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Vol. 16(14), pp. 749-762, 5 April, 2017
DOI: 10.5897/AJB2016.15901
Article Number: BDA6FB363601
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

*Full Length Research Paper*

# Micropropagation of two species of *Micranthocereus* (Cactaceae) with ornamental potential native to Bahia, Brazil

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Received 18 January, 2017, Accepted 8 March, 2017

**The two target cactus species in this study, *Micranthocereus flaviflorus* subsp. *densiflorus* (Buining and Brederoo) P.J.Braun and Esteves and *Micranthocereus. polyanthus* subsp. *alvinii* M. Machado and Hofacker, are endemic to the state of Bahia and have ornamental value. Therefore, this work proposed to establish *M. flaviflorus in vitro*, as well as to micropropagate both species. The temperature regimes 25°C and 30°C and the alternating temperatures of 15 to 25°C were tested for *in vitro* germination of *M. flaviflorus* seeds, which achieved higher germination rates at 25°C. Regarding nutrient media, the lower water potentials of MS and MS/2 media, when compared to agar, allowed the germination of *M. flaviflorus* seeds. Furthermore, the test of plant growth regulators for *in vitro* multiplication consisted of regulator concentrations in a factorial arrangement (2x4): Naphthaleneacetic acid (NAA) (0 and 1.34 µmol $L^{-1}$) and Kinetin (KIN) (0, 6.74, 20.22 and 40.44 µmol $L^{-1}$) in MS/2 media. Shoots of different lengths derived from *in vitro* propagation were removed from explants and directly planted in plastic cups for survival appraisal. In the *in vitro* propagation of the studied species, the association of the highest KIN concentration (40.44 µmol $L^{-1}$) to 1.34 µmol $L^{-1}$ of NAA significantly reduced the number of shoots per explant. The use of 1.34 µmol $L^{-1}$ of NAA without cytokinin is suggested for *in vitro* multiplication in both species. Shoot size was determinant for survival during acclimatization, and so, the usage of shoots at least 0.6 and 1.5 cm long for *M. flaviflorus* and *M. polyanthus*, respectively, is recommended for *ex vitro* establishment.**

**Key words:** Acclimatization, Brazilian flora, Cactaceae, conservation.

## INTRODUCTION

The family Cactaceae encompasses 100 genera and 1500 to 2000 species distributed in the American continent, with the exception of genus *Rhipsalis*, which occurs also in tropical Africa, Madagascar, islands in the Indian Ocean and Sri Lanka (Rojas-Aréchiga and Vázquez-Yanes, 2000; Pérez-Molphe-Balch et al., 2015). Its species possess great ecological and economic importance, and are under several threats of anthropic origin such as habitat destruction, predatory collection and illegal trading (Oldfield, 1997; Machado, 1999; Taylor

and Zappi, 2004; Pérez-Molphe-Balch et al., 2015).

The two target species of this study, *Micranthocereus flaviflorus* subsp. *densiflorus* (Buining and Brederoo) P.J.Braun and Esteves and *M. polyanthus* subsp. *alvinii* M. Machado and Hofacker, are endemic to the state of Bahia, Brazil, and have ornamental potential (Charles, 2009). *M. flaviflorus* subsp. *densiflorus* has shrub bearing with ramifications from the base, dense yellowish spines and reddish tubular flowers with yellow center, which appear in great quantity in lateral pseudocephalia, composed of wool and hairs, and their fruits are also reddish (Machado, 1999). *M. polyanthus* subsp. *alvinii* also possesses shrub-like bearing with branching from the base, but its spines are whitish. Its small and numerous tubular flowers are white in the center and rosy around. They are organized into lateral pseudocephalia, composed of wool and hairs, and have rosy fruits.

Both species are threatened and listed in Appendix II of CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) and IUCN (International Union for Conservation of Nature) Red List of Threatened Species (Machado et al., 2013; Machado and Braun, 2013). Given their precarious *in situ* conservation, endeavors to achieve *ex situ* preservation were made, consisting of seed storage and cryopreservation studies (Veiga-Barbosa et al., 2010; Civatti et al., 2015a,b).

*In vitro* germination, for some species can provide higher germination rates than *ex vitro* germination, as was the case for *Mammillaria mathildae* Kraehenb and Krainz (García-Rubio and Malda-Barrera, 2010). This approach can also be used to supply *in vitro* germinated plants for plant tissue culture enterprises such as micropropagation and somatic embryogenesis protocols, and maintenance of *in vitro* germplasm banks (Dias et al., 2008).

With the advent of plant tissue culture, the problems of traditional cultivation like low germination rates or difficulty in obtaining seeds, can be evaded and cacti can be multiplied in large scale and/or conserved *in vitro* (Lema-Rumińska and Kulus, 2014; Pérez-Molphe-Balch et al., 2015). One of the approaches provided by tissue culture is the *in vitro* multiplication of plants previously established in laboratory, meeting the demand of the ornamental market and indirectly protecting the integrity of natural populations (Malda et al., 1999; Pence, 2010).

Clonal propagation from tissues of *ex vitro* adult plants may not represent the real variability of species, besides often resulting in loss of material due to fungal or bacterial contamination during plant *in vitro* establishment. This can be avoided by applying propagation techniques on plants introduced in the *in vitro* environment through their seeds, as was done with *Pelecyphora aselliformis* Ehrenb. (Santos-Díaz et al., 2003) and eight species of *Turbinicarpus* (Dávila-Figueroa et al., 2005). The micropropagation of cacti, however, has demonstrated that protocols developed so far are species-specific, and that even for closely related species requirements for *in vitro* multiplication may vary (Lema-Rumińska and Kulus, 2014).

This work aimed to establish both species *in vitro,* evaluating ideal media and temperature conditions for *in vitro* germination of *M. flaviflorus*, as well as to multiply *in vitro M. polyanthus* and *M. flaviflorus,* subsequently acclimatizing the shoots obtained in this manner.

## MATERIAL AND METHODS

### Plant materials

Ripe fruits of *Micranthocereus flaviflorus* subsp. *densiflorus* and *Micranthocereus polyanthus* subsp. *alvinii* were collected in Morro do Chapéu, Bahia, Brazil. The seeds obtained were processed, dried and stored in paper bags at room temperature for 2 weeks until the setup of experiments. Seed disinfestation was performed in laminar flow cabinet immersing seeds in absolute alcohol for 1 min followed by soaking in sodium hypochlorite at 2.5% for 15 min, and then rinsing 3 times with sterile water before sowing.

Given the restricted distribution and threatened status of *M. polyanthus,* not enough seeds were obtained for germination tests. The *in vitro* germination protocol established for *M. flaviflorus* was later applied to *M. polyanthus* seeds, and allowed for the generation of plants, used in the micropropagation tests of the latter. Germinated plants were kept *in vitro* on half-strength Murashige and Skoog (1962) media (MS/2) until the multiplication experiment was assembled at the Biology Institute of the Federal University of Bahia (Salvador, BA) in 2015, with periodical transfers, to allow plant growth and sustainment.

### Effect of nutrient media and temperature over *in vitro* germination

*M. flaviflorus* seeds were sowed in agar (distilled water with Agar at 6 $gL^{-1}$), Murashige and Skoog (1962) nutrient media (MS) supplemented with 30 g $L^{-1}$ of sucrose or MS/2 supplemented with 15 g $L^{-1}$ of sucrose. Seeds were submitted to constant temperatures of 30±1 and 25±3°C, and to temperatures alternating between 15±1 and 25 ±1°C, under white fluorescent light (60 µmol $m^{-2}$ $s^{-1}$). The design was completely randomized using a factorial arrangement of 3x3 (media x temperature) with 4 replicates of 20 seeds in each treatment.

Germination was evaluated daily for 21 days after the first seed germinated, considering the emission of radicle as evidence of germination (Brasil, 2009). The variables used in the analysis were germination (%G), mean germination time (MGT = $(\sum n_i\ t_i)/\sum n_i$, where $n_i$ = number of seeds that germinated in day "i", and $t_i$ = day "i" in evaluated time), germination speed index (GSI = $\sum(n_i/t_i)$, where $n_i$ = number of seeds that germinated in day "i", and $t_i$ = day "i" in evaluated time), germination uniformity coefficient (GUC =

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$(\sum n_i)/\sum(MGT\text{-}t_i)^2 n_i$, where $n_i$ = number of seeds that germinated in day "i") (Santana and Ranal, 2000) and absolute frequency of germination over time. As germination (%G) was expressed in %, it was arcsine-transformed.

#### Seed viability testing

The viability of seeds that did not germinate until the end the experiments was tested using tetrazolium (2,3,5 triphenyl tetrazolium chloride) (ISTA, 2007). In order to do so, 25 non-germinated seeds had their opercula removed and were placed in Petri dishes lined with Germ test paper moistened with distilled water and wrapped in aluminum foil (adapted from Veiga-Barbosa et al*.,* 2010). After 24 h of soaking, seeds were immersed in tetrazolium solution at 0.6% and remained in the dark at room temperature (25±3°C). Followed 24 h, the seed coats were broken and embryos analyzed based on their staining using a magnifying glass.

#### *In vitro* multiplication of *Micranthocereus flaviflorus* subsp. *densiflorus* and *Micranthocereus polyanthus* subsp. *alvinii*

*In vitro* germinated plants of *M. flaviflorus* and *M. polyanthus*, 18 and 15 months old, respectively, were used as explant source to test the influence of plant growth regulators on their multiplication. The nutrient media used for such test was MS/2 supplemented with sucrose at 15 g $L^{-1}$ and gelled with 6 g $L^{-1}$ of agar. This nutrient media was chemically sterilized using sodium hypochlorite containing 2.5% of active chloride (adapted from Teixeira et al., 2006). The design was completely randomized with a factorial arrangement of 2x4 plant growth regulator concentrations: α-naphthaleneacetic acid (NAA, synthetic plant hormone of the auxin family) (0 and 1.34 µmol $L^{-1}$) and Kinetin (KIN, a type of cytokinin) (0, 6.74, 20.22 and 40.44 µmol $L^{-1}$), totaling 8 treatments for each studied species. These regulators were added to the nutrient media during its preparation, and each test tube was inoculated with only one horizontal explant. The explants were cut from the *in vitro* germinated plants of *M. flaviflorus* and *M. polyanthus*, disregarding apexes and roots of the mother plants, through transversal cross-sections of the cladode to obtain segments with 30 to 40 areoles, approximately.

Five replicates of 9 explants were used for each treatment, summing 45 explants per treatment per species. The variables analyzed were, the number of shoots per explant, shooting explants (%), rooting explants (%), oxidizing explants (%), and explants that formed callus (%). The multiplication experiment lasted 120 days in a 16 h photoperiod, under fluorescent light (60 µmol $m^{-2}$ $s^{-1}$) at room temperature (25 ± 3°C).

#### Acclimatization of micropropagated shoots

Shoots of different lengths, derived from *in vitro* multiplication with NAA at 1.34 µmol $L^{-1}$ were detached from their explants and directly planted in plastic cups containing earth and sand (1:1).

The shoot length classes tested were ± 0.2 to 3.0 cm, ± 0.4 to 5.0 cm and ± 0.6 to 0.7 cm for *M. flaviflorus*, while for *M. polyanthus* they were intervals of 0.3 to 0.5 cm, 0.6 to 0.9 cm and 1.0 to 1.5 cm, with 3 replicates of 6 shoots per class for each species. Culture conditions were established at greenhouse with daily watering for 60 days, at the end of which the variables rooting (%) and survival of shoots (%) were calculated for both species

#### Statistical analysis

For all experiments, ANOVA was performed and means were compared by the Tukey test at 5% probability. The statistics program used was Sisvar 5.3 (Ferreira, 2008).

## RESULTS AND DISCUSSION

### *In vitro* germination of *Micranthocereus flaviflorus*

*M. flaviflorus* seeds took 12 days average to germinate (Figure 1), the first germination being observed 3 days after the assembly of the experiment. The different nutrient media did not interfere in germination for any of the analyzed variables (Table 1), and there was no interaction between nutrient media and temperature regime in the evaluated variables: %G (p=0.7436), GSI (p=0.9103), MGT (p=0.3632) and GUC (p=0.3493). Under the constant temperature of 25°C, *M. flaviflorus* seeds achieved higher %G when compared to the other temperature regimes, while the GSI of said temperature was significantly higher than that of the alternating temperatures (Table 1). The average %G found, considering all treatments, was 20.4%. The low %G of *M. flaviflorus* (20.4%) suggests that this species may present some kind of dormancy or need a post-maturation period to achieve higher %G, which is consistent with the germination behavior of species that form seed banks on their natural environments and has been documented for other cacti (Rojas-Arechiga and Vazquez-Yanes, 2000; Montiel and Montaña, 2003; Benítez-Rodríguez et al., 2004; Mandujano et al., 2005; Ortega-Baes and Rojas-Aréchiga, 2007).

Taking into account the values of GUC and GSI, which were average 0.238 and 0.424, respectively, it is possible to assume that the germination of *M. flaviflorus* was not concentrated, but dispersed throughout the evaluated time in the experiment. The germination frequency obtained for *M. flaviflorus* (Figure 1) at different temperatures also demonstrated a dispersed pattern in germination over time, in which germination seems to occur at random during the evaluated timeframe regardless of the tested treatments (Ferreira and Borghetti, 2004). Seeds of this species show a lack of synchronism that may be caused by the heterogeneity of the used seed lot, a result that is characteristic of native undomesticated species and species capable of generating lasting seed banks (Ferreira and Borghetti, 2004). Similar results were found for other cacti, such as *Pilosocereus gounellei* (F.A.C.Weber ex K.Schum.) Byles and G.D.Rowley, *Stephanocereus luetzelburgii* (Vaupel) N.P.Taylor and Eggli and *Discocactus zehntneri* Britton and Rose (Marchi, 2012). In other species, however, germination is concentrated in time, as is the case of *Melocactus ernestii* Vaupel*, M. glaucescens* Buining and Brederoo and *M. xalbicephalus* Buining and Brederoo*,* for which germination peaks appeared on the second day after sowing (Cruz, 2011).

Temperature is a determining factor in the germination of several plant species, altering enzyme activity and, consequently, the velocity of reactions associated with

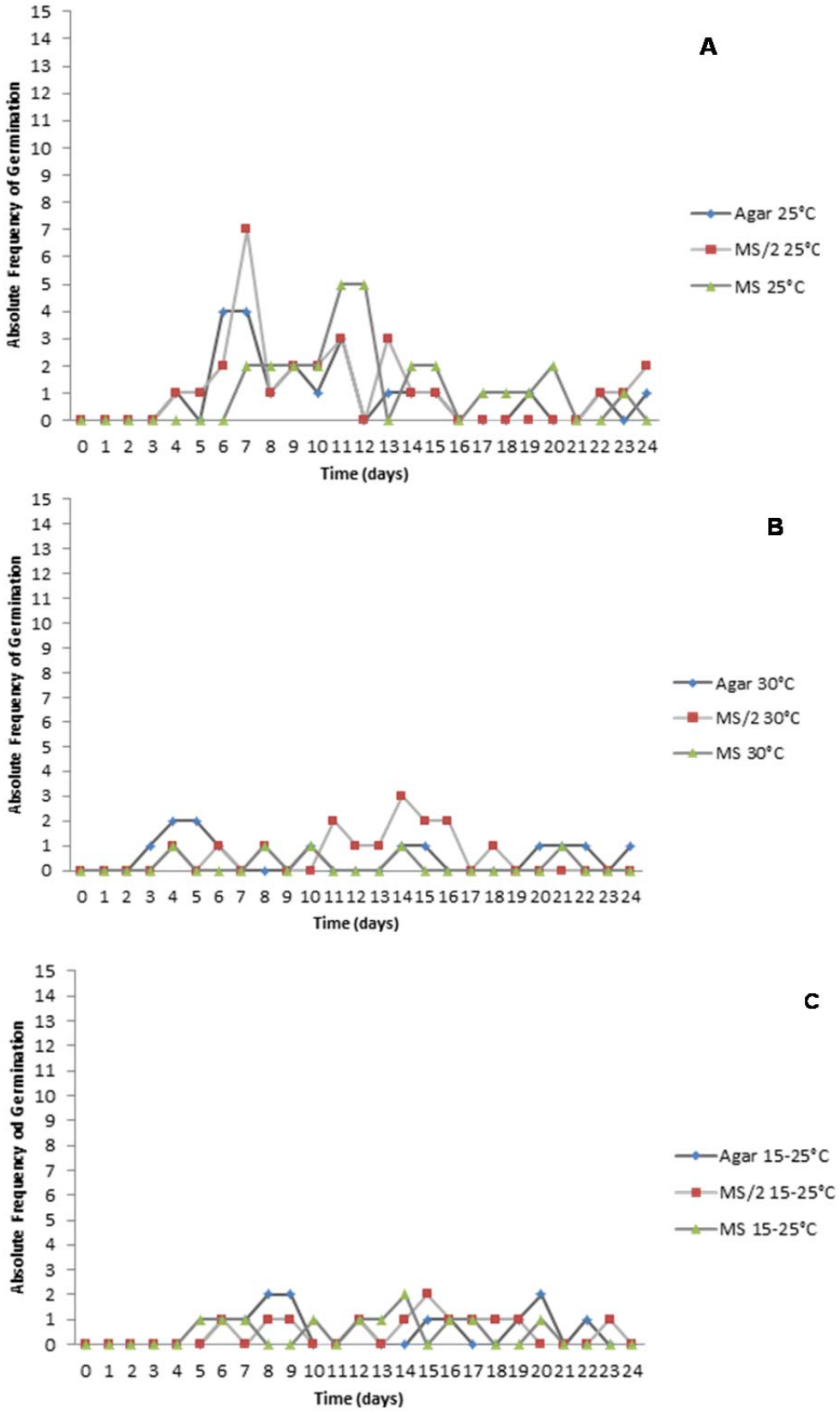


**Figure 1.** Absolute frequency of germination of *Micranthocereus flaviflorus* over time under different temperature regimes and nutrient media: A) Constant temperature of 25°C in Agar, MS/2 and MS; B) Constant temperature of 30°C in Agar, MS/2 and MS; C). Alternating temperature of 15-25°C in Agar, MS/2 and MS.

**Table 1.** Effect of nutrient medium and different temperature regimes on germination (%G), mean germination time (MGT), germination speed index (GSI) and germination uniformity coefficient (GUC) of *Micranthocereus flaviflorus* seeds.

| Temperature (°C) | Germination (%) | | | | Mean Germination Time (days) | | | |
|---|---|---|---|---|---|---|---|---|
| | Agar | MS/2 | MS | Means | Agar | MS/2 | MS | Means |
| 25 | 27.5 | 35.0 | 35.0 | 32.5 a | 10.2 | 13.7 | 13.0 | 12.3 a |
| 30 | 16.2 | 18.7 | 6.2 | 13.7 b | 14.5 | 12.7 | 8.1 | 11.8 a |
| 15 – 25 | 16.2 | 15.0 | 13.7 | 15.0 b | 12.9 | 15.2 | 10.8 | 13.0 a |
| Means | 20.0 a | 22.9 a | 18. 3 a | | 12.6 a | 13.8 a | 10.6 a | |
| **Temperature (°C)** | **GSI** | | | | **GUC** | | | |
| | **Agar** | **MS/2** | **MS** | **Means** | **Agar** | **MS/2** | **MS** | **Means** |
| 25 | 0.642 | 0.795 | 0.605 | 0.680 a | 1.152 | 0.035 | 0.072 | 0.420 a |
| 30 | 0.455 | 0.355 | 0.150 | 0.320 ab | 0.070 | 0.335 | 0.065 | 0.157 a |
| 15 – 25 | 0.297 | 0.245 | 0.270 | 0.270 b | 0.042 | 0.287 | 0.082 | 0.137 a |
| Means | 0.465 a | 0.465 a | 0.342 a | - | 0.422 a | 0.219 a | 0.073 a | - |

Means followed by the same lower case letter do not differ significantly by the Tukey test (p>0.05).

germination, as well as denaturing proteins and causing phase transition in membranes, all of which can limit germination when temperature is unfavorable (Ferreira and Borghetti, 2004). Cacti, as most tropical species, have favorable germination temperatures ranging around 25°C, whilst numerous genera have optimum temperatures of 20°C (Rojas-Arechiga and Vazquez-Yanes, 2000). Thereby, it is necessary to evaluate the germination behavior of a species to determine optimum germination temperature, as it varies among species (Rojas-Arechiga and Vazquez-Yanes, 2000; Hartmann Et Al., 2011; Kerbauy, 2012).

Some plant species have seeds that need an increase in thermal variation in a short period of time (e.g. 24h) in order to germinate (Ferreira and Borghetti, 2004). Certain cactus species exhibit higher germination rates when submitted to alternating temperatures (Rojas-Aréchiga and Vázquez-Yanes, 2000). Even so, the alternating regime of 15-25°C did not promote germination in the present study (Table 1), so other thermal variations may be tested in different time intervals to try promoting germination in future works.

In Morro do Chapéu temperatures during the day may reach 27°C, and during the night they may stabilize around 14°C, depending on the time of the year (Hong Kong Observatory, 2012; INMET, 2016). It would be expected that alternating temperatures reproducing the environmental conditions of where *M. flaviflorus* naturally occurs exerted influence over the germination of this species. In *Stenocereus stellatus* (Pfeiff.) Riccob., similarly to *M. flaviflorus*, higher germination rates were observed under constant temperature of 25°C when compared to alternating temperatures of 20-35°C (Rojas-Aréchiga et al., 2001). *Melocactus bahiensis* (Britton and Rose) Luetzelb. did not germinate in alternating temperatures of 20-30ºC (Lone et al., 2007), although for *Melocactus ernestii* Vaupel, *M. glaucescens* and *M. xalbicephalus* the best germination response was observed under the regime of 25-30ºC (Cruz, 2011). *Stenocereus queretaroensis* (F.A.C.Weber ex Mathsson) Buxb. achieved a mean germination of 84% under the regimes of 25-15°C, 25-25°C and 35-25°C (De La Barrera and Nobel, 2003). *D. zehntneri*, on the other hand, did not have its germination significantly altered by constant (25 and 30°C) or alternating (25-30°C) temperatures (Marchi, 2012).

*M. flaviflorus*, *P. gounellei* and *Cereus jamacaru* DC. germinated with rates higher than 80% in constant temperatures of 20°C, 25°C and 30°C, and alternating temperatures of 15-25°C (Veiga-Barbosa et al., 2010), as did *Trichocereus terscheckii*'s seeds in constant temperatures of 15°C, 20°C and 25°C (Ortega-Baes and Rojas-Arechiga, 2007), while none of these species germinated in the extreme temperature of 10°C (Ortega-Baes and Rojas-Arechiga, 2007; Veiga-Barbosa et al., 2010). Such results disagree with those found for *M. flaviflorus* (Figure 2) in the present study, which did not achieve the high values of %G shown in those species, aside from displaying significant differences between constant and alternating temperature regimes (Table 1). For the *in vitro* establishment of this species 25°C is suggested, as it is the standard temperature of growth rooms.

Veiga-Barbosa et al. (2010) obtained high %G rates for *M. flaviflorus* under the regimes of 20°C (96%), 25°C (87%), 30°C (93%) and 15-25°C (91%) in Petri dishes lined with wet Germ test. The differences between the *in vitro* and *ex vitro* environments may have been the cause of the divergence between these results and those of the present work. Since water availability in the substrate is crucial for the hydration of seed tissues, allowing seed metabolism to restart and cells to expand (Kerbauy, 2012), it would be expected that media with higher water potential, e.i. media with lower solute concentrations,

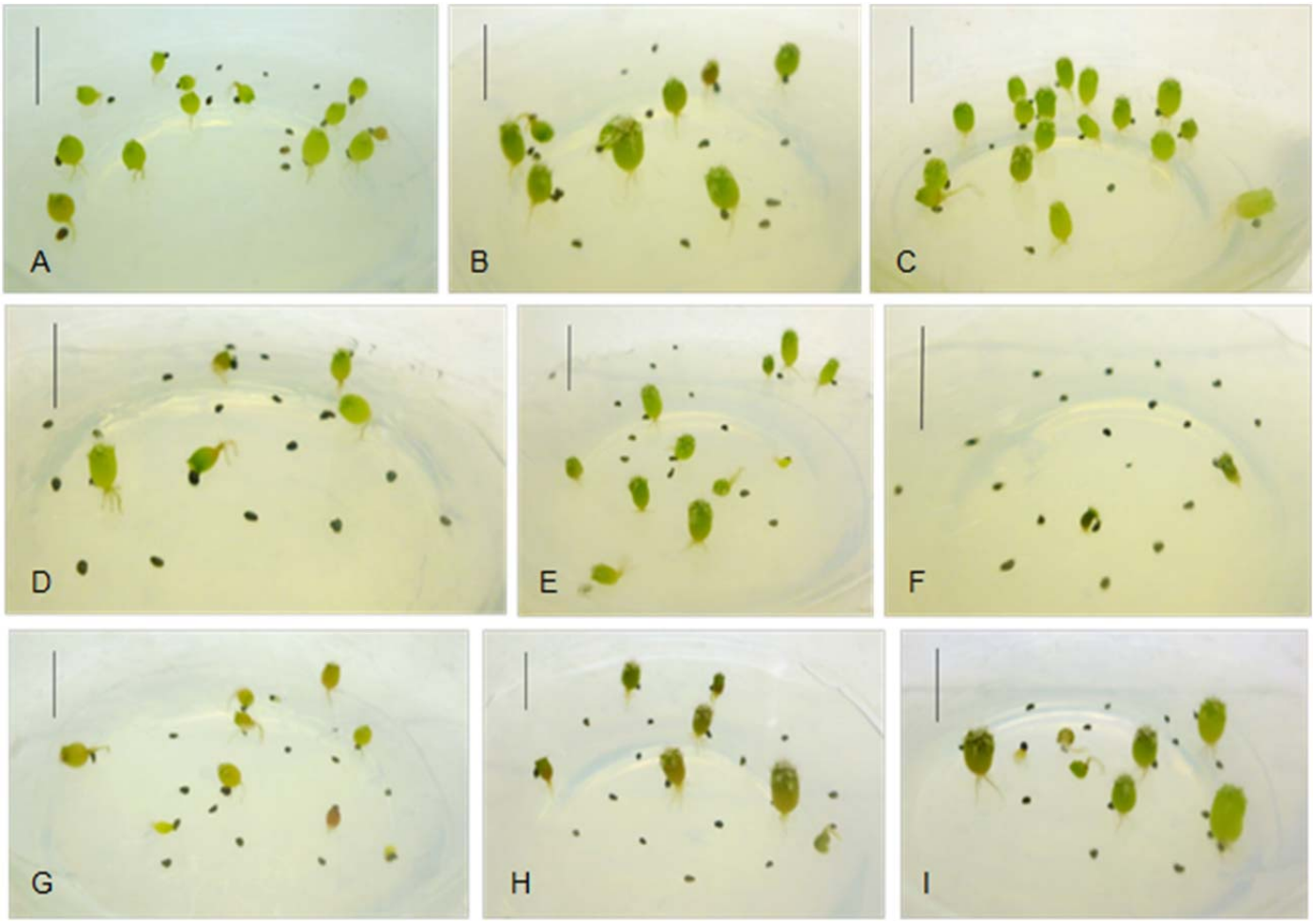


**Figure 2.** *Micranthocereus flaviflorus* plants germinated at 25°C in Agar, MS and MS/2 (A, B and C, respectively), at 30°C in Agar, MS/2 and MS (D, E and F, respectively) and alternating temperatures of 15-25°C in Agar, MS and MS/2 (G, H and I, respectively).

showed better results for germination (Marchi, 2012). Notwithstanding, in the present study different media saline and sucrose concentrations did not induce differences in germination, which means that the lower water potentials of MS/2 and MS media permitted the germination of *M. flaviflorus* seeds. The nutrient media also did not interfere in the *in vitro* germination of *Arrojadoa* spp. (Dias et al., 2008). The water potential of such media did not prevent *in vitro* germination of *D. zehntineri* either, whereas seeds of *S. luetzelburgii* and *P. gounellei* achieved higher G% in MS and MS/2 media than in agar (Marchi, 2012). The nutrient media recommended for *in vitro* germination of *M. flaviflorus* is, therefore, MS/2, since it is the most economical option while also having nutrients to allow healthy initial development of seedlings.

In the tetrazolium test conducted for *M. flaviflorus*, embryo viability of non-germinated seeds reached an average of 90.69%, and so an experiment to promote dormancy break in this species is underway.

### *In vitro* multiplication of *Micranthocereus flaviflorus* and *M. polyanthus*

*M. flaviflorus* and *M. polyanthus* exhibited high averages of explant shooting (89.8% and 96.3%, respectively), rooting (98.0% in both) and survival (99.0% in both), while oxidation rates varied between 20.0% and 82.5% in *M. flaviflorus* (Table 2) and from 21.1% to 77.8% in *M. polyanthus* (Table 3). None of the explants of either species presented callogenesis or hyperhydricity after 4 months in culture.

NAA and KIN concentrations interacted significantly in the variables shoots per explant (p=0.0060), shooting (p=0.0000) and oxidation (p=0.0023) for *M. flaviflorus.* Explant oxidation was more frequent in the two highest concentrations of KIN when combined with NAA (82.5% and 56.3%), two treatments that also showed the lowest percentages of shooting explants (72.5% and 61.4%). Concerning number of shoots obtained per explant, values found for KIN concentrations did not differ from

**Table 2.** Influence of different concentrations of KIN and NAA on number of shoots per explant, as well as on explant shooting (%), rooting (%), oxidation (%) and survival (%) in *Micranthocereus flaviflorus.*

| NAA (µmol $L^{-1}$) | KIN (µmol $L^{-1}$) | | | | |
|---|---|---|---|---|---|
| | **0** | **6.74** | **20.22** | **40.44** | **Means** |
| **Shoots per explant** | | | | | |
| 0 | $4.8^{aB}$ | $4.5^{aA}$ | $3.4^{aA}$ | $3.8^{aA}$ | $4.2^{A}$ |
| 1.34 | $6.4^{aA}$ | $4.6^{bA}$ | $3.7^{bcA}$ | $2.4^{cA}$ | $4.2^{A}$ |
| Means | $5.6^{a}$ | $4.6^{ab}$ | $3.6^{bc}$ | $3.1^{c}$ | - |
| **Shooting (%)** | | | | | |
| 0 | $100.0^{aA}$ | $95.6^{aA}$ | $97.8^{aA}$ | $100.0^{aA}$ | $98.3^{A}$ |
| 1.34 | $93.3^{aA}$ | $97.8^{aA}$ | $72.5^{bB}$ | $61.4^{bB}$ | $81.2^{B}$ |
| Means | $96.7^{a}$ | $96.7^{a}$ | $85.1^{b}$ | $80.7^{b}$ | - |
| **Rooting (%)** | | | | | |
| 0 | $100.0^{aA}$ | $95.5^{aA}$ | $100.0^{aA}$ | $100.0^{aA}$ | $98.9^{A}$ |
| 1.34 | $95.5^{aA}$ | $100.0^{aA}$ | $100.0^{aA}$ | $97.8^{aA}$ | $98.3^{A}$ |
| Means | $97.8^{a}$ | $97.8^{a}$ | $100.0^{a}$ | $98.8^{a}$ | - |
| **Oxidation (%)** | | | | | |
| 0 | $20.0^{aA}$ | $29.1^{aA}$ | $33.9^{aB}$ | $25.8^{aB}$ | $27.2^{B}$ |
| 1.34 | $27.2^{cA}$ | $30.4^{cA}$ | $82.5^{aA}$ | $56.3^{bA}$ | $49.1^{A}$ |
| Means | $23.6^{c}$ | $29.8^{bc}$ | $58.2^{a}$ | $41.1^{b}$ | - |
| **Survival (%)** | | | | | |
| 0 | $100.0^{aA}$ | $97.8^{aA}$ | $100.0^{aA}$ | $100.0^{aA}$ | $99.4^{A}$ |
| 1.34 | $97.8^{aA}$ | $100.0^{aA}$ | $100.0^{aA}$ | $100.0^{aA}$ | $99.4^{A}$ |
| Means | $98.9^{a}$ | $98.9^{a}$ | $100.0^{a}$ | $100.0^{a}$ | - |

Means followed by the same letter, lowercase in rows and uppercase in columns, do not differ significantly by the Tukey test ($p>0.05$).

the control in the absence of NAA (Table 2). On the other hand, in the presence of NAA and at the higher concentrations of KIN in the nutrient media, the lowest number of shoots per explant in *M. flaviflorus* was observed. Explant survival and rooting were not affected by the different combinations of plant growth regulators, as *M. flaviflorus* achieved high rates in all of them (Table 2).

There was no interaction between NAA concentrations and KIN concentrations in any of the analyzed variables for *M. polyanthus*, but isolated these regulators interfered significantly in the number of shoots per explant ($p=0.0016$ and $p=0.0000$, respectively) and in explant oxidation ($p=0.0001$ and $p=0.0000$, respectively). As it was observed in *M. flaviflorus*, in the presence of NAA the two highest KIN concentrations contributed to explant oxidation (Table 3). In regards to *M. polyanthus* shoot production, in the absence of KIN or at its lowest concentration (6.74 µmol $L^{-1}$), together with NAA, shooting was favored. The percentages of explant survival, rooting and shooting in this species were high in all the treatments and were not significantly affected by them (Table 3).

From germination until the propitious moment for multiplication of the targeted cacti species it took 15 or 18 months. Then, from each cactus two cross-cut explants could be obtained. Starting out with 20 plants of each species and taking into account the mean multiplication rates of each species in the media supplemented with NAA, which were 6.4 shoots/explant for *M. flaviflorus* and 3.6 shoots/explant for *M. polyanthus*, within 120 days 256 shoots of *M. flaviflorus* and 144 of *M. polyanthus* can be produced.

Shoots generated from *M. flaviflorus'* explants took longer to be emitted during the experiment of *in vitro* multiplication than those of *M. polyanthus*. The latter, thus, reached greater lengths than those of *M. flaviflorus* (Figure 3), which were, in turn, smaller and more numerous in comparisons at the end of the evaluated period (Figure 4).

**Table 3.** Influence of different concentrations of KIN and NAA on number of shoots per explant, as well as on explant shooting (%), rooting (%), oxidation (%) and survival (%) in *Micranthocereus polyanthus*.

| NAA (µmol $L^{-1}$) | KIN (µmol $L^{-1}$) | | | | |
|---|---|---|---|---|---|
| | **0** | **6.74** | **20.22** | **40.44** | **Means** |
| **Shoots per explant** | | | | | |
| 0 | $2.5^{aB}$ | $2.9^{aB}$ | $2.2^{aA}$ | $1.9^{aA}$ | $2.4^{B}$ |
| 1.34 | $3.6^{abA}$ | $4.6^{aA}$ | $2.6^{bcA}$ | $1.9^{cA}$ | $3.2^{A}$ |
| Means | $3.1^{ab}$ | $3.7^{a}$ | $2.4^{bc}$ | $1.9^{c}$ | - |
| **Shooting (%)** | | | | | |
| 0 | $100.0^{aA}$ | $97.8^{aA}$ | $95.6^{aA}$ | $91.1^{aA}$ | $96.1^{A}$ |
| 1.34 | $97.8^{aA}$ | $100.0^{aA}$ | $95.6^{aA}$ | $92.5^{aA}$ | $96.4^{A}$ |
| Means | $98.9^{a}$ | $98.9^{a}$ | $95.5^{a}$ | $91.8^{a}$ | - |
| **Rooting (%)** | | | | | |
| 0 | $100.0^{aA}$ | $97.8^{aA}$ | $95.6^{aA}$ | $95.6^{aA}$ | $97.2^{A}$ |
| 1.34 | $100.0^{aA}$ | $100.0^{aA}$ | $100.0^{aA}$ | $97.5^{aA}$ | $99.4^{A}$ |
| Means | $100.0^{a}$ | $98.9^{a}$ | $97.8^{a}$ | $96.5^{a}$ | - |
| **Oxidation (%)** | | | | | |
| 0 | $43.3^{bA}$ | $21.1^{cB}$ | $47.8^{bB}$ | $71.1^{aA}$ | $45.8^{B}$ |
| 1.34 | $51.1^{bA}$ | $45.3^{bA}$ | $77.8^{aA}$ | $76.4^{aA}$ | $62.6^{A}$ |
| Means | $47.2^{b}$ | $33.2^{b}$ | $62.8^{a}$ | $73.7^{a}$ | - |
| **Survival (%)** | | | | | |
| 0 | $100.0^{aA}$ | $97.8^{aA}$ | $100.0^{aA}$ | $95.6^{aA}$ | $98.3^{A}$ |
| 1.34 | $100.0^{aA}$ | $100.0^{aA}$ | $100.0^{aA}$ | $97.5^{aA}$ | $99.4^{A}$ |
| Means | $100.0^{a}$ | $98.9^{a}$ | $100.0^{a}$ | $96.5^{a}$ | - |

Means followed by the same letter, lowercase in rows and uppercase in columns, do not differ significantly by the Tukey test ($p>0.05$).

Apical dominance break through apex removal is still the most efficient and widely used mode of achieving areolar activation and cactus *in vitro* multiplication (Rubluo et al., 2002; Pérez-Molphe-Balch et al., 2015). The usage of plant growth regulators, however, must be accommodated to each cactus species, varying according to specific genotypes (Lema-Rumińska and Kulus, 2014).

Several cacti are propagated *in vitro* using cytokinins as the sole regulator added to the nutrient media (Dabekaussen et al., 1991; Pérez-Molphe-Balch and Dávila-Figueroa, 2002; Sriskandarajah and Serek, 2004; Ramirez-Malagon et al., 2007; Estrada-Luna et al., 2008; Quiala et al., 2009). The presence of cytokinin increased the *in vitro* production of shoots in cacti such as *Opuntia lanigera* Salm–Dyck, *Pilosocereus robinii* (Lemaire) Byles et Rowley, *Pelecyphora aselliformis* Eerhenberg and *Pelecyphora strobiliformis* Werdermann (Pérez-Molphe-Balch and Dávila-Figueroa, 2002; Estrada-Luna et al., 2008; Quiala et al., 2009). These results differ from those obtained with *M. flaviflorus* and *M. polyanthus*, for which the addition of KIN singly to the medium did not favor an increased number of shoots per explant. In the case of the cultivars *Rhipsalidopsis gaertneri* X *R. rosea* and *Schlumbergera russeliana* X *S. truncata* it was necessary to exclude auxins and include BAP (6-benzylaminopurine), TDZ (thidiazuron) and zeatin together (all at 27 µmol $L^{-1}$) to generate the highest number of shoots per explant (Sriskandarajah and Serek, 2004).

The application of NAA on the propagation of *M. polyanthus* and *M. flaviflorus*, nevertheless, promoted the induction of shoots when applied alone (in *M. flavilforus* and *M. polyanthus*) or in combination with low concentrations of KIN (6.74 µmol $L^{-1}$) (in *M. polyanthus*). Other cacti did not have their micropropagation enhanced by the presence of auxins in the nutrient media (Dabekaussen et al., 1991; Ramirez-Malagon et al., 2007). This was the case for 10 species of *Mammillaria*, for which higher concentrations of KIN (27.8 µmol $L^{-1}$ and 46.5 µmol $L^{-1}$), in the presence or absence of indole-3-acetic acid (IAA), increased shoot formation (Ramirez-

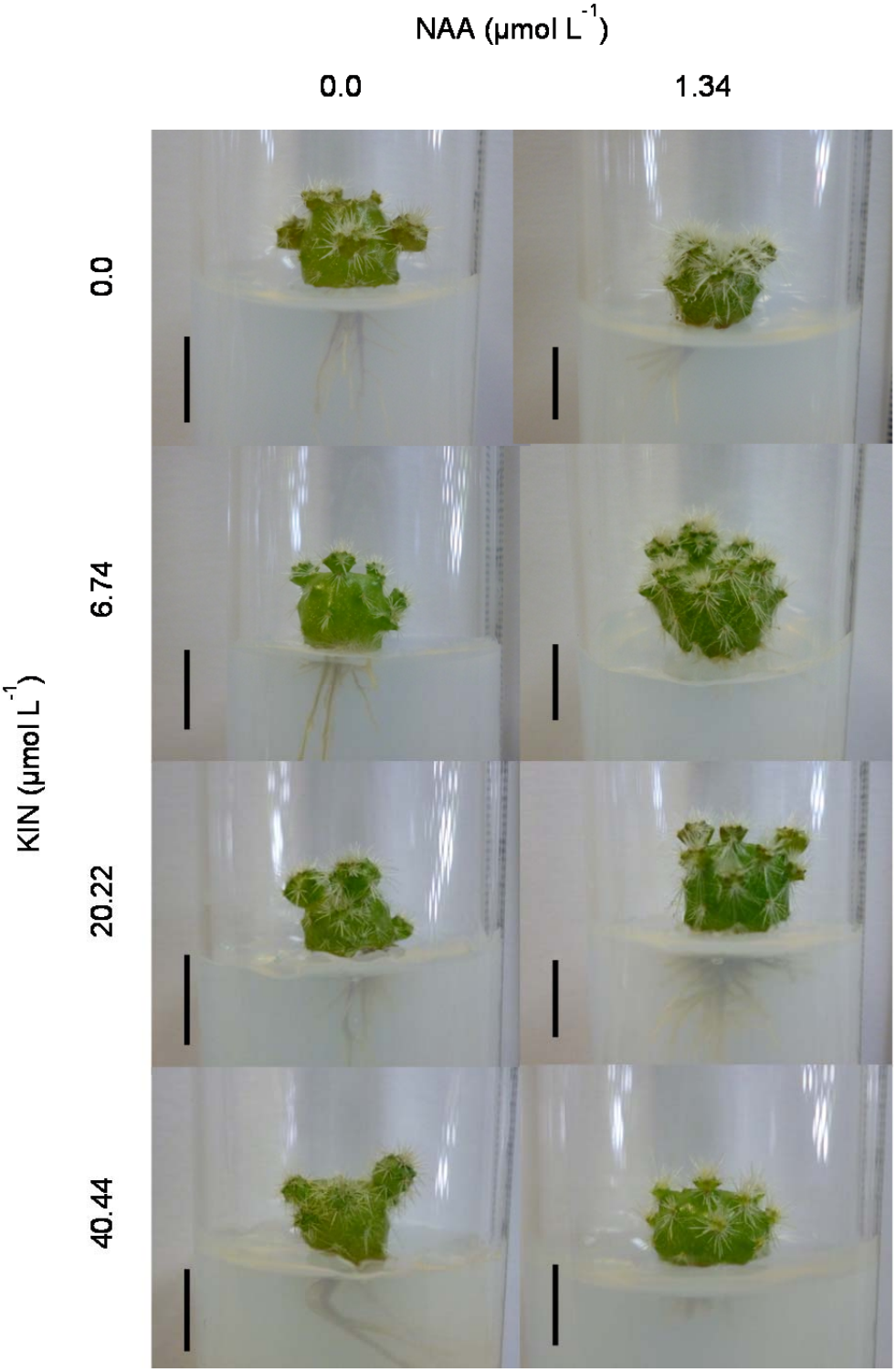


**Figure 3.** Organogenic response of explants from *Micranthocereus flaviflorus* to different concentrations of KIN and NAA combined (Scale indicates 1 cm).

Malagon et al., 2007), and for *Sulcorebutia alba* Rausch, in which auxin did not cause significant changes in propagation (Dabekaussen et al., 1991). However, *M. flaviflorus* and *M. polyanthus* were not the only cacti to have their *in vitro* multiplication benefited by the presence of auxins in the nutrient media: Rubluo et al. (2002) also observed areole activation in *Mammillaria san-angelensis* under the influence of exogenous auxins. The use of 1.34

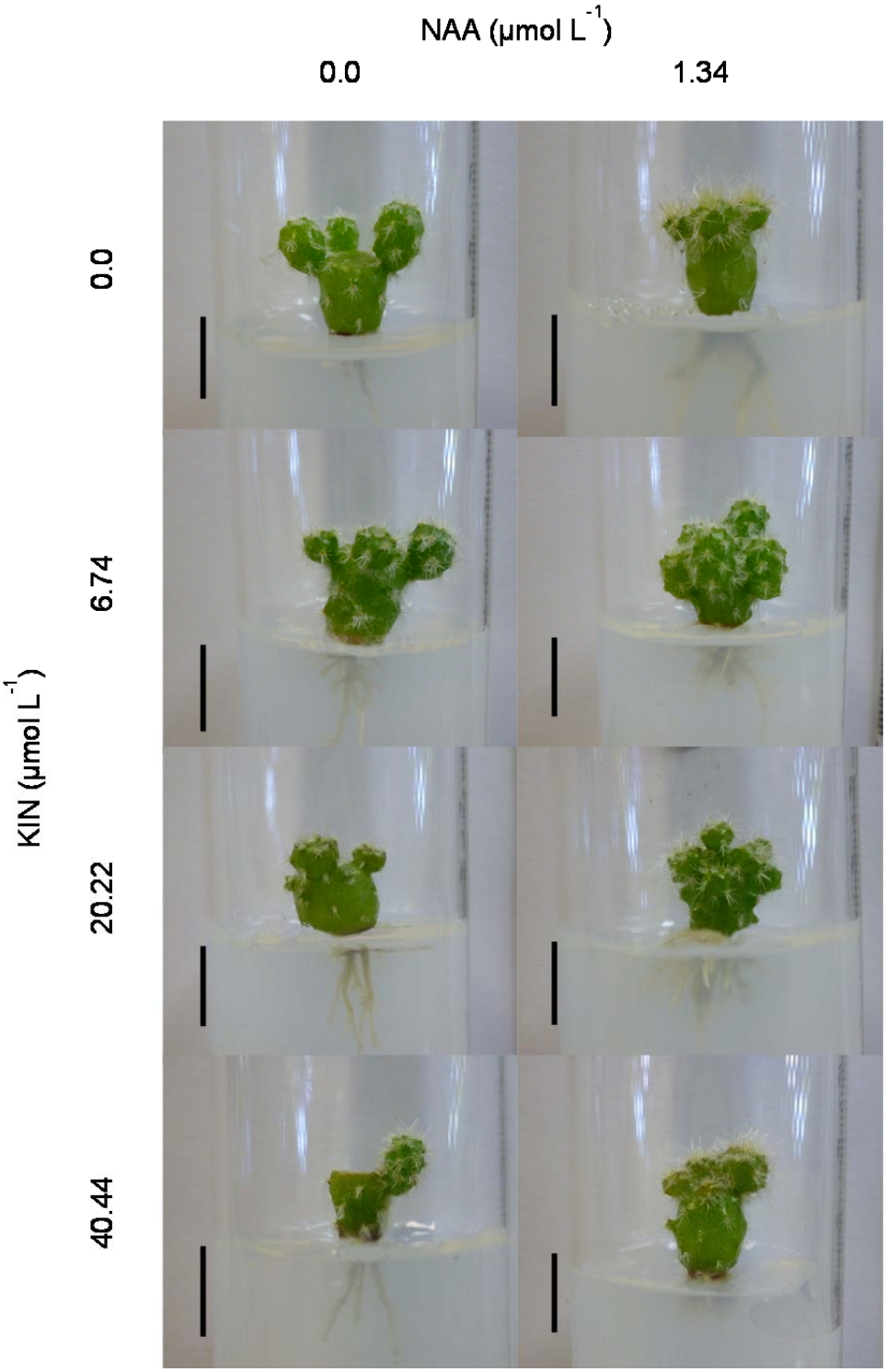


**Figure 4.** Organogenic response of explants from *Micranthocereus polyanthus* to different concentrations of KIN and NAA combined (Scale indicates 1 cm).

µmol $L^{-1}$ of NAA potentiated the multiplication of *P. gounellei* and *S. luetzelburgii* as well (Marchi, 2012). Furthermore, similarly to *M. polyanthus*, another 11 rare or threatened species of cacti from different genera [*Escobaria missouriensis* (Sweet) D.R. Hunt, *Mammillaria wrightii* Engelm., *Sclerocactus spinosior* (Engelm.) D Woodruff and L.D.Benson, *Pediocactus despainii* S.L.Welsh and Goodrich, *P. paradinei* B.W.Benson, *P.*

**Table 4.** Survival (%) and rooting (%) of acclimatized *Micranthocereus flaviflorus* shoots.

| Shoot size (cm) | Survival (%) | Rooting (%) |
|---|---|---|
| 0.2 - 0.3 | 0[b] | 0[b] |
| 0.4 - 0.5 | 44[a] | 17[b] |
| 0.6 - 0.7 | 85[a] | 67[a] |

Means followed by the same letter do not differ significantly by the Tukey test (p>0.05).

*winklerii* K.D.Heil., *Toumeya papyracantha* Britton and Rose, *E. robbinsorum* (W.H.Earle) D.R.Hunt, *S. mesae-verdae* (Boissev. and C.Davidson) L.D.Benson, *P. bradyi* L.D.Benson and *P. knowltonii* L.D.Benson] achieved higher shooting rates with cytokinin combined with low concentration of auxin in the nutrient medium (Clayton et al., 1990).

For the *in vitro* multiplication of *M. flaviflorus* and *M. polyanthus*, the increase in cytokinin concentration did not increment shooting, since in both species the increase in KIN concentrations in nutrient media in the presence of NAA coincided with a decrease in the number of shoots per explant. Studies suggest, however, that in plant tissue culture a balance in auxin and cytokinin concentrations is favorable to cacti *in vitro* multiplication (Lema-Rumińska and Kulus, 2014). Shoot and root differentiation is the outcome of the interaction between cytokinin and auxin hormones, while said interaction depends upon the concentrations produced by the plant tissues themselves and those of exogenous regulators added to the nutrient media (Juárez and Passera, 2002). The highest concentrations of auxins in the propagation of *Cereus peruvianus* Mill. induced superior rates of adventitious shooting only in the presence of cytokinins (Machado and Prioli, 1996), analogously to the micropropagation of *Opuntia ellisiana* Griff., in which a higher percentage of activated areoles was obtained with the employment of BAP and indole-3-butyric (IBA) combined (Juárez and Passera, 2002).

Exogenous growth regulators are used aiming to promote areolar activation and induce shooting in explants, but as endogenous levels of plant hormones may vary from one species to the next, a species-specific tissue response is expected to exogenous growth regulators, seeing as concentrations of these regulators affect individual homeostasis and physiological processes (Juárez and Passera, 2002). Therefore, the addition of cytokinins may be unnecessary or even detrimental to shooting in *M. flaviflorus* and *M. polyanthus*, which would explain the obtained results for these cacti. Studies indicate that cacti have naturally high levels of endogenous auxins and cytokinins (especially those derived from isopentenyl), which could influence their regenerative potential (Clayton et al., 1990; Sriskandarajah et al., 2006). Adding auxin in low concentration to the nutrient media, with or without KIN at 6.74 µmol $L^{-1}$, may have been efficient in acting together with the hormones present in explant tissues and stimulating shooting in both *M. flaviflorus* and *M. polyanthus*.

Hormonal homeostasis in plants is modulated by enzyme activity which is also species-specific (Sriskandarajah et al. 2006). The measurement of such activity, as well as endogenous concentrations of cytokinins and auxins, in tissues of *M. polyanthus* and *M. flaviflorus* may elucidate the questions here arisen, since the balance between these hormones is more propitious to shooting when there is a higher proportion of cytokinins in relation to auxins (Phillps, 2004; Sriskandarajah et al. 2006**;** Lema-Rumińska and Kulus, 2014). Tests with other auxins are also suggested to investigate if this stimulus can be optimized. Combinations with other cytokinins and auxins could be tested as well, although it could end up compromising explant metabolism and induce callus formation and hyperhydricity, neither of which occurred in the present work

Micropropagation is, therefore, an alternative to produce plants of *M. flaviflorus* and *M. polyanthus,* being recommended 1.34 µmol $L^{-1}$ of NAA for both species.

## Shoot acclimatization of *Micranthocereus flaviflorus* and *M. polyanthus*

The longest shoots from each species achieved the highest survival and rooting rates, whereas the smallest shoots did not survive in either species (Tables 4 and 5). The low rooting percentage of *M. flaviflorus* shoots did not prevent their survival (Table 4). Concomitantly, *M. polyanthus* shoots showed similar survival and rooting rates within each size class (Table 5), which indicates that rooting, besides size, could be a determining factor in the acclimatization of these shoots.

Numerous factors can affect acclimatization success of *in vitro* derived shoots, such as temperature and humidity *ex vitro*, substrate, previous *in vitro* rooting of plants, stomata functionality, cuticle presence and amount of water and nutrient reserves in plant tissues (Malda et al., 1999; Cavalcanti and Resende, 2006). In the *in vitro* environment plants usually do not retain normal control of the opening and closing of stomata, neither do they

**Table 5.** Survival (%) and rooting (%) of acclimatized *Micranthocereus polyanthus* shoots.

| Shoot size (cm) | Survival (%) | Rooting (%) |
|---|---|---|
| 0.3 - 0.5 | 0[c] | 0[c] |
| 0.6 - 0.9 | 33[b] | 28[b] |
| 1.0 - 1.5 | 67[a] | 61[a] |

Means followed by the same letter do not differ significantly by the Tukey test (p>0.05).

produce a significant quantity of cuticle, and their photosynthesis does not surpass a basal level (Malda et al., 1999). These factors can interfere in the transition from *in vitro* to *ex vitro*. It is generally necessary to perform gradual changes in humidity and luminosity in order for plants to survive (Rout et al., 2006).

Shoots from *M. flaviflorus* and *M. polyanthus* did not go through a stage of previous *in vitro* rooting, something that has be demonstrated as an unnecessary stage for the acclimatization of some cacti such as *Melocactus glaucescens* (Resende et al., 2010) and *Stephanocereus luetzelburgii* (Marchi, 2012, 2016). *In vitro* produced roots do not possess the same quality as those generated *ex vitro*, which develop in contact with the substrate and tend to form a more complete root system with a larger number of secondary roots (Grattapaglia and Machado, 1998). Nonetheless, various species have had success in acclimatization with shoots previously rooted *in vitro*, like *Turbinicarpus laui* Glass et Foster (Rosas et al., 2001), *Opuntia ellisiana* (Juárez and Passera, 2002), *Pelecyphora aselliformis* (Pérez-Molphe-Balch and Dávila-Figueroa, 2002), *Pelecyphora* strobiliformis (Pérez-Molphe-Balch and Dávila-Figueroa, 2002), *Ariocarpus kotschoubeyanus* (Lem.) K.Schum. (Moebius-Goldammer et al., 2003), *Opuntia lanígera* Salm–Dyck (Estrada-Luna et al., 2008), *Pilosocereus robinii* (Quiala et al., 2009), *Melocactus glaucescens* (Resende et al., 2010) and *Pilosocereus gounellei* (Marchi, 2012).

Rooting may be directly related to shoot size and survival. Acclimatized shoots of *Melocactus glaucescens* without any previous rooting were significantly smaller than those *in vitro* rooted, achieving a significantly lower survival rate (63.33%) than that of previously rooted shoots (86.67% to 90.00%) (Resende et al., 2010). Consequently, the assumption can be made that a previous stage of rooting can increase survival rates in acclimatization. Simultaneously to *in vitro* root formation, however, happens shoot growth, which causes an uptake in mass and, therefore, in the shoot's reserve tissues. Thus, it is possible that not only *in vitro* rooting of cacti may affect their survival during acclimatization, but that *in vitro* growth may also be a crucial factor in this matter. Seedlings of *C. jamacaru* with a higher number of lateral buds (i.e. taller plants) developed better in the field, exhibiting higher values for phytomass and dry weight (Cavalcanti and Resende, 2006).

Regarding *M. flaviflorus* and *M. polyanthus*, the longer the shoots, the greater their *ex vitro* rooting and survival rates. The smallest class tested did not survive acclimatization, which was also observed in shoots of *S. luetzelburgii* ≤ 2 mm long (Marchi, 2012). Such result was attributed to their difficulty in attaching themselves to the substrate and emitting roots (Marchi, 2012). In this same species, however, other size classes tested (8 to 11 mm, 5 to 7 mm and 3 to 4 mm) obtained high survival rates (85% to 100%), which is in accordance with the results of the biggest size classes tested for *M. flaviflorus* (0.6 to 0.7 mm; 85%) and *M. polyanthus* (1.0 to 1.5 mm; 67%).

## Conclusions

The nutrient media recommended for *in vitro* germination of *M. flaviflorus* is MS/2 supplemented with sucrose at 15 g $L^{-1}$, and the temperature regime of 25°C is suggested as it is standard in growth rooms. For *in vitro* multiplication of *M. flaviflorus* and *M. polyanthus*, the employment of 1.34 µmol $L^{-1}$ of NAA is recommended. Moreover, shoot size is determinant in acclimatization survival, so the use of shoots with lengths of ≥0.6 cm and ≥1.5 cm for *M. flaviflorus* and *M. polyanthus*, respectively, is suggested..

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

## ACKNOWLEDGEMENTS

The authors would like to thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for financial support.

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