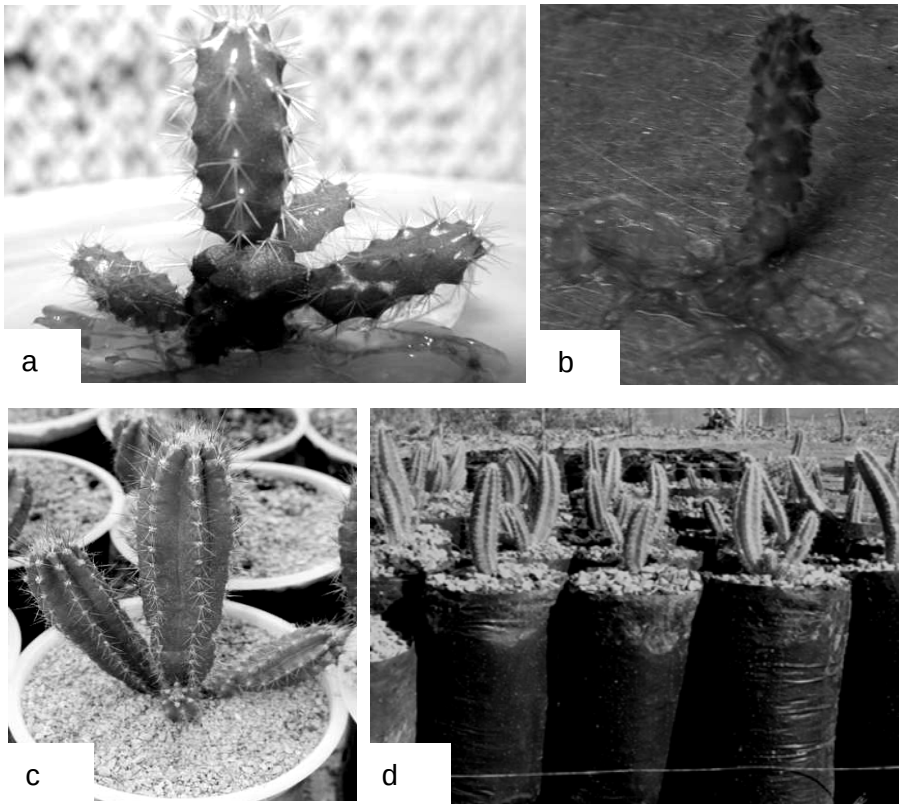

Figure 4. *In vitro* propagation of *Pilosocereus robinii*, a) Shoot production by areole activation with 6.66 µM 6-BAP after 7 wk of culture initiation, b) Shoots rooted after 8 wk culture in full-strength MS basal media free of growth regulators, c) In vitro-generated plants growing in 100% cattle manure with eight months of decomposition covered with a 2.0 cm layer of zeolite after 70 days, d) In vitro-generated plants in nursery conditions after six months.

Acknowledgements

This work was supported by the Union of Universities of Latin America (UDUAL). We express our sincere thanks to Ph. D. Yelenys Alvarado Capó and M. Sc. Osmildo Fernández for critical reading of this manuscript. We would also like to thank Dr. Martín Mata Rosas and Ph. D. Eugenio Pérez Molphe Balch for the scientific literature they supplied.

References

Borhidi, A. and Muñiz, O. 1983. Catálogo de Plantas cubanas amenazadas o extinguidas. Editorial de la Academia de Ciencias de Cuba, La Habana, Cuba. p. 17.

Elias-Rocha M.A., Santos-Días M.S., Arredondo-Gómez, A. 1998. Propagation of *Mammillaria candida* (Cactaceae) by tissue culture technique. Haseltonia 6:96–101.

González-Benito, M.E., Martín, C., Iriondo, J.M., Pérez, C. 1999. Conservation of the Rare and Endangered Plants Endemic to Spain. In: Plant Conservation Biotechnology. Benson, E. (Ed.). Taylor & Francis, London. pp. 251-264.

Oliveira, S.A., Machado, M.F.P.S., Prioli, A.J. 1995. In vitro propagation of *Cereus peruvianus* Mill (Cactaceae). In Vitro Cell. Dev. Biol. Plant 31:47–50.

Machado, M.F.P.S. and Prioli, A.J. 1996. Micropropagation of *Cereus peruvianus* Mill. (Cactaceae) by areole activation. In Vitro Cell. Dev. Biol. Plant 32:199–203.

Mata-Rosas, M., Monroy de la Rosa, M.A., Goldammer, K.M., Chávez-Avila, V.M. 2001. Micropropagation of Turbinicarpus laui, an endemic and endangered species. In Vitro Cell. Dev. Biol. Plant 37: 400–404.

Moebius-Goldammer, K. G., Mata-Rosas, M. and Chávez-Avila, V. M. 2003. Organogenesis and somatic embryogenesis in *Ariocarpus kotschoubeyanus* (Lem.) K. Schum. (Cactaceae), an endemic and endangered Mexican species. In Vitro Cell. Dev. Biol. Plant. 39: 388-393.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473–497.

Pérez-Molphe-Balch E,, Pérez-Reyes, M. E., Villalobos-Amador, E., Meza-Rangel, E., Morones-Ruiz, L.R., Lizalde-Viramontes, H. 1998. Micropropagation of 21 species of Mexican Cacti by axillary proliferation. In Vitro Cell. Dev. Biol. Plant 34:131–135.

Pérez-Molphe-Balch, E., Dávila-Figueroa, C.A. 2002. *In vitro* propagation of *Pelecyphora aselliformis* Ehrenberg and *P. strobiliformis* Werdermann (Cactaceae). In Vitro Cell. Dev. Biol. Plant 38:73–78.

Pinheiro da Costa, S., Soares, A.A., Arnholdt-Schmitt, B. 2001. Studies on the induction of embryogenic globular structures in *Opuntia ficus-indica*. Journal of the Professional Association for Cactus Development 4: 66-74.

Wyka, P.T., Hamerska, M. and Wrablewska, M. 2006. Organogenesis of vegetative shoots from *in vitro* cultured flower buds of *Mammillaria albicoma* (Cactaceae). Plant Cell, Tissue and Organ Culture 87: 27-32.

Ziv, M. 1991. Vitrification: morphological and physiological disorders of *in vitro* plants. In: Debergh, P.C. and Zimmerman, R.H. (Eds.). Micropropagation Technology and application. Dordrecht: Kluwer Academic Publisher. pp. 45–6.