In Vitro Mass Micropropagation of *Mammillaria Vetula* ssp. *Gracilis* var. Arizonica Snowcap

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Abstract

A mass micropropagation method was developed for *Mammillaria vetula* ssp. *gracilis* cv. Arizonica Snowcap, a cactus species with high ornamental value. A culture media based in 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} GA_3 + 30 g l^{-1} sucrose (MSM-1), was developed specifically for the establishment and multiplication of *Mammillaria vetula* cv. Snowcap offshoots, let us to produce a proliferation rate of 34.9 ± 5.9 new offshoots per explant. 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.

Introduction

The Cactaceae family is native from America and it comprises more than 2000 species. More than 600 species are from Mexico, especially at the Queretaro-Hidalgo Arid Zone (Pérez-Molphe-Balch, E., et al., 2015). *Mammillaria* is the largest genus among the family Cactaceae and it has been largely studied (Vyskot and Jára, 1984; Rubluo, 1997). 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 on maintenance and preservation of many species of cactus, that are endangered because of his 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, 2014; Pérez-Molphe-Balch et al., 2015). It is obviously important to avoid over-collection in nature, and the use of efficient micropropagation methods for these species could overcome this problem.

To date, the use of in vitro methods to propagate cacti, have been mostly developed to preserve endangered *Mammillaria* species, risking for extinction, such as *M. mathildae* (García-Rubio et al, 2010); M. *bocasana*, *M. densipina*, *M. orcutii*, etc. (Ramirez-Malagon et al. 2007); *M. schiedeana* (Soria-Campos et al, 2013); *M. pectinifera* (Giusti et al., 2002); *M. san-angelensis* (Martinez-Vazquez and Rubluo, 1989); *M. luethyi* (Escobedo et al., 2002).

Our goal species, *Mammillaria vetula ssp. gracilis* var. Arizonica Snowcap is a species of Cactaceae, endemic from 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 sides and upper part of 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 pinkish or brownish

midstripe in early spring. It is used as a very popular ornamental cactus highly demanded for international markets.

There are only few previous studies about *M. gracilis* (Poljuha et al., 2003; Balen et al., 2004), despite just talking about metabolism, they also noted that *M. gracilis* is suitable for micropropagation. To date the unique micropropagation method available for in vitro propagation of cv. Snowcap, is a micropropagation method through callus regeneration, developed by Balen et al. (2004).

In this work, we have developed a mass micropropagation method 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, able to produce severe morphological changes and progenies out of type.

Material And Methods

Plant material and disinfection

Young offshoots 0.5-1 cm diameter of *Mammillaria vetula* ssp. *gracilis* var. Arizonica Snowcap, provided by Solisplant Nurseries (El Ejido, Almeria, Spain), were used as explants for in vitro aseptic establishment. These explants were cleaned, disinfected, isolated, dissected and disinfected again to obtain the primary explants for micropropagation as follows:

The initial pieces of cacti were thoroughly washed with tap water and then roots were cut away and discarded. After first isolation, the explants consisting in a small cactus specimen (young offshoots) were carefully washed again with a soap solution and tap water and then treated with fungicide (Benomyl 0.3%) for 15 min under shaking and after the explants were rinsed several times in sterile distilled water to eliminate the fungicide, and then were disinfected in a 2% sodium hypochlorite 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.

Offshoots induction and proliferation

Culture media and culture conditions

For explant development, disinfected explants were incubated in a culture room at 25 ± 1°C in light conditions (under 16 h photoperiod under cool white fluorescent tubes (F40 tubes Gro-lux, Sylvania) with 45 µmol m^{-2} s^{-1} (400–700 nm) Photosynthetic Active Radiation). on MS salts (Murashige and Skoog, 1962) supplemented with 100 mg l^{-1} i-inositol, 30 g l^{-1} sucrose and 8 g l^{-1} of Agar A-1296 (Sigma), and after the aseptic establishment and due to the null growth of explants this medium was supplemented with different concentrations of plant growth regulators.

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, 05, 0.6) mg l^{-1}; IBA, IAA, Picloram, pCPA (0, 0.1, 0.5) mg l^{-1}; multiple combinations cytokinins and auxins to select the best combination possible and later, when the optimal combination of auxins plus cytokinins was stablished, gibberellic acid (1, 5, 10 mgl^{-1} during 3 days) was tested. After the selection of the best combination of PGRs, different levels of sucrose (0, 10, 20, 30, 40, 50, 60) g l^{-1} as well as 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.

In all the experiments 50 explants were used per treatment and the experiments were repeated twice.

For micropropagation three different media have been developed MSM-1 (Mammillaria Snowcap Medium-1, for explant and offshoots growth and maintenance), MSM-2 (Mammillaria Snowcap Medium-2, for offshoots induction and prolifereation) and MSM-3 (Mammillaria Snowcap Medium-3, for rooting). These media consisting on basal Murashige Skoog medium (Murashige and Skoog, 1962) supplemented as follows:

MSM-1: MS plus 0.5 mg l^{-1} NAA + 0.3 mg l^{-1} KIN + 20 g l^{-1} sucrose.

MSM-2: MS plus 0.5 mg l^{-1} NAA + 0.3 mg l^{-1} KIN + 5.0 mg l^{-1} GA_3 + 20 g l^{-1} sucrose.

MSM-3: MS plus 0.5 mg l^{-1} NAA + 0.3 mg l^{-1} KIN + 5 mg l^{-1} ANC + 10g l^{-1} sucrose.

The pH of all the culture media was adjusted to 5.7 before autoclaving. For culture initiation, rooting and multiplication experiments, 75 ml of medium per jar were aliquoted into 120 mL culture jars covered with polypropylene caps (Magenta Corp., Chicago, IL) and autoclaved for 20 min at 121°C and 1.05 Kg cm^{-2}. 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.

Bud sprouting, multiplication, rooting

Adult specimens maintained in MSM-1 are used to produce offshoots. It is necessary using explants 4 months old and large enough (3 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 just two months. After the subculture of adult 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 are large enough (0.3–0.5 cm in length) to be harvested (around 6 weeks after the bud sprouting). 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, as single plantlets, subcultured in fresh media (MSM-1). For subculturing medium involving big

sized plantlets, sometimes it is necessary to dissect the basal part of the plants, eliminating necrotic tissue and the callus surrounding the bottom of the plant, 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 growth and proliferate, reaching in our containers until 7–8 cm long (Fig. 7). After 6–8 weeks the percentage of survival, rooting, shoot and root number and length were recorded. Each treatment consists on a minimum of 100 explants.

Acclimation of Snowcap plantlets

Plantlets with well-developed roots were thoroughly washed in tap water and transplanted to polyethylene trays with 4 x 4 cm alveolus 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 19ºC and 30ºC, with a mean temperature of 25ºC. 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 and maintained for two months at 50% relative humidity. During the experiment, plants were lightly watered and fertilized periodically. Plants grew under 80% shade. Data on survival rate correspond to four months acclimation.

Statistical analysis

All data were analyzed using SPSS software package (version 19.0; SPPS 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, pairwise comparisons among groups were performed by LSD Fisher's least significant difference (LSD) test. The number of roots, 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 culture medium.

Results

Disinfection

For the disinfection process, a total of 100 initial samples were used. And just one primary explant was totally cleaned and disinfected. No bacteria, yeast or fungi were detected in this explant. So, every explant has been obtained from the same mother plant: all the plantlets used in this work are clones. The low rate of disinfection obtained (1%) appears to be due to the general presence of endogenous contaminant bacteria in the primary explants.

Explant growth, offshoots induction and rooting

Cytokinins

BA induced rooting (40%), but unfortunately, producing a big amount of callus and abnormal tissue. This callus is a problem, so the use of BA has been discarded for explants micropropagation. Otherwise, BA shows an important increase in size of the explants during the incubation period, significantly bigger than the other cytokinins tested (Fig. 1).

KIN rooting results 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 shows a good growth of the primary explants but with a low rate of rooting 18%, lower than kinetin and BA, clearly insufficient for our purposes and AS was discarded for further uses (Table 1). Less than 1% of primary explants generate a minimal number of offshoots after two months of incubation with these compounds.

Table 1
Effects of BA, KIN and AS on growth and development of explants.

Medium	PGR (mgl^{-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 significantly differences in each parameter between different culture media and initial explant.

The best value obtained for KIN (0.3 mg l^{-1}) together with the nearest values (0.1 and 0.5 mg l^{-1}) were used to carry out the next experiments involving the combination of auxins and cytokinins.

Auxins

Table 2 shows the effect of NAA, IAA and IBA (0.1 and 0.5 mg l^{-1}) on growth, rooting and callus induction. 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%). The best rooting rates (73, 54 and 49%), correspond to IBA treatments. The results on growth of the primary explant do not show significant differences respect the treatments with the other auxins, but the level of callus proliferation is too high for the treatments with IBA and IAA showing the better results on rooting, otherwise the treatments with NAA showed very low levels of callus proliferation with medium levels of rooting (37, 40%).

Table 2
Effects of NAA, IBA and IAA on growth, rooting and callus induction after 8 weeks of incubation.

Auxins	(mgl^{-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 significantly differences by HSD-Tukey at α = 0.05 in each parameter between different culture media and initial explant.

Again, less than 2% of primary explants generate a minimal number of offshoots after two months of incubation with these auxins.

Auxins + Cytokinins

Table 3 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 growth, rooting and callus induction. The higher values on callus development correspond to combinations of KIN plus IAA and IBA as well as in No. of roots and root length but better results in explant growth and lower percentages of callus growth together

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.

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

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

Different letters indicate significantly differences by HSD-Tukey at α = 0.05 in each parameter between different culture media and initial explant.

But unfortunately the combination of these auxins and cytokinins only promote a scarce number of offshoots in the 2–3% of primary explants, as happen when these auxins and cytokinins were included alone in the medium.

Gibberellic acid

When we supplement the medium, already supplemented with the best concentrations for cytokinins plus auxins (0.5 mg l^{-1} NAA + 0.3 mg l^{-1} KIN) medium MSM-1, with 5 mg l^{-1} GA_3 applied for 3 days we are able to induce 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 4). The average production per explant was 34.9 ± 5.9 offshoots (Fig. 2). With 10mg l^{-1} GA_3 also a 100% of explants generate offshoots but in a lower number than the treatment with 5 mg l^{-1} GA_3. Once the offshoots are cut from the mother explant, new offshoots are generated. The medium (MSM-2) for offshoot induction consist in MSM-1 medium plus 5 mg l^{-1} GA_3 applied for 3 days (Table 4).

Table 4
Effects of GA_3 treatments on offshoots induction and development.

GA_3 ($m\ l^{-1}$)	No. of offshoots per explant	Explants generating offshoots (%)	Callus induction (%)
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 significantly differences by HSD-Tukey at α = 0.05 in each parameter between different culture media and initial explant.			

Rooting Induction

Auxins

The tests with other auxins such as Picloram and pCPA. give negative results for rooting (data not shown). Picloram even generated an undesirable big amount of abnormal tissues and callus and both auxins were discarded to induce rooting on Snowcap explants.

Sucrose

Best rooting results are produced with 1% sucrose, reaching 53% of good quality rooted explants (Table 5, Fig. 3). A 43% rooting rate was recorded without sucrose in the medium (Table 5), but the explants show an abnormal color and lower quality. Between 1–4% sucrose, no abnormal tissue was found, but for higher doses than 4%, explants started to dry and finally died. No significant differences on offshoot

growth were recorded along the treatments and for callus proliferation only the higher value of sucrose showed significant differences respect the rest of concentrations assayed.

Table 5
Effects of different levels of sucrose on growth, rooting and callus induction.

Sucrose (mgl^{-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 significantly differences by HSD-Tukey at α = 0.05 in each parameter between different culture media and initial explant. Different letters indicate groups that were significantly different by LSD at α = 0.05

Ancymidol

Full rooting results were recorded in MSM-3. This rooting medium contains the growth retardant ancymidol (5 mg l^{-1}) and the use of this compound results in a 100% of rooting rate (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% success. Callus proliferation detected was low (Table 6).

Table 6
Effects of diverse doses of ancymidol on growth rooting and callus induction.

Ancymidol (mgl^{-1})	Length of offshoots (cm)	Rooting (%)	No. of roots	Callus (%)
0	1.9 ± 0.4a	40 ± 6c	1.3 ± 0.2b	10 ± 4a
1	1.5 ± 0.2b	70 ± 8b	1.2 ± 0.2b	14 ± 3a
2	1.6 ± 0.2b	70 ± 5b	1.4 ± 0.1b	14 ± 4a
3	1.5 ± 0.2b	77 ± 1b	1.4 ± 0.2b	12 ± 5a
4	1.5 ± 0.3b	92 ± 4a	1.9 ± 0.2a	10 ± 8a
5	1.5 ± 0.3b	100 ± 0a	2.1 ± 0.1a	10 ± 7a
Different letters indicate significantly differences by HSD-Tukey at α = 0.05 in each parameter between different culture media and initial explant.				

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 stays always insufficient for mass micropropagation requirements, except for the treatment including 5 mg l^{-1} of ancymidol (100%).

Acclimation

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

Discussion

Previous works had been done on *Mammillaria ssp. gracilis*, focused on basic studies on microscopy and on callus regeneration, paying limited attention to micropropagation (Balen et al., 2004; Poljuha et al., 2003).

Working with *M. ssp. gracilis* cv Snowcap, we had developed an efficient method for mass micropropagation through axillary sprouting of cactus offshoots; using growth regulators. This method avoids callus development or regeneration of plants from callus.

It is important to remark that all the experiments and treatments have been developed with clonal material obtained from a unique explant.

Offshoots induction and proliferation

Many authors had micropropagated different cacti by callus regeneration: *Mammillaria pectinifera, Pelecyphora aselliformis* (Giusti et al., 2002), *Cereus peruvianus* (De Oliveira et al., 1995), *M. mathildae* (García-Rubio, and Malda-Barrera, 2010).

Another method of micropropagation consists in obtaining plants through direct organogenesis from different plant sections; such as shoot tips, bases and longitudinal sections of cactus (Papafotiu 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*, Martinez-Vazquez 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*, Martinez-Vazquez and Rubluo, 1989; *Mammillaria wrightii*, Clayton et al., 1990; *M. elongata*, Papafotiou et al., 2001, *M. candida*, Elias-Rocha, 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 never 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 et al. (1998) reported for 6 *Mammillaria* species) we always used small whole plants of cv Snowcap as explants, and treated them unsuccessfully 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 working with other *Mammillaria* species.

Gibberellic acid

GA_3 combined with NAA and KIN, is the only PGR that had been able to activate the sprouting of the axillary buds existing in the areoles, for all treatments tested, inducing mass proliferation, reaching in two of the three treatments assayed until a 100% of explants generating offshoots, reaching a maximum number of 34.9 ± 5.9 offshoots per explant. That agrees with Giusti et al. (2002), which confirmed that different cactus species respond very differently to different types of growth regulators, because most of 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 GA_3 plus NAA and KIN), instead of just cytokinins, in agreement with Mauseth (1976), which reported that cytokinins and GA_3 are able to activate the apical meristem and cause the continual initiation of lateral primordia in *Opuntia polyacantha*, we had also used GA_3 together with KIN and NAA, to induce the massive initiation of lateral primordia to produce offshoots. The strong effect over he 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* ssp *gracilis* 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) or by halving MS salts (Ramirez-Malagon, 2007, rooting rates data not available). We had reached 100% of rooted explants

(small 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 Pinus strobus (Burkhart and Meyer, 1991) or several species of Asparagus (Desjardins et al, 1987, Stajner et al, 2002, Carmona et al., 2014, 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 6, Fig. 4).

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*, and after that, that range had been generally adopted in most cacti in vitro works (M. *bocasana*, *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 previous cacti media. Our results show no significant differences in growth for 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 of number of roots developed (4.2 vs 1.2) along the incubation process, so we selected 1% sucrose as optimal level for cv. Snowcap rooting medium (MSM-3). In fact, we have found that the treatment 4% sucrose have 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 over the explants, which is in clear disagreement with Dabekaussen et al. (1991).

As far as we know, it is the first time that mass micropropagation for *M. vetula* ssp. *gracilis* var. Arizonica cv. Snowcap had been reported.

Conclusion

In conclusion, we have developed a method for mass micropropagation of *M. vetula ssp. gracilis* var. Arizonica cv. Snowcap that improves previous results for the same subspecies, allowing the commercial micropropagation of cv. Snowcap, an ornamental cactus species highly appreciated in plant markets.

This reliable micropropagation system has been used routinely in our laboratory during 5 years without problems and this system is also versatile and has been applied successfully to other *Mammillaria* species.

Abbreviations

ANC
Ancymidol α-Cyclopropyl-α-(4-methoxyphenyl)-5-pyrimidylmethanol
AS
6-Aminopurine hemisulfate
BA

benzylaminopurine
IAA
indole-3-acetic acid
KIN
6-furfurylaminopurine
IBA
indole-3-butyric acid
MS
Murashige and Skoog medium
NAA
1-naphthaleneacetic acid
pCPA
para-chlorophenylalanine
GA_3
Gibberellic acid
PGR
Plant Growth Regulator

Declarations

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Ethics approval (Not applicable)

Consent to participate: All authors have given their consent to participate in this work.

Consent for publication: All authors have given their consent for the publication of this work.

Availability of data and material (Not applicable)

Code availability (Not applicable)

Authors' contributions: ECL designed and wrote the manuscript. RJJ collaborated in the design and writing of the manuscript. LGM and AA carry out the development and data collection of the in vitro tests.

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Figures

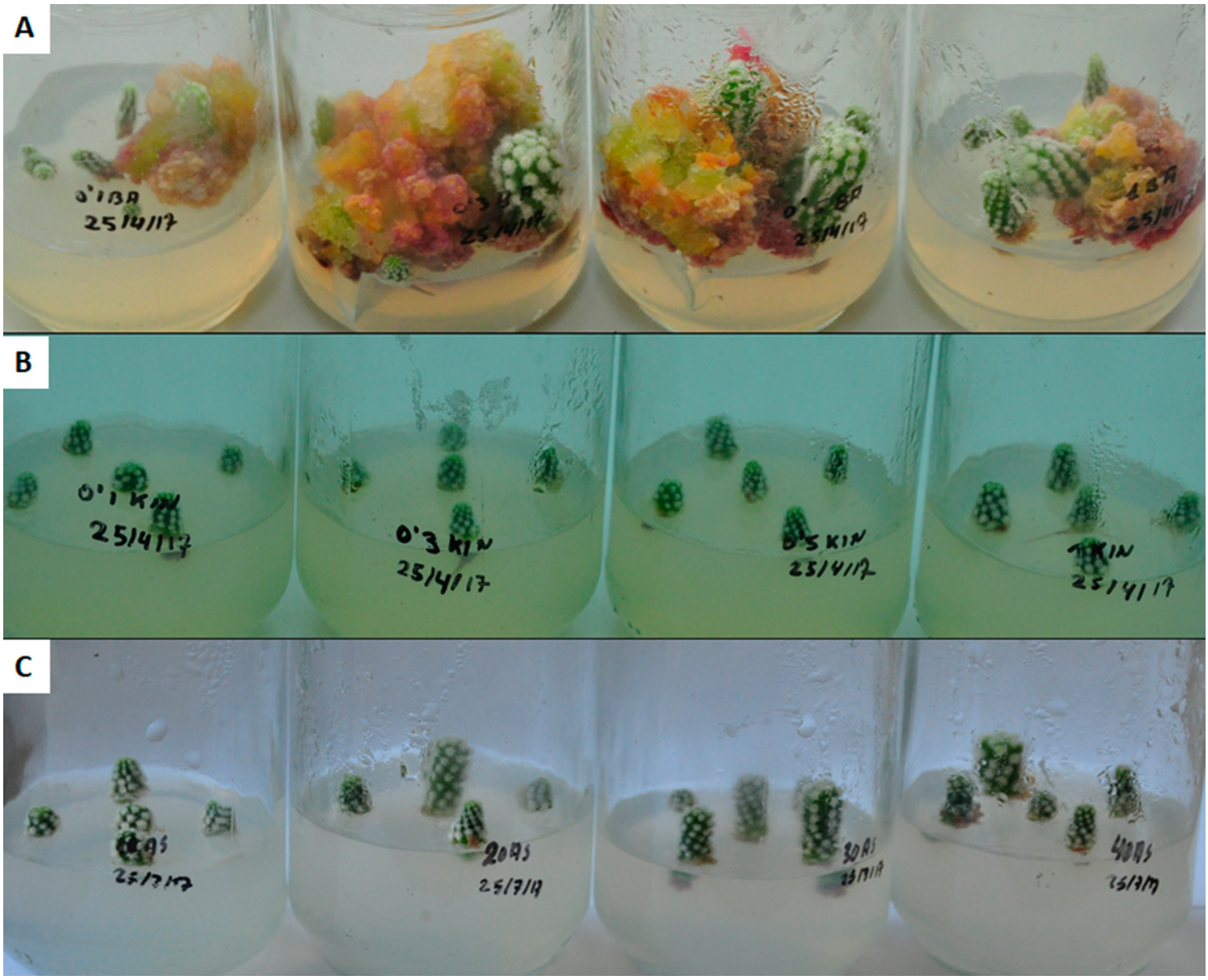

Figure 1

A. Effect of BA concentrations (0.1, 0.3, 0.5, 1 mg l-1; from left to right). B. Effect of KIN (0.1, 0.3, 0.5, 1 mg l-1; from left to right). C. Effect of AS (10, 20, 30, 40 mg l-1) on growth and rooting.

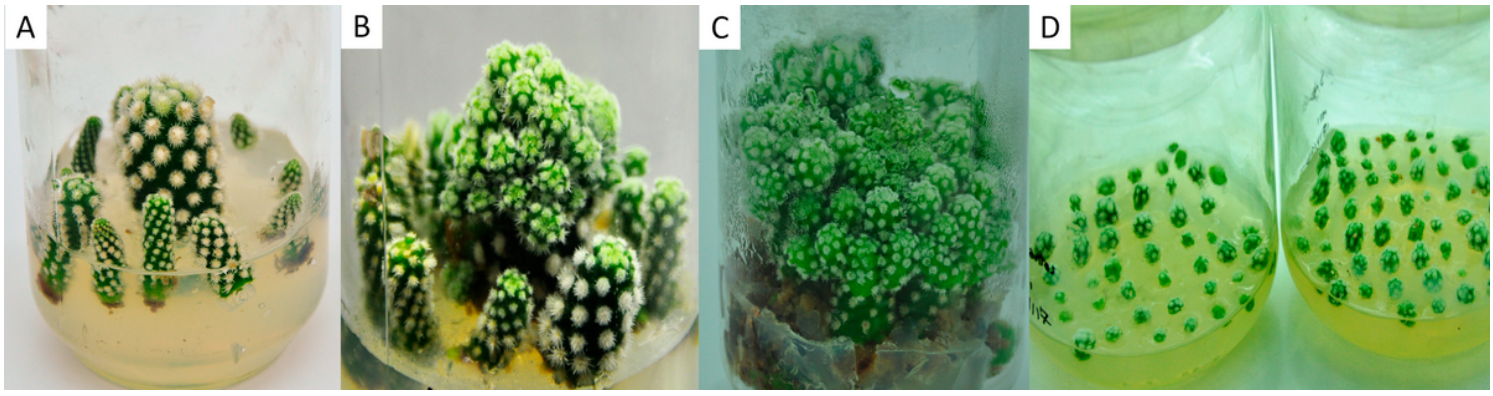

Figure 2

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

Figure 3

Rooted explants in medium with a low sucrose level (1%).

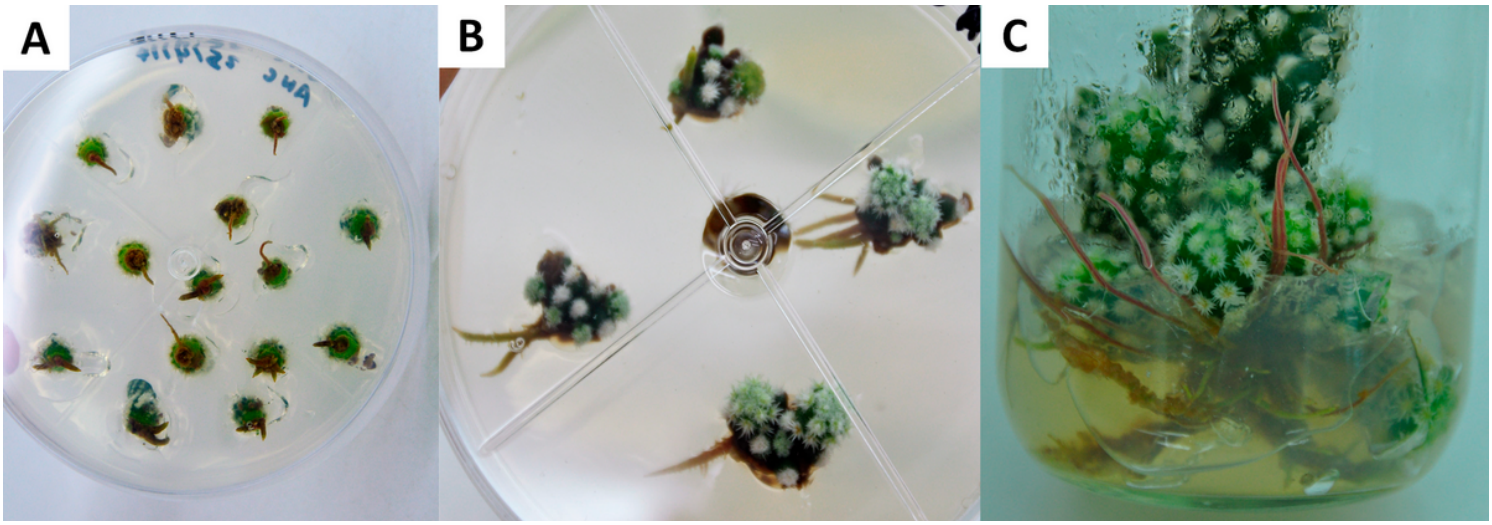

Figure 4

Effect of ancymidol on rooting on young and adult explants.