Micropropagation and Reintroduction of the Endemic *Mammillaria mathildae* (Cactaceae) to Its Natural Habitat

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Abstract. A rapid shoot multiplication protocol was established for the endangered cactus *Mammillaria mathildae* to reintroduce it to its natural habitat. In vitro-germinated seedlings were used as the source of explants. Three explant sources (apical, lateral, and basal excised from in vitro-germinated seedlings) were tested. Shoot multiplication was induced in Murashige and Skoog (MS) medium supplemented with different 6-benzylaminopurine/indole-3-acetic acid (BA/IAA) combinations (0, 22.19, 44.39 and 0, 1.43, 2.85, 5.71, respectively). Explants developed abundant callus in the presence of any BA/IAA concentration, whereas hormone-free media produced 0.59 ± 0.11 new shoots (with a 41% callus development) from basal explants. Apical and lateral explants produced 1.14 ± 0.07 and 4.09 ± 0.13 new shoots, respectively, without callus generation. Plantlets originating from lateral explants developed a vigorous rooting system after 2 months growing on MS medium supplemented with $30 \text{ g} \cdot \text{L}^{-1}$ sucrose. Under greenhouse conditions, 98% of micropropagated *M. mathildae* survived. Plantlets were reintroduced in an experimental plot near to Juriquilla's wild population of *M. mathildae*; over 52% of the outplanted *M. mathildae* lot declined after 5 months. Water availability was associated with the decline of outplanted populations during the first month (43%).

México has an exceptional diversity in the Cactaceae family, from which 80% are endemic species (Arias, 1993). Most cacti are endangered as a result of low growth and reproduction rates and anthropogenic threats such as habitat destruction, overcollection, livestock and agriculture, and others. Many of these plants are protected by the Mexican Federal Government NOM-059-ECOL-2001 (SEMARNAT, 2002). *Mammillaria* species in particular are collected from the wild, generating strong pressure on natural populations. *M. mathildae* is an endemic cactus to Querétaro State in central México. Its populations are confined to eight localities (García and Malda, 2010), and only three reside in natural protected areas. The largest population of *M. mathildae* is found at Cañada Juriquilla with 209 plants registered in 2003 but declining to 133 in 2007 (García and Malda, 2010).

In the last decades, tissue culture has been implemented to propagate many threatened

and endangered cacti, e.g., *Obregonia denegrii* (Malda et al., 1999); *Coryphantha elephantidens* (Wakhlui and Bahu, 2000); *Mammillaria elongata* (Papafiotou et al., 2001); *Pelecypophora* spp. (Pérez-Molphe-Balch and Dávila-Figueroa, 2002); *Ariocarpus kotschoubeyanus* (Moebius-Goldammer et al., 2003); *Turbidnicarpus* spp. (Dávila-Figueroa et al., 2005); *Notocactus magnificus* (de Medeiros et al., 2006); 10 different *Mammillaria* spp. (Ramírez-Malagon et al., 2007); and so on. Although a general goal is that in vitro propagation may reduce overcollecting by commercialization of plants, no reports have shown a reduction of collection of wild specimens. Micropropagation techniques can also be used to re-establish wild populations that have been decimated or extirpated (Sarasan et al., 2006).

Micropropagation protocols commonly use an exogenous growth regulator to induce morphogenetic responses, particularly for the *Mammillaria* family (Papafiotou et al., 2001; Poljuha et al., 2003; Ramírez-Malagon et al., 2007). Growth regulator morphogenetic response commonly varies, so auxin–cytokinin ratio must be empirically determined for each species. It is known that the addition of exogenous growth regulators, the prolonged in vitro cultivation, and indirect organogenesis are factors that induce somaclonal or epigenetic variations in micropropagated plants (Podwyszyńska, 2005). In contrast, micropropagation systems based on areole activation (direct organogenesis) are considered to obtain more genetically stable plants (Machado

and Prioli, 1996) and therefore are a better plant material to achieve successful reintroduction. This study aimed to develop a protocol for in vitro propagation of *M. mathildae* followed by greenhouse acclimatization and reintroduction to Cañada Juriquilla's natural population to assess cactus' short-term survival rate.

Materials and Methods

Plant material. *M. mathildae* seeds were collected from 62 plants (57.4% of the population) in the Cañada Juriquilla locality, Querétaro, México; and in vitro culture was initiated from a seed mix to maximize the representation of the gene pool. Seeds were vigorously washed in a vortex (2500 rpm) with 1% of a commercial liquid surfactant (containing alkyl ether sulfate ethoxylate) and rinsed three times with distilled water and then disinfected under aseptic conditions for 3 min in 3% H_2O_2 followed by three washes in sterile distilled water, 2 min in 1% AgNO_3 , and finally three washes in sterile distilled water. Seeds were aseptically germinated in 120-mL jars capped with polypropylene caps (Magenta Corp., Chicago, IL) containing 30 mL half-strength Murashige and Skoog (MS) salts (Murashige and Skoog, 1962) supplemented with $15 \text{ g} \cdot \text{L}^{-1}$ sucrose, $0.05 \text{ g} \cdot \text{L}^{-1}$ myo-inositol, $0.5 \text{ mg} \cdot \text{L}^{-1}$ thiamine hydrochloride, $0.25 \text{ mg} \cdot \text{L}^{-1}$ nicotinic acid, $0.25 \text{ mg} \cdot \text{L}^{-1}$ pyridoxine hydrochloride, and $8 \text{ g} \cdot \text{L}^{-1}$ agar (Sigma-Aldrich, Química S.A., Mexico). Culture media was adjusted to pH 5.7 with NaOH. The culture jars were sterilized in an autoclave at $121^\circ\text{C}/103 \text{ KPa}$ for 15 min. After 1 month, seedlings were transferred into full-strength MS salts supplemented with $30 \text{ g} \cdot \text{L}^{-1}$ sucrose, $0.1 \text{ g} \cdot \text{L}^{-1}$ myo-inositol, $1 \text{ mg} \cdot \text{L}^{-1}$ thiamine hydrochloride, $0.5 \text{ mg} \cdot \text{L}^{-1}$ nicotinic acid, $0.5 \text{ mg} \cdot \text{L}^{-1}$ pyridoxine hydrochloride, and $8 \text{ g} \cdot \text{L}^{-1}$ agar. Seedlings were maintained at $26 \pm 1^\circ\text{C}$ under a total photosynthetic photon flux (PPF) of 120 to 130 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, provided by cool fluorescent lamps, in a 16-h photoperiod. Seedlings were transferred every 4 weeks to fresh medium.

Shoot induction. Three-month-old *M. mathildae* seedlings (15 mm) germinated in vitro were used as the source of explants for shoot induction experiments. Three explant types were tested: apical (5 mm), lateral (longitudinally cut 7-mm shoots without apex), and basal segments ($\approx 3 \text{ mm}$ wide). The cut-exposed areas of explants were placed in MS medium supplemented with $30 \text{ g} \cdot \text{L}^{-1}$ sucrose, $0.1 \text{ g} \cdot \text{L}^{-1}$ myo-inositol, $1 \text{ mg} \cdot \text{L}^{-1}$ thiamine hydrochloride, $0.5 \text{ mg} \cdot \text{L}^{-1}$ nicotinic acid, $0.5 \text{ mg} \cdot \text{L}^{-1}$ pyridoxine hydrochloride, and $8 \text{ g} \cdot \text{L}^{-1}$ agar and with different cytokinin–auxin concentrations: 0, 22.19, and $44.39 \mu\text{M}$ 6-benzylaminopurine (BA) combined with 0, 1.43, 2.85, and $5.71 \mu\text{M}$ indole-3-acetic acid in a 3×4 factorial design to select the most efficient combination for shoot production. Eleven apical and basal as well as 22 lateral replicates per treatment were tested. New shoot proliferation per explant and callus generation were recorded after 60 d of induction.

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Rooting of shoots. Rooting was induced in 10- to 15-mm long new shoots cultured using previously described half- or full-strength MS medium supplemented with various concentrations of indole-3-butyric acid (IBA) (0, 4.9, 9.8, 19.7, and 29.5 μM) in a 2×5 factorial design. Fifteen shoots originating from lateral explants per treatment were used. Root production was assessed every 2 weeks after initiation of experiment. Shoots developing at least three roots 15 mm long were scored positively.

Greenhouse acclimatization and field reintroduction. Rooted plants were transplanted to plastic containers with a mixture of commercial potting soil (1:1 Pro-Moss, Premier Tech Ltd., Québec, Canada and Hortiperl, Termolita S.A. de C.V., Nuevo León, México), in 2.5-cm diameter pots, covered for the first week with a plastic canopy, and watered every third day to prevent desiccation and allow acclimatization. Plants were maintained at $26 \pm 1^\circ\text{C}$ under a total PPF of 120 to 130 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ in a 16-h photoperiod and were transferred to a greenhouse by the end of the second week. For acclimatization, plantlets were watered every week during 2 months before transfer into the field. Reintroduction was performed in October at the end of the rainy season; 100 plantlets were transferred to Cañada Juriquilla in a $75 \times 10\text{-m}$ experimental plot. Plants were measured (diameter and height) and systematically tagged. Survival percentages were determined every other day the first week followed by weekly observations for a month, and finally monthly observations along a 6-month period.

Reintroduction area. From the five known populations of *M. mathildae*, only two are under federal protection (García and Malda, 2010). Cañada Juriquilla locality, a natural protected area, was selected as a result of favorable conditions to achieve reintroduction (because deciduous tropical forest presents a good conservation status). This site is located north of Querétaro City in the province of Juriquilla (long. $20^\circ 41' 35.2''$ N, lat. $100^\circ 27' 16.4''$ W; alt. 1890 m). It encloses the largest registered population of *M. mathildae* (133 individuals in 2007). To study the effect of daily precipitation on survival rate, we compared *M. mathildae*'s decay in situ against rain data. Precipitation patterns were obtained from CONAGUA's (National Ministry of Water) in meteorological station "Querétaro" (long. $20^\circ 41' \text{ N}$, lat. $100^\circ 27' \text{ W}$).

Statistical analysis. New shoot production and rooting percentage were analyzed with analysis of variance ($P < 0.05$). Micropropagation experiments were performed twice. Statistical analyses were carried out using JMP 6.0 statistical software (SAS Institute, Inc., Cary, NC).

Results

Shoot induction. In vitro germination was 91% in contrast to the 40% germination rate registered in its natural habitat (García, 2009). Highest germination rates for *M. mathildae* were observed during the second and third weeks. Morphogenetic responses of the three

explant types (basal, lateral, and apical portions of 3-month-old seedlings) are presented in Table 1. All explant types developed a profuse, compact, greenish callus with light pink portions in the surface as a response to growth regulators combination (Fig. 1A). Meanwhile 18%, 11%, and 9% of apical, lateral, and basal explants, respectively, yielded new shoots under low BA concentrations (Table 1). In contrast, without any exogenous growth regulators, apical and lateral explants spontaneously produced new shoots, although explant type resulted in different shoot yields ($F = 237.7$ Prob. $> F < 0.0001$). Basal explants formed fewer shoots (0.59 ± 0.11), although they also developed callus (41%). Apical and lateral explants produced 1.14 ± 0.07 and 4.09 ± 0.13 new shoots, respectively (Fig. 1B) without callus generation after 2 months in culture (Table 1).

Rooting induction. Full-strength MS and half-strength MS medium with and without IBA tested for rooting new shoots is shown in Table 2. Explants cultured in full-strength MS medium showed a significant difference in rooting percentage compared with half-strength MS ($F = 12.2$ Prob. $> F < 0.0009$), although they did not show significant differences in root length ($F = 0.6817$ Prob. $> F < 0.5106$). A vigorous rooting system was observed in 7 to 8 weeks (Fig. 1C–D).

Reintroduction. Establishment of plantlets to ex vitro conditions presented no significant problems, and 98% of in vitro-derived plants survived in greenhouse conditions (Fig. 1E), during 8 weeks, before their transplant to the wild.

M. mathildae grows in pronounced slopes; hence, medium-intensity precipitation could dislodge reintroduced plants. In addition, when rainfalls reached maximum intensity, herbivory resulted in deterioration of young plants. Our observations show that 34.2% of Cañada Juriquilla's population experienced terrestrial isopods herbivory (Fig. 2C). For these reasons, *M. mathildae* reintroduction was performed in early Oct. 2007, at the end of the rainy season, when insect populations also decrease. Reintro-

duced plants diminished 43% through the first month (Fig. 1F), and subsequently the lot decreased only 9% in the next 3 months. After 5 months, 52% of reintroduced plants died (Fig. 3).

Discussion

***Mammillaria mathildae* micropropagation.** Growth regulator-free MS medium was enough for in vitro shoot production of *M. mathildae*, contrary to several results reported for other *Mammillaria* species (Papafotiou et al., 2001; Poljuha et al., 2003; Ramirez-Malagon et al., 2007), where growth regulator complementation, especially cytokinins, is crucial for shoot generation.

These findings suggest that endogenous levels of growth regulators present in *M. mathildae* juvenile tissues are sufficient enough to promote shoot growth. Bertsouklis et al. (2003) found that 100% of *Globularia alypum* excised explants, from 2-month-old seedlings grown in vitro, yield shoots on MS supplemented with $20 \text{ g}\cdot\text{L}^{-1}$ sucrose without exogenous plant growth regulators. Additionally, rooting was observed in 50% of the shoots maintained in the same free growth regulator medium during a 2-month period. In addition, it is known that cacti have the capacity to synthesize auxins autonomously in vitro (Clayton et al., 1990); therefore, the addition of exogenous auxins often stimulates callus production. This could be a disadvantage for micropropagation systems because hormone supplementation could promote genetic changes in plants (Lakshmanan et al., 2007). Although such variation is of particular concern for conservation (Sarasan et al., 2006), because it is possible that genetic variability induced by tissue culture might be beneficial, favoring the survival of the species on restoration in their natural environment (Giusti et al., 2002). Palomino et al. (1999) demonstrated karyological stability of tissue-cultured *M. san-angelensis* despite their long-term in vitro subculturing and auxin supplementation. However, karyological analysis cannot reveal alterations in specific genes or small chromosomal

Table 1. Effect of 6-benzylaminopurine (BA) and indole-3-acetic acid (IAA) on shoot and callus formation on explants of *M. mathildae*.^z

Concn (μM)		Shoot no. per explant cultured (mean $\pm$ SE)			Callogenesis (%)		
BA	IAA	Apical	Lateral	Basal	Apical	Lateral	Basal
0	0	1.14 ± 0.07 a1 ^y	4.09 ± 0.13 a2	0.59 ± 0.11 a1	0 a1	0 a1	40.9 a2
0	1.43	0 b	0 b	0 b	100 b	100 b	100 b
0	2.85	0 b	0 b	0 b	100 b	100 b	100 b
0	5.71	0 b	0 b	0 b	100 b	100 b	100 b
22.19	0	0.18 ± 0.08 bc	0.11 ± 0.05 bc	0.09 ± 0.06 bc	77.3 c	88.6 c	90.9 c
22.19	1.43	0 b	0 b	0 b	100 b	100 b	100 b
22.19	2.85	0.05 ± 0.05 bc	0 b	0 b	95.5 b	100 b	100 b
22.19	5.71	0 b	0 b	0 b	100 b	100 b	100 b
44.39	0	0 b	0 b	0 b	100 b	100 b	100 b
44.39	1.43	0 b	0 b	0 b	100 b	100 b	100 b
44.39	2.85	0 b	0 b	0 b	100 b	100 b	100 b
44.39	5.71	0 b	0 b	0 b	100 b	100 b	100 b

^zResults are the average of two separate experiments.

^yMeans followed by the same letters within columns are not significantly different according to analysis of variance ($P < 0.05$). Means followed by the same number between columns are not significantly different according to analysis of variance ($P < 0.05$).

Fig. 1. Micropropagation and reintroduction of *M. mathildae*. (A) Callus proliferation in the presence of growth regulators; (B) spontaneous shoot proliferation from lateral explants on Murashige and Skoog (MS) medium after 2 months in culture; (C) rooting of shoots after 8 weeks in culture on MS medium plus 30 g·L⁻¹ sucrose; (D) 7-month-old micropropagated plantlets; (E) plantlet acclimatization in commercial potting soil; and (F) reintroduced plants after 5 months in the experimental plot.

Table 2. Effect of Murashige and Skoog (MS) medium and indole-3-butyric acid (IBA) on root formation from lateral explants.^z

MS strength	IBA (μM)	Root formation (mean ± SE)	Root length (cm) (mean ± SE)	Callogenesis (%)
Full-strength	0.0	0.97 ± 0.03 c ^y	6.99 ± 0.12 a	0 a
Full-strength	4.9	0.10 ± 0.06 a	6.83 ± 0.38 a	90 b
Full-strength	9.8	0 d	—	100 b
Full-strength	19.7	0 d	—	100 b
Full-strength	29.5	0 d	—	100 b
Half-strength	0.0	0.63 ± 0.09 b	6.77 ± 0.15 a	0 a
Half-strength	4.9	0 d	—	100 b
Half-strength	9.8	0 d	—	100 b
Half-strength	19.7	0 d	—	100 b
Half-strength	29.5	0 d	—	100 b

^zResults are the average of two separate experiments.

^yMeans followed by the same letters within columns are not significantly different according to analysis of variance ($P < 0.05$).

rearrangements (Lakshmanan et al., 2007; Rout et al., 1998). Nevertheless, long-term effects of possible genetic variations are indeterminate and their repercussions over ecosystems are largely unknown. In addition, *M. mathildae* mean shoot production by direct organogenesis was low in contrast to other species like *M. san-angelensis*, which reached yields of 21 to 35 shoots per callus explants (Martínez-Vázquez and Rubluo, 1989). Therefore, shoot proliferation derived from direct organogenesis in the absence of external growth regulators may be a better strategy to obtain parent material to restore *M. mathildae* or other species into natural environments. On this basis, using low yields of genetically stable

plants versus high yields of possibly genetically altered plants for reintroduction represents an affordable long-term cost–benefit strategy.

Although growth regulators have been used for root induction of cacti (Fay and Gratton, 1992), *M. mathildae* rooted spontaneously in growth regulator-free MS medium. This is a frequent result reported for many other cacti rooting in vitro on auxin-free media, e.g., *Coryphantha elephantidens* (Wakhlú and Bahu, 2000); *Turbinicarpus* spp. (Dávila-Figueroa et al., 2005); *Notocactus magnificus* (de Medeiros et al., 2006); and in particular those species that root freely in vivo such as *Opuntia amyclaea* (Escobar et al., 1986); *Agave parrasana* (Santacruz-Ruvalcaba et al., 1999);

Hylocereus undatus (Mohamed-Yasseen, 2002); and *Agave tequilana* (Valenzuela-Sánchez et al., 2006). Conversely, any hormone addition induced profuse callus formation in *M. mathildae*.

M. mathildae exhibits a profuse shoot and root regeneration capability in the wild. An interesting phenomenon observed during in vitro rooting was the spontaneous shoot development from roots (Fig. 2A). This phenomenon is not extraordinary, because *M. mathildae* is capable of regenerating a whole plant from its roots after complete shoot removal (Fig. 2B). After some mechanical damage, *M. mathildae* produces many ramets from both roots and shoots (personal observation). Sriskandarajah et al. (2006) studied the regenerative capacity in two cacti, *Rhipsalidopsis* and *Schlumbergera*. They found that during in vitro subcultures, an enhanced auxin metabolism in combination with the increased cytokinin oxidase–dehydrogenase activity shifts the auxin and cytokinin pool, favoring adventitious shoot formation in *Rhipsalidopsis*. Meanwhile, a low level of peroxidase activity and auxin autotrophy–conjugation makes *Schlumbergera* more recalcitrant. *M. mathildae* regeneration capacity enables this plant to persist in the wild, because they augment their biomass and eventually increase their seed yield. It is possible that root formation could be associated with high endogenous hormone levels in *M. mathildae*. However, further studies will be necessary to confirm this suggestion.

Reintroduction. Successful reintroductions such as those of *M. san-angelensis* reporting 91% survival (Rubluo et al., 1993) or those of *Mammillaria pectinifera* and *Pelecypora aselliformis* (Giusti et al., 2002) were achieved in botanical gardens where specimens are constantly maintained. In contrast, when cacti reintroduction into a natural habitat takes place, significant losses (sometimes the whole lot) occurs (Contreras and Valverde, 2002; Leirana-Alcocer and Parra-Tabla, 1999). Decruze et al. (2003) reported a successful reintroduction of the orchid *Vanda spathulata* in the wild with a survival rate between 50% and 70%; and population decline was caused by abiotic factors (direct sunlight and high wind velocity). In a different study, Stiling et al. (2000) established that *Opuntia corallicola* restoration reached 65% of surviving individuals, and the principal cause of decline was stem browning (bacterial origin).

Reintroduced plants abruptly decreased 15 d after the last rain (Fig. 3). Absence of rain after reintroduction may have had an impact on survival of cactus. Under water stress, stomatal and cuticular leaf components play a significant role in plant water balance. Suppression of wax yield in tissue culture plants is associated with high humidity and low light intensity conditions (Shepherd and Griffiths, 2006). Malda et al. (1999) showed that in vitro-derived cacti registered low amounts of epicuticular waxes; and only after 3 months of acclimatization did these plantlets reach similar wax levels to those of mature plants in the

greenhouse. Furthermore, no correlation was found between survival percentages and epicuticular wax content (Malda et al., 1999). However, acclimatization was performed under greenhouse conditions with regular watering (every 5 d). Early observation of *M. mathildae* persistence in the wild suggests that

Fig. 2. (A) *M. mathildae* plantlet showing the appearance of shoots (S) from roots 3 months after root emergence; (B) plant (P) regenerated from root (R) in the wild; and (C) plant damaged by herbivorous (H) terrestrial isopods (sow bugs).

absence of rainy days led to poor field establishment of micropropagated plants.

Generally, under wild conditions, plants associate with a wide diversity of bacteria and fungi symbionts (Brundrett, 2002). Mycorrhizal fungi play a fundamental role in plant health by increasing phosphate absorption and water uptake (Sylvia et al., 2003). Goicoechea et al. (2004) found that mycorrhizal fungi conferred greater drought responsiveness in *Anthyllis cytisoides*, by inducing both epicuticular wax deposition and leaf abscission. The authors stated that this phenomenon may constitute an ecological adaptation to cope with severe drought.

Water uptake capacity of reintroduced plants is low compared with wild plants naturally colonized by mycorrhizal fungi. Therefore, it is probable that lack of such beneficial symbionts in *M. mathildae* micropropagated plants resulted in an unfavorable condition when they were reintroduced. For these reasons, if micropropagated plants are destined to reduce overcollection of wild populations by their distribution to collectors, then watering would not be an important issue. However, if the fate of such plants is field reintroduction, then water availability becomes a limiting factor. Therefore, for conservation purposes, further research is needed to explore the role of beneficial microorganisms on *M. mathildae* establishment as a factor to increase outplanting survival.

Finally, in vitro morphogenesis observed for *M. mathildae* in our protocol allows plantlet production with a minor risk of possible genetic alterations resulting from free growth regulator culture. Proliferation rates and in vitro plant growth assure an average of five plantlets/seed (1.79 ± 0.04 cm tall, 1.37 ± 0.02 cm wide, and 25.47 ± 0.82 areoles) after 7 months in culture, the equivalent to a 3-year-old wild plant. Therefore, it is expected that this biotechnological approach becomes a useful method for mass production of plants for ecological restoration practices.

Fig. 3. Survival of *M. mathildae* plantlets after 5 months of reintroduction to their natural habitat. Solid line represents precipitation patterns at Cañada Juriquilla.

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