



Mass micropropagation and in vitro flowering of *Mammillaria vetula* ssp. *gracilis* var. *Arizonica* Snowcap

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Abstract

A mass micropropagation method was developed for *Mammillaria vetula* ssp. *gracilis* (Pfeiff.) var. *Arizonica* cv. Snowcap, a cactus species with high ornamental value. A culture media based on MS medium (Murashige and Skoog, 1962) supplemented with 0.1 mg l⁻¹ NAA + 0.3 mg l⁻¹ KIN + 5.0 mg l⁻¹ GA₃ + 30 g l⁻¹ sucrose (MSM-1), was developed specifically for the establishment and multiplication of *Mammillaria* cv. Snowcap offshoots, let us to produce a proliferation rate of 34.9 ± 5.9 new offshoots per explant in 6–7 weeks. The non-rooted offshoots were rooted in MSM-2, consisting in MS salts supplemented with 0.1 mg l⁻¹ NAA + 0.3 mg l⁻¹ KIN plus 5 mg l⁻¹ ancymidol and a lower dose (10 g l⁻¹) of sucrose, with a rooting rate of 100%. The rate of acclimatization of the rooted offshoots was 100%. The full process of micropropagation, from aseptic establishment in vitro to the end of plant acclimation, takes approximately between 5 and 6 months and the plant production can be maintained continuously during all year. The in vitro flower induction was successful (66%) when in vitro plantlets of cv. Snowcap, previously under starvation and vernalization, were pretreated by changing the photoperiod progressively from short/day to long/day conditions during four months, and then incubated during 1 month in MS supplemented with Paclobutrazol 1 mg l⁻¹. Flowers started to bloom when plantlets were moved to fresh MSM-1.

Key message

The first complete method for in vitro mass micropropagation and in vitro flower induction has been developed for *Mammillaria vetula* ssp. *gracilis* var. *Arizonica* Snowcap, an economically important ornamental cactus species.

Keywords Cactus · Flower induction · Ornamental · Propagation in vitro · Regeneration · Rooting

Abbreviations

ANC	Ancymidol, α -cyclopropyl- α -(4-methoxyphenyl)-5-pyrimidylmethanol
AS	6-Aminopurine hemisulfate
BA	Benzylaminopurine
GA ₃	Gibberellic acid

IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
KIN	6-Furfurylaminopurine
MS	Murashige and Skoog medium
NAA	1-Naphthaleneacetic acid
PAR	Photosynthetic active radiation
pCPA	Para-chlorophenylalanine
PGR	Plant growth regulator
PGRT	Plant growth retardant

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Introduction

The Cactaceae family is native from America and it comprises more than 2000 species. More than 600 species are from Mexico, mainly from the Queretaro-Hidalgo Arid Zone (Gomez-Hinostrosa et al. 2017; Pérez-Molphe-Balch et al. 2015). *Mammillaria* is the largest genus in Cactaceae family and it has been largely studied (Vyskot and Jára 1984;

Rubluo 1997; Rubluo et al. 2002; Ramirez-Malagon et al. 2007; Sanchez-Martinez et al. 2009; García-Rubio et al. 2010; Maruri-Aguilar et al. 2012; Balen et al. 2009, 2012; Soria-Campos et al. 2013; Lázaro-Castellanos et al. 2018; Song et al. 2021a; Bouzroud et al. 2022). A total of 392 species were described by Hunt (1992), and in the last update Guzman et al. (2003) catalogued a number of new subspecies.

The development of efficient methods of micropropagation in vitro of different species of cactus has become very important for the maintenance and preservation of many species of cactus, being these endangered because of their ornamental value and the over-collection due to the high demand of the markets and also by the degradation of their natural habitats (Lema-Rumińska and Kulus 2014; Pérez-Molphe-Balch et al. 2015; Bouzroud et al. 2022).

To date, the use of in vitro methods to propagate cacti, have been mostly developed to preserve endangered *Mammillaria* species, at risk of extinction, such as *M. mathildae* Kraehenb. & Krainz (García-Rubio and Malda-Barrera 2010); *M. bocasana* Poselg., *M. densispina* (J.M.Coult.) Orcutt, *M. orcutii* Boed. (Ramirez-Malagon et al. 2007); *M. schiedeana* Ehrenb. ex Schltdl. (Soria-Campos et al. 2013); *M. pectinifera* F.A.C.Weber (Giusti et al. 2002); *M. san-angelensis* (R.T.Craig) D.R.Hunt (Martinez-Vazquez and Rubluo 1989); *M. luethyi* G.S.Hinton (Escobedo et al. 2002).

Our target species, *Mammillaria vetula* ssp. *gracilis* var. *Arizonica* is a species of Cactaceae, endemic of the states of Hidalgo and Queretaro, Mexico, being the cv. Snowcap a monstrous form of *M. vetula* ssp. *gracilis* var. *Arizonica* which is characterized by densely-packed, separate clusters of white and short spines. This cultivar offsets generously from the sides and the upper part of the plant, which gives it a snowball appearance. Mature plants can reach 10–12 cm in diameter and 6–8 (10) cm in height. The flowers are small, about 12 mm long in diameter and cream-yellow color, with a pinkish or brownish mid stripe in early spring. It is used as a very popular ornamental cactus on high demand for international markets.

There are only a few previous studies about *M. gracilis* (Poljuha et al. 2003; Balen et al. 2003). Apart from talking about metabolism, they also noted that *M. gracilis* is suitable for micropropagation. To date, the only micropropagation method available for in vitro propagation of cv. Snowcap, is a micropropagation method through callus regeneration, developed by Balen et al. (2003).

There is little information about methods of in vitro flowering induction in cactus species: *Turbinicarpus valdeianus* (Song et al. 2021b). The only information about flowering induction corresponds to *ex vitro* studies in different cactus species: in *Rhipsalidopsis gaertneri* (K.Schum.) Lindling. (Boyle 1991, 1995a, 1995b; Boyle et al. 1994; Karle and

Boyle 1999; Rohwer and Heins 2007); in *Schlumbergera truncata* (Haw.) Moran (Ho et al. 1985; Larsen et al. 1998); in *Hylocereus* (A.Berger) Britton & Rose and *Selenicereus* (A.Berger) Britton & Rose (Khaimov and Mizrahi 2006; Khaimov-Armoza et al. 2012) and in *Opuntia ficus-indica* (L.) Mill. (Inglese et al. 1998; Aounallah et al. 2009; Arba et al. 2017). All these studies involve the application of treatments of vernalization/starvation (temperature, light, fertilizers, watering), together with changes in photoperiod trying to mimic the natural environmental conditions existing during the winter/spring months, that induce *Mammillaria* sp. flowering in nature. In *Mammillaria* sp. flowering takes place after a winter period of cold weather and short days, normally during spring or early summer, with higher temperatures and gradually longer days.

We cannot find any report about in vitro flower induction in *Mammillaria* cv. Snowcap or any other species of *Mammillaria*. In commercial plantations *ex vitro* of this species, natural flowering occurs at random in an uncontrolled way, and as far as we know there is a lack of methods for flower induction in *Mammillaria* species.

In this work, we have developed an innovative mass micropropagation method, consisting on micropropagation of offshoot explants through direct axillary branching and rooting, avoiding an intermediate step of callus induction and regeneration, and the inherent problems of genetic variation of this kind of methods, that can produce severe morphological changes and progenies out of type. From what we can tell, we have developed for the first time a method for in vitro flower induction in a *Mammillaria* species (cv. Snowcap).

Material and methods

Plant material and disinfection

Young offshoots (0.5–1 cm diameter) of *Mammillaria vetula* ssp. *gracilis* var. *Arizonica* cv. Snowcap, provided by Solis-plant Nurseries (El Ejido, Almería, Spain), were used as explants for in vitro aseptic establishment.

For the disinfection process, a total of 100 initial samples were used. These explants were isolated, cleaned and disinfected to obtain the primary explants for micropropagation as follows: after the isolation of the mother plant, the explants consisting in a small cactus specimen (young offshoots) were carefully washed with a soap solution and tap water and then treated with fungicide (Thiophanate-methyl 0.5%) for 20 min, under shaking, and then the explants were rinsed several times in sterile distilled water to eliminate the fungicide. Later, the explants were disinfected in a 1% povidone-iodine solution for 20 min, under vacuum conditions, and rinsed three times in aseptic conditions with

sterile distilled water. The last step consisted in the establishment in vitro of the disinfected primary explants in a basic medium consisting in MS medium (Murashige and Skoog 1962) supplemented with 100 mg l⁻¹ i-inositol, 3% sucrose and 0.8% of Agar A-1296 (Sigma), and 200 mg l⁻¹ cefotaxime.

Offshoots establishment, growth and development

Culture media and culture conditions

For growth and development, disinfected explants were incubated in a culture room on MS medium as indicated above, without Cefotaxime, and after the aseptic establishment we increased the number of offshoots in vitro (explants) through preliminary assays during several months, until we reached a very big amount (> 3000) of offshoots, because they were necessary to carry out all the tests of proliferation detailed below.

Different concentrations of cytokinins and auxins were tested: KIN (0, 0.1, 0.3, 0.4, 0.5, 0.6, 1.0) mg l⁻¹; BAP (0, 0.1, 0.3, 0.5, 1.0) mg l⁻¹; Adenine hemisulfate (0, 10, 20, 30, 40) mg l⁻¹ and various levels of NAA (0, 0.1, 0.4, 0.5, 0.6), IBA, IAA, (0, 0.1, 0.5, both) mg l⁻¹; also multiple combinations of cytokinins and auxins were tested to select the best possible combination. Data on length of explants, rooting %, number and length of roots and callus %, were scored.

Later, when the optimal combination of auxins plus cytokinins was established for the adequate growth of the offshoots, gibberellic acid at different doses (0, 1, 5, 10 mg l⁻¹), was applied in pulses for 3 days; for this, offshoots were incubated during 3 days in the optimal gelled growth medium plus the gibberellic acid, and its effects on multiplication were evaluated, after a new incubation during 6 weeks in optimal gelled growth medium without GA₃. Data on the number of offshoots per explant, % of explants generating offshoots and % of callus induction were scored.

After the selection of the best combination of PGRs, different levels of sucrose (0, 10, 20, 30, 40, 50, 60) g l⁻¹, and the growth retardant Ancymidol (0, 1.0, 2.0, 3.0, 4.0, 5.0) mg l⁻¹ were tested to evaluate growth, rooting rate and callus proliferation. Data on the length of offshoots, rooting percentage, number of roots and callus percentage were scored.

After the initial tests of micropropagation, the results allow us to select three different media adequate for different purposes (Table 1): MSM-1 (*Mammillaria* Snowcap Medium-1, for offshoots growth and maintenance), MSM-2 (*Mammillaria* Snowcap Medium-2, for offshoots induction and proliferation) and MSM-3 (*Mammillaria* Snowcap Medium-3, for rooting).

The pH of all the culture media was adjusted to 5.7 before autoclaving. For culture initiation, multiplication, rooting and flowering experiments, 75 ml of medium per

jar were aliquoted into 120 ml culture jars covered with Magenta caps, and autoclaved for 20 min at 121 °C and 1.05 kg cm⁻². For micropropagation, all cultures were incubated at 25 ± 1 °C under a 16/8 photoperiod under cool white fluorescent tubes (F40 tubes Gro-lux, Sylvania) with 45 µmol m⁻² s⁻¹ (400–700 nm) Photosynthetic Active Radiation (PAR), and for in vitro flowering induction explants were incubated at 450 µmol m⁻² s⁻¹ (400–700 nm) PAR, under different photoperiods (8/16, 12/12 and 16/8). These growing photoperiods were applied together with conditions of starvation and vernalisation, consisting in incubating the explants in media depleted of nutrient during two months combined with daily periods of incubation in dark conditions (overnight) at low temperatures (4–6 °C) followed by incubation at 25 °C in light conditions.

Offshoots induction, multiplication, rooting

Large specimens, obtained in preliminary experiments, and maintained in active growth in MSM-1 during 3 months, are the best source of material used to produce offshoots, after incubating them in MSM-2. It is necessary to use explants large enough (3–4 cm in length and at least 1 cm in width) to be able to obtain the average number of offshoots recorded in this article, and besides, after the harvest of the offshoots, the explants can produce a new batch of offshoots in approximately two months. After the subculture of large explants in MSM-2, it was necessary to wait for three weeks until offshoots' sprouting took place, and 3–4 additional weeks until the offshoots generated were large enough (0.3–0.4 cm in length) to be harvested. The typical pattern of growth of the offshoots consists in a general sprouting of axillary buds covering the surface of the mother plant like a helmet. (Fig. 2).

After development, individual offshoots were easily separated mechanically from the mother plant and then subcultured in fresh medium (MSM-1) as single secondary explants. For subculturing to fresh medium big sized plantlets, sometimes it was necessary to dissect the basal part of the plants, eliminating damaged tissue and the callus surrounding the bottom of the plant, in order to maintain the quality of the plantlets obtained.

In our method of micropropagation, offshoots are established and incubated in a MSM-3 for growth and rooting for 8–12 weeks. Rooting occurs between the third and the fourth weeks. Usually, one or two roots appear from the bottom of the offshoot with a width of 0.1 cm and a length of approximately 1 cm. When the explant grows, roots also grow and proliferate, reaching up 7–9 cm long in our containers (Fig. 4). After 6–8, weeks the percentage of survival, rooting, shoot and root number and length were recorded.

Acclimation of snowcap plantlets

Plantlets with well-developed roots were thoroughly washed in tap water and transplanted to polyethylene trays with 4 × 4 cm alveoli containing a mixture of autoclaved sand:peat:perlite (2:1:1). Potted plantlets were maintained for one month in a polyethylene tunnel with 80% relative humidity. The temperature inside the tunnel ranged between 19 °C and 30 °C, with an average temperature of 25 °C. Plants were transferred to 9 cm diameter pots containing the same substrate, and maintained at 60% relative humidity for another month, before a final transplantation into 12 cm diameter pots containing peat:sand:soil (1:1:1) mixture, where they were maintained for two more months at 50% relative humidity. During the experiment, plants were lightly watered and fertilized periodically. Plants grew under 80% shade. After four months in the glasshouse, data on survival rate was recorded.

In vitro flowering induction

Plant material, culture media and culture conditions

The explants for flowering induction were selected by size, and for experimental purposes only big size explants (BSZ, 4–6 cm long) were considered. For *Mammillaria* cv Snowcap flowering induction we tested the effect of different growth retardants such as Ancymidol and Paclobutrazol at different doses (0, 0.5, 1, 2, 4 mg l⁻¹) over a basal medium MSFM (*Mammillaria* Snowcap Flowering Medium) consisting in MS supplemented with KIN (0.2 mg l⁻¹), NAA (0.2 mg l⁻¹) and 3% sucrose.

Treatments of starvation/vernalisation plus a sequence of photoperiod consisting in: short day conditions (8/16 h for 3 months), followed by medium day conditions (12/12 h, for 1 month) and long day conditions (16/8 h for 2 months) were also assayed to produce flowers in Snowcap, using medium size explants (MSZ) or big size explants (BSZ).

We also applied treatments with Plant Growth Retardants (PGRT) such as ancymidol (0, 0.3, 0.5, 1, 3, 5 mg l⁻¹) and Paclobutrazol (0, 0.3, 0.5, 1, 3, 5 mg l⁻¹) after the treatment of starvation/vernalisation/photoperiod.

Data on percentage of flowering, and number of flowers per explant were recorded after 8 weeks of incubation in MSFM.

Statistical analysis

In all the experiments, 35 explants were used per treatment and the experiments were repeated three times.

All data were analyzed using SPSS software package (version 19.0; SPSS INC., Chicago, IL, USA). The percentage of rooting was analyzed by Generalized Linear Models using Logit as the link function and Binomial as the probability distribution. The

number of roots and flowers, plant length and root length were analyzed by one-way ANOVA, using a HSD-Tukey test in the post-hoc analysis for comparisons between the different sucrose concentrations and different culture media.

Pairwise comparisons among groups were performed by LSD Fisher's least significant difference (LSD) test.

Results

Disinfection

The rate of disinfection obtained using a refined method reach 80%, and the 20% of resilient contamination appears to be due to some endogenous bacteria.

Explant growth, offshoots induction and rooting

Cytokinins

Results on offshoots induction are poor with cytokinins treatments, less than 10% of primary explants generated a small number of offshoots after two months of incubation with BA, KIN and AS.

Benzylaminopurine induces an important increase in the size of the explants during the incubation period, being the explants incubated in BA significantly bigger than the explants incubated in KIN and AS (Fig. 1). The treatment with BA induced rooting in a 40% of explants, but stimulating the development of a big amount of callus and abnormally swollen tissue. Therefore, the use of BA has been discarded for cv. Snowcap micropropagation.

Rooting rates obtained with KIN treatments are similar (34%) to the obtained with BA, but producing a minimal amount of callus and an acceptable growth of the explants, allowing to obtain good quality plantlets. Adenine hemisulfate (AS) also induced some amount of callus and showed a good growth of the primary explants, but with a low rate of rooting (18%), lower than kinetin and BA. Therefore, AS was discarded for further uses (Table 2).

The best values on shoot growth, rooting rate, together with the lack of callus growth were obtained for KIN (0.3 mg l⁻¹) and the nearest values of KIN tested (0.1 and 0.5 mg l⁻¹), and these doses were used to carry out the next experiments involving the combination of auxins and cytokinins.

Auxins

Table 3 shows the effect of NAA, IAA and IBA (0, 0.1, 0.3 and 0.5 mg l⁻¹) on the growth, rooting and callus induction

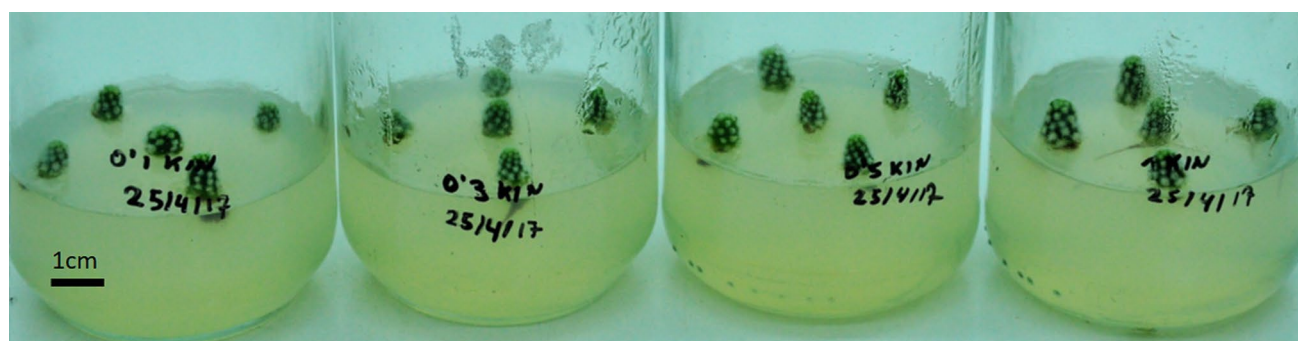


Fig. 1 Effect of KIN (0.1, 0.3, 0.5, 1 mg l⁻¹; from left to right). on the growth, proliferation and rooting

Table 1 Composition of the three culture medium selected for different purposes during the micropropagation of *Mammillaria vetula* ssp. *gracilis* var. *Arizona* Snowcap: MSM-1 (*Mammillaria* Snowcap

Medium-1), MSM-2 (*Mammillaria* Snowcap Medium-2) and MSM-3 (*Mammillaria* Snowcap Medium-3)

Media*	Growth regulators (mg l ⁻¹)				Sucrose	Agar	Purposes
	NAA	KIN	GA ₃	ANC			
MSM-1	0.5	0.3	—	—	2%	0.8%	Offshoots growth and maintenance
MSM-2	0.5	0.3	5	—	2%	0.8%	Offshoots induction and proliferation
MSM-3	0.5	0.3	—	5	1%	0.8%	Offshoots rooting

*All media containing MS salts and vitamins (Murashige and Skoog 1962)

Table 2 Effects of different doses of BA, KIN and AS on the growth, proliferation and rooting of offshoot explants of *M. cv. Snowcap*

Media	PGR (mg l ⁻¹)	Length of primary explants (cm)	Rooting %	Callus induction (%)
BA	0	0.2 ± 0.1c	2 ± 1c	0 ± 0c
	0.1	0.7 ± 0.3b	0 ± 0c	18 ± 2b
	0.3	1.0 ± 0.4a	40 ± 3a	32 ± 2a
	0.5	1.1 ± 0.4a	18 ± 2b	25 ± 7a
	1	0.9 ± 0.2b	12 ± 9b	26 ± 8a
KIN	0	0.1 ± 0.1c	1 ± 1c	0 ± 0c
	0.1	0.7 ± 0.3b	32 ± 7a	0 ± 0c
	0.3	0.8 ± 0.2b	34 ± 5a	0 ± 0c
	0.5	0.8 ± 0.2b	32 ± 4a	6 ± 0c
	1	0.9 ± 0.2b	40 ± 9a	16 ± 1b
AS	0	0.1 ± 0.1c	2 ± 1c	2 ± 1c
	10	1.2 ± 0.4a	0 ± 0c	14 ± 5b
	20	1.1 ± 0.3a	0 ± 0c	14 ± 6b
	30	1.2 ± 0.5a	12 ± 6b	18 ± 2b
	40	1.1 ± 0.2a	18 ± 1b	18 ± 1b

Different letters indicate significant differences by the HSD-Tukey test at $\alpha=0.05$ in each parameter between different culture media

BA benzylaminopurine, KIN 6-furfurylaminopurine, AS 6-aminopurine hemisulfate

in *Mammillaria vetula* cv. Snowcap. No significant differences were detected on primary explant growth among the auxins assayed. All the auxins tested show a low level of callus proliferation (3–24%) and, less than 10% of primary explants generated a minimal number of offshoots after two months of incubation with these auxins.

The best rooting rates (73, 54 and 49%, depending on the concentration), correspond to IBA treatments. The results on growth of the primary explant do not show significant differences with respect to the treatments with the other auxins, but the level of callus proliferation is too high for the treatments with IBA and IAA showing better results on rooting. Otherwise, the treatments with NAA showed very low levels of callus proliferation with medium levels of rooting (37, 40%).

Auxins + cytokinins

Table 4 shows the effects of the test combining three doses of KIN (0.1, 0.3, 0.5 mg l⁻¹) with NAA (0.3, 0.5 mg l⁻¹), IAA (0.5 mg l⁻¹) and IBA (0.1 mg l⁻¹) on the growth, rooting and callus induction.

Unfortunately, the combination of these auxins and cytokinins only promote the development of a small number of offshoots in 10–12% of primary explants, as happen when

Table 3 Effects of NAA, IBA and IAA on growth, rooting and callus induction after 8 weeks of incubation

Auxins	(mg l ⁻¹)	Length of explant (cm)	Rooting (%)	No. of roots	Length of roots (cm)	Callus (%)
NAA	0	1.0±0.2a	11±3d	0.0±0.0d	0.2±0.1c	5±3b
	0.1	1.2±0.1a	30±8bc	1.1±0.1c	0.5±0.2bc	4±3b
	0.3	1.2±0.2a	37±6bc	1.7±0.3b	0.5±0.3bc	3±2b
	0.5	1.0±0.1a	40±9bc	2.0±0.1b	0.5±0.2bc	6±1b
IAA	0	1.0±0.1a	6±2d	0.2±0.1d	0.3±0.2c	6±3b
	0.1	1.0±0.1a	27±8c	1.0±0.0c	0.2±0.2c	14±4a
	0.3	1.2±0.2a	32±6bc	1.2±0.2c	0.3±0.1c	6±4b
	0.5	1.1±0.1a	53±9ab	1.9±0.1b	0.7±0.4ab	20±2a
IBA	0	1.0±0.1a	10±5d	0.2±0.2d	0.1±0.1a	4±4b
	0.1	1.1±0.1a	73±8a	2.9±0.4a	0.8±0.6a	16±2a
	0.3	1.1±0.2a	49±7ab	2.5±0.4a	0.7±0.4ab	21±4a
	0.5	1.1±0.1a	54±9ab	2.0±0.2b	0.6±0.1ab	24±4a

Different letters indicate significant differences by the HSD-Tukey test at $\alpha=0.05$ in each parameter between different culture media

NAA 1-naphthaleneacetic acid, IAA indole-3-acetic acid, IBA indole-3-butyric acid

Table 4 Effects of KIN combined with NAA, IBA and IAA on the growth, rooting and callus induction after 8 weeks of incubation

Cytokinins + (mg l ⁻¹)	Auxins (mg l ⁻¹)	Length of explant (cm)	Rooting (%)	No. of roots	Length of roots (cm)	Callus (%)
KIN 0	0 NAA	1.0±0.2b	11±3d	0.0±0.0d	0.2±0.1c	5±3c
	0.3 NAA	1.2±0.2ab	37±6bc	1.7±0.3b	0.5±0.3bc	3±2c
	0.5 NAA	1.0±0.1b	40±9bc	2.0±0.1b	0.5±0.2bc	6±1c
	0.5 IAA	1.1±0.1b	53±9ab	1.9±0.1b	0.7±0.4ab	20±2ab
	0.1 IBA	1.1±0.1b	73±8a	2.9±0.4a	0.8±0.6a	16±2b
KIN 0.1	0 NAA	0.7±0.3b	32±7d	0.0±0.0d	0.2±0.1c	0±0c
	0.3 NAA	0.9±0.1b	38±2d	1.3±0.1c	0.6±0.2b	3±3c
	0.5 NAA	1.1±0.1b	46±4c	1.3±0.1c	0.5±0.2 b	6±1c
	0.5 IAA	0.7±0.1a	43±6c	1.7±0.3b	0.4±0.4b	24±2a
	0.1 IBA	0.9±0.1b	45±2c	2.3±0.4b	0.9±0.6a	26±2a
KIN 0.3	0 NAA	0.9±0.2b	34±5d	0.2±0.1d	0.3±0.2c	0±0c
	0.3 NAA	1.2±0.6ab	40±3c	1.9±0.4b	0.6±0.2b	1±0c
	0.5 NAA	1.8±0.5a	57±4b	1.8±0.5b	0.7±0.3b	2±0c
	0.5 IAA	1.0±0.1b	43±1c	1.3±0.1c	0.4±0.4b	32±2a
	0.1 IBA	0.9±0.1b	50±2b	2.2±0.4b	1.2±0.6a	28±2a
KIN 0.5	0 NAA	0.8±0.3b	32±4d	0.2±0.2d	0.1±0.1c	5±2c
	0.3 NAA	1.1±0.4b	29±6d	1.7±0.5b	0.6±0.2b	3±2c
	0.5 NAA	1.3±0.4ab	55±3b	2.1±0.3b	0.8±0.4a	8±4c
	0.5 IAA	1.0±0.1b	43±3c	2.0±0.1b	0.3±0.4c	25±6a
	0.1 IBA	1.0±0.1b	51±3b	3.5±0.4a	1.1±0.6a	32±9a
Cytokinins + (mg l ⁻¹)	Auxins (mg l ⁻¹)	Length of explant (cm)	Rooting (%)	No. of roots	Length of roots (cm)	Callus (%)
	0 NAA					
	0.3 NAA					
	0.5 NAA					
	0.5 IAA					
	0.1 IBA					

Different letters indicate significant differences by the HSD-Tukey test at $\alpha=0.05$ in each parameter between different culture media

KIN 6-furfurylaminopurine

Table 5 Effects of GA₃ treatments on offshoots induction and development

GA ₃ (m l ⁻¹)	No. of offshoots per explant	Explants generating offshoots (%)	Callus induction (%)
0	5.3 ± 2.4c	4 ± 2c	12 ± 3a
1	9.7 ± 6.4b	40 ± 2c	14 ± 7a
5	34.9 ± 5.9a	100 ± 0a	10 ± 2a
10	13.3 ± 5.6b	100 ± 0a	16 ± 7a

Different letters indicate significant differences by HSD-Tukey at $\alpha=0.05$ in each parameter between different culture media

GA₃ gibberellic acid

these auxins and cytokinins were included alone in the medium.

The higher values on callus development correspond to combinations of KIN plus IAA and IBA, as well as in the number of roots and root length, but the better results in explant growth together with lower percentages of callus growth and with acceptable rooting rates pointed to a combination of 0.3 mg l⁻¹ KIN with 0.5 mg l⁻¹ NAA as the most suitable combination.

Gibberellic acid

The incubation of big explants of *Mammillaria* cv Snowcap in the medium MSM-1 supplemented with 5 mg l⁻¹ GA₃ during three days, induce the sprouting and growth of offshoots in a 100% of explants, generating a great amount of new offshoots from areoles, obtaining from 28 to 46 offshoots per explant after 8 weeks of incubation (Table 5). The average production of offshoots per explant was 34.9 ± 5.9 (Fig. 2). With 10 mg l⁻¹ GA₃, also 100% of explants generated offshoots but in a lower number than on the treatment with 5 mg l⁻¹ GA₃. Once the offshoots are removed from the mother explant, new offshoots regrow. The medium

Table 6 Effects of different levels of sucrose on the growth, rooting and callus induction

Sucrose (mg l ⁻¹)	Length of offshoots (cm)	Rooting (%)	No. of roots	Callus (%)
0	1.8 ± 0.1a	43 ± 9ab	3.0 ± 0.2b	10 ± 9b
10	1.8 ± 0.1a	53 ± 9a	4.2 ± 0.2a	12 ± 7b
20	1.6 ± 0.1a	23 ± 8bc	1.3 ± 0.1c	8 ± 7b
30	1.8 ± 0.1a	20 ± 7c	1.2 ± 0.2c	14 ± 5b
40	1.5 ± 0.1a	17 ± 7c	1.5 ± 0.2c	8 ± 8b
50	1.5 ± 0.1a	20 ± 7c	1.2 ± 0.1c	12 ± 9b
60	1.8 ± 0.1a	13 ± 6c	1.7 ± 0.2c	22 ± 3a

Different letters indicate significant differences by the HSD-Tukey test at $\alpha=0.05$ in each parameter between different levels of sucrose. Different letters indicate groups that were significantly different by LSD at $\alpha=0.05$

(MSM-2) for offshoot induction consists of MSM-1 medium plus 5 mg l⁻¹ GA₃ applied for 3 days (Table 5).

Auxins

The best percentages of rooting correspond to IBA treatments, (0.1 mg l⁻¹, 73%) but in cv. Snowcap, for mass proliferation, this rooting rate is insufficient. We have developed another rooting method involving a treatment with ancymidol (5 mg l⁻¹) with perfect rooting rate (100%). For this reason, we discarded the use of auxins for Snowcap rooting.

Sucrose

The best rooting results are produced with 1% sucrose, reaching 53% of good quality rooted explants (Table 6, Fig. 3). A 43% rooting rate was recorded without sucrose in the medium (Table 6), but the explants showed an abnormal color and poor quality. Between 1 and 4% sucrose, no abnormal tissue was found, but for higher doses than 4%, explants began to dry out and eventually died. No significant

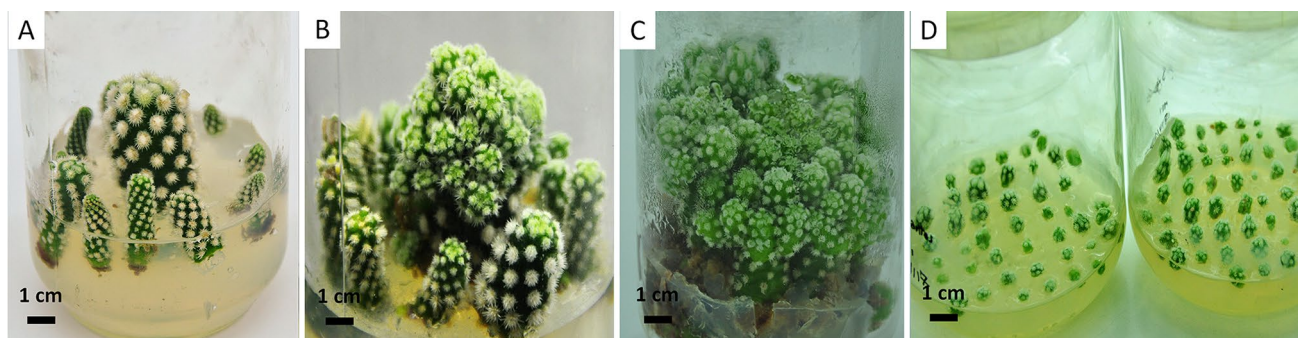


Fig. 2 **A** Initiation of multiplication of *M. cv. Snowcap* explants in MSM-2. **B** Offshoots growth. **C** Development of offshoots. **D** Total offshoots production corresponding to two adult explants

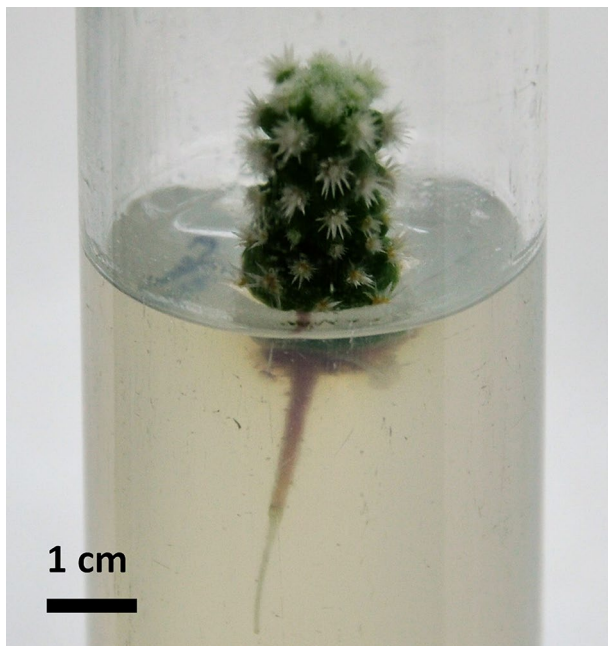


Fig. 3 Rooted explant of *M. cv. Snowcap* in a culture medium with a low sucrose level (1%)

differences on offshoot growth were recorded during the treatments, and for callus proliferation only the higher value of sucrose showed significant differences with respect to the rest of the concentrations assayed.

Ancymidol

Best rooting results were recorded in MSM-3 supplemented with Ancymidol (5 mg l^{-1}) reaching a rooting rate of 100% (Fig. 4). This protocol has been tested and validated for three different sizes of explants: 1, 3 and 6 cm in length, always with a 100% rate of success. Callus proliferation detected was low (Table 7).

Acclimatization

After two months of acclimatization on mini-tunnels at a glasshouse, the survival rate of rooted Snowcap plantlets was 100%, and the acclimated plantlets developed without problems.

Flower induction

Starvation/vernalization treatments

Our preliminary flowering assays involving plant starvation of groups of big plantlets (BSZ) of *Mammillaria cv. Snowcap* are useless for in vitro flowering induction, even

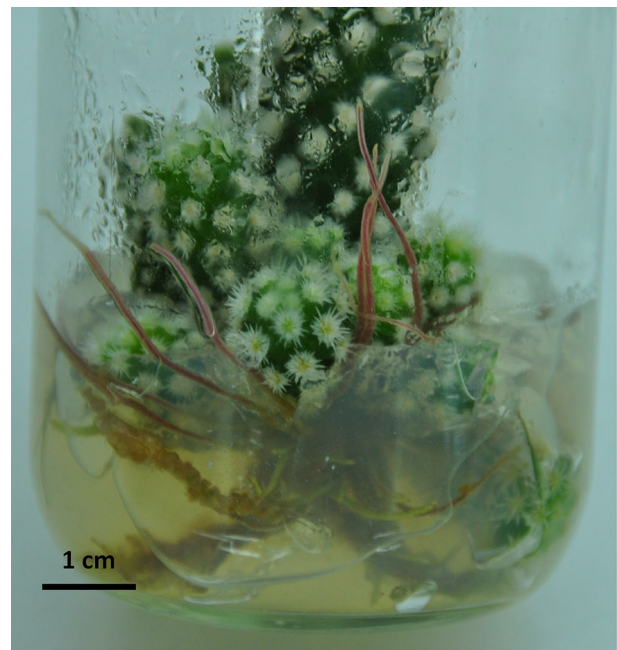


Fig. 4 Effect of ancymidol on rooting of explants

Table 7 Effects of different doses of ancymidol on the growth, rooting and callus induction

Ancymidol (mg l^{-1})	Length of offshoots (cm)	Rooting (%)	No. of roots	Callus (%)
0	$1.9 \pm 0.4a$	$40 \pm 6c$	$1.3 \pm 0.2b$	$10 \pm 4a$
1	$1.5 \pm 0.2b$	$70 \pm 8b$	$1.2 \pm 0.2b$	$14 \pm 3a$
2	$1.6 \pm 0.2b$	$70 \pm 5b$	$1.4 \pm 0.1b$	$14 \pm 4a$
3	$1.5 \pm 0.2b$	$77 \pm 1b$	$1.4 \pm 0.2b$	$12 \pm 5a$
4	$1.5 \pm 0.3b$	$92 \pm 4a$	$1.9 \pm 0.2a$	$10 \pm 8a$
5	$1.5 \pm 0.3b$	$100 \pm 0a$	$2.1 \pm 0.1a$	$10 \pm 7a$

Different letters indicate significant differences by the HSD-Tukey test at $\alpha=0.05$ in each parameter between different culture media

when starvation treatments were combined with vernalization treatments (daily cold treatments in the dark, at $4\text{--}6^\circ\text{C}$), overnight, during 3 months. Plantlets never developed flowers.

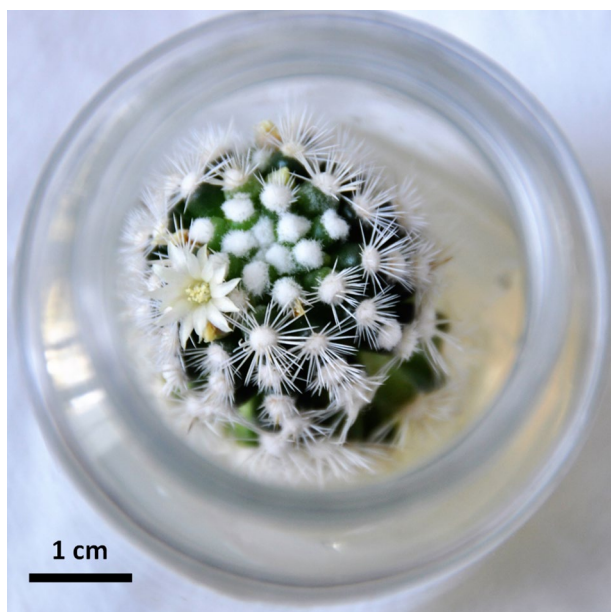
Photoperiod treatments

The treatments of starvation/vernalization plus a sequence of photoperiod combining short day conditions (8/16 h for 3 months), followed by medium day conditions (12/12 h, for 1 month) and long day conditions (16/8 h for 4 months) never produce flowers in Snowcap in medium size explants (MSZ) or big size explants (BSZ).

Table 8 Effects of different concentrations of Paclobutrazol, after 6 weeks, on the in vitro flowering induction of *Mammillaria* cv. Snowcap

Paclobutrazol (mg l ⁻¹)	Number offshoots	Flower initiation (%)	Number of flowers
0	0.11 ± 0.04b	0 ± 0d	0 ± 0c
0.3	0.21 ± 0.06b	0 ± 0d	0 ± 0c
0.5	0.24 ± 0.07b	0 ± 0d	0 ± 0c
1	0.26 ± 0.07b	66 ± 6a	1.40 ± 0.16a
3	0.77 ± 0.09a	21 ± 5b	0.37 ± 0.10b
5	1.00 ± 0.10a	11 ± 4c	0.24 ± 0.09b

Different letters indicate significant differences by the HSD-Tukey test at $\alpha=0.05$ in the number of offshoots and flowers between different paclobutrazol concentrations. Different letters indicate groups that were significantly different by LSD at $\alpha=0.05$ in flower initiation rate between different paclobutrazol concentrations

**Fig. 5** In vitro flower induction of *Mammillaria* cv. Snowcap

Growth retardants treatments

The treatments with Ancymidol are useless for flowering induction, but the treatments with Paclobutrazol are able to induce flowering in BSZ explants of *Mammillaria* cv. Snowcap, after the treatments of starvation/vernalization/photoperiod indicated above. Our best result in flower induction (66%) was obtained from the treatment of 1 mg l⁻¹ paclobutrazol. Higher doses also induce flowering but in lower percentages, as paclobutrazol doses increase (Table 8, Fig. 5).

Discussion

Previous works had been done on *Mammillaria vetula* ssp. *gracilis* that were focused on basic studies on microscopy and on micropropagation by callus regeneration, working with *Mammillaria pectinifera*, *Pelecypora aselliformis* Ehrenberg (Giusti et al. 2002), *Cereus peruvianus* Mill. (De Oliveira et al. 1995), *M. mathildae* (García-Rubio and Malda-Barrera 2010), paying limited attention to micropropagation by direct organogenesis (Balén et al. 2003; Poljuha et al. 2003).

Another alternative method of micropropagation consists in obtaining plants through direct organogenesis from different plant sections; such as shoot tips, bases and longitudinal sections of cactus (Papafotiou et al. 2001; García-Rubio and Malda-Barrera 2010). It is well-known that in *Mammillaria* and other Cactaceae, cytokinins, such as BAP or KIN, are able to induce the development of lateral shoot primordia (*M. mathildae*, García-Rubio and Malda-Barrera 2010; *M. san-angelensis*, Martínez-Vázquez and Rubluo 1989) through direct organogenesis. Therefore, the combination of cytokinins plus auxins has been also used to promote the growth of lateral offshoots in these species (*M. san-angelensis*, Martínez-Vázquez and Rubluo 1989; *Mammillaria wrightii*, Engelm, Clayton et al. 1990; *M. elongata* DC, Papafotiou et al. 2001, *M. candida* (Scheidt) Britton & Rose (Elías-Rocha et al. 1998). For *Mammillaria* cv. Snowcap the combination of auxins (NAA) and cytokinins (KIN) is able to maintain alive and promote the vegetative growth of explants (small offshoots), but it is useless for inducing offshoots, probably due to the strong apical dominance of the apical meristem and the deep dormancy of axillary offshoots.

It is important to remark that contrasting with the standard types of explants used in other methods of micropropagation of *Mammillaria*, (generally germinating seeds or pieces of plants, as Perez-Molphe-Balch et al. (1998) reported for six *Mammillaria* species, we always used small whole plants (offshoots) of cv Snowcap as primary explants, and treated them with auxins and cytokinins to break apical dominance and activate the growth of the axillary buds located in the areoles, as indicated for Vyskot and Jara (1984) working with shoot pieces as explants in other *Mammillaria* species, but unsuccessfully.

Working with *M. vetula* ssp. *gracilis* cv Snowcap, we have developed an efficient method for mass micropropagation through direct axillary sprouting of cactus offshoots, using plant growth regulators. This innovative method avoids callus development or regeneration of plants from callus, reducing the possibilities of somaclonal variation at the end of the micropropagation process.

Offshoots induction and proliferation

Gibberellic acid

Gibberellic acid combined with NAA and KIN, is the only PGR that has been able to activate the full sprouting of the axillary buds existing in the areoles of *Mammillaria* cv. Snowcap for all treatments tested, inducing mass proliferation, and reaching in two of the three treatments assayed up to 100% of explants generating offshoots, obtaining a maximum number of 34.9 ± 5.9 offshoots per explant. That agrees with Giusti et al. (2002), who confirmed that different cactus species respond very differently to different types of growth regulators and, most of the studied cacti species respond to cytokinins breaking apical dominance. Unfortunately, cv. Snowcap only broke apical dominance when subcultured in MSM-2 (MS medium supplemented with GA₃ plus NAA and KIN), instead of just cytokinins, in agreement with Mauseth (1976), who reported that cytokinins and GA₃ are able to deactivate the apical meristem and cause the continual initiation of lateral primordia in *Opuntia polyacantha* Haw. We had also used GA₃ together with KIN and NAA in *Mammillaria vetula* cv. Snowcap to induce the massive initiation of lateral primordia to produce offshoots, and an offshoot regrow of the initial explants happens after the harvesting of the first batch of new offshoots, something similar occurs in *Pelecyphora* sp. (Perez-Molphe-Balch and Davila-Figueroa 2002), both species are able to produce several batches of new offshoots when the initial explants were under successive cycles of proliferation. The strong effect over the offshoots' induction and development obtained by auxins, cytokinins and gibberellic acid working together, and the high rate of proliferation recorded, seems to indicate a synergetic effect of these PGRs in shoot organogenesis of *Mammillaria* cv. Snowcap.

Rooting induction

In vitro rooting had been generally induced by auxins in many *Mammillaria* species (92.5% for *M. pectinifera*, Giusti et al. 2002; 90% for *M. candida*, Perez-Molphe-Balch et al. 1998; 67% *M. elongata*, Papafiotiou et al. 2001; 97% *M. mathildae* Garcia-Rubio and Malda-Barrera 2010), *Mammillaria* sp. Ramirez-Malagon 2007, rooting data not provided). We reached 100% of rooted explants (small, medium and big size explants maintained in MSM-1) incubating these explants during 4–5 weeks in MSM-3, a medium supplemented with a low level of sucrose (1%) plus 5 mg l⁻¹ ancymidol, in agreement with positive effect on rooting induction obtained in other species such in *Aloe arborescens* Mill. (Velcheva et al. 2005), *Echeveria* sp DC (Encina, unpublished) and asparagus (Regalado et al 2015). This growth retardant, an inhibitor of gibberellin biosynthesis, is able to

reduce the growth of the aerial part of the plants and promote rooting in Snowcap plants, developing a good root system in each explant (Table 7, Fig. 4). This is an innovative method never described before in cactus micropropagation.

Dabekaussen et al. (1991) developed a study about the role of sucrose levels in micropropagation of cacti, concluding that the optimal levels were between 2.5 and 5.5% for *Sulcorebutia alba* Rausch, and after that, that range has been generally adopted in most cacti in vitro works (*M. bocasana* Poselg., *M. densispina*, *M. orcutii*, Ramirez-Malagon et al. 2007; *M. mathildae*, Garcia-Rubio and Malda-Barrera 2010; *M. schiedeana*, Soria-Campos et al. 2013). Sucrose levels of MSM-1 medium are lower than most of the previous cacti media. Our results show no significant differences in growth for *Mammillaria* cv. Snowcap between 1 and 3% of sucrose levels. This low sucrose level also offers an additional advantage on rooting rate (50% vs 20%) and an increase in the number of roots developed (4.2 vs 1.2) during the incubation process, so we selected 1% sucrose as the optimal level for the *Mammillaria* cv. Snowcap rooting medium (MSM-3). In fact, we have found that the treatment with 4% sucrose has a negative effect on growth, and the explants start to get dry, and the higher doses of sucrose tested (5% and 6%) have a lethal effect on the explants, which is in clear disagreement with Dabekaussen et al. (1991).

After the analysis of rooting treatments, it is clear that rooting can be induced by a wide range of growth regulators, but the rooting rate is always insufficient for mass micropropagation requirements, except for the treatment including 5 mg l⁻¹ of ancymidol (100%).

Flowering induction

Flowering assays *ex vitro* involving starvation and vernalization and natural changes in photoperiod result in positive flower induction in different cactus species such as: *Opuntia* spp. (Arba et al. 2017) and *Rhipsalidopsis gaertneri* (Boyle 1995a, b). In vitro flower bloom was successful in *Hatiora gaertneri* (Regel) Barthlott (Boyle and Marcotrigiano 1997), and in *Turbinicactus* (Backeb) Buxb. & Backeb. (Song et al. 2021b). In *Mammillaria vetula* cv. Snowcap, all the treatments assayed in vitro involving starvation/vernalization/photoperiod with a final incubation at high light intensity, alone or together failed to induce flowering directly in explants of medium size and big size.

In previous experiments, we have detected that a plant growth retardant, the ancymidol, has a big impact on rooting the explants of cv. Snowcap, so we tested this plant growth retardant at different doses trying to verify if the inhibition of the biosynthesis of gibberellins induced by Ancymidol was able to produce changes and to induce in vitro flowering, unsuccessfully. However, when we applied another PGRT, the Paclobutrazol, at different doses, it was able to

induce in vitro flowering in *Mammillaria* cv. Snowcap with an average rate of 66% of explants (Fig. 5). It seems that the combination of PGRs with Paclobutrazol could modify the balance of cytokinins/abscisic acid by inhibiting the biosynthetic pathway of gibberellins, resulting in the flower induction. More work is required to confirm this hypothesis.

As far as we know, it is the first time that a mass micropropagation method for *M. vetula* ssp. *gracilis* var. *Arizona* cv. Snowcap through direct organogenesis has been reported. It is also the first time that an innovative method for in vitro flower induction has been reported for a *Mammillaria* species.

Conclusion

We have developed a method for mass micropropagation through direct organogenesis of *M. vetula* ssp. *gracilis* var. *Arizona* cv. Snowcap that improves previous results for the same subspecies, allowing the commercial micropropagation in any season of *Mammillaria* cv. Snowcap, an ornamental cactus species on high demand in plant markets.

This reliable micropropagation system has been used routinely in our laboratory during five years without problems, and this system is also versatile and has been applied successfully to other *Mammillaria* species.

We have also developed for the first time a method for in vitro flower induction for a *Mammillaria* species (cv. Snowcap) combining treatments of starvation plus vernalization plus photoperiod with a Plant Growth Retardant (Paclobutrazol).

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Authors contributions JJR and CLE contributed to the study conception and design. Material preparation, data collection and analysis were performed by ML-G and AA. The manuscript was written by CLE and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript. CLE supervised and coordinated the work.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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