In Vitro Cell.Dev.Biol.—Plant (2014) 50:326–336
DOI 10.1007/s11627-014-9600-x

MICROPROPAGATION

In vitro conservation of *Mandevilla moricandiana* (Apocynaceae): short-term storage and encapsulation–dehydration of nodal segments

Sandra Zorat Cordeiro · Naomi Kato Simas · Anaize Borges Henriques · Alice Sato

Received: 28 February 2013 / Accepted: 17 February 2014 / Published online: 8 March 2014 / Editor: Randy Niedz

Abstract *In vitro* conservation of *Mandevilla moricandiana* was performed by slow-growth storage and encapsulation–dehydration. For slow-growth storage, half- and full-strength Murashige and Skoog (MS) medium and Woody Plant Medium, with or without sorbitol, mannitol, or glucose, were used to test the development of nodal segments and maintenance of plant viability after 6 mo. Recovery was performed using MS medium. The basal medium and carbon source did not interact, and only the carbon source had a significant effect on slow-growth storage and recovery. Sorbitol and glucose, individually or in combination, promoted development of plants with a low multiplication rate during the slow-growth period and a high multiplication rate during the recovery period. For encapsulation–dehydration, nonencapsulated and sodium alginate-encapsulated nodal segments were evaluated to determine their viability after storage at different temperatures. Nonencapsulated nodal segments gave 16.6% recovery after 60 d at 25°C. The effects of preculturing encapsulated nodal segments in MS medium with 0.4 or 0.75 M sucrose followed by dehydration were also tested. Capsules precultured for 48 h in the presence of 0.40 M sucrose and dehydrated to 40% moisture content showed 93.3% recovery. These conditions were then used to store capsules under different temperatures and for different lengths of time. The precultured capsules showed ca. 30% recovery after storage for 30 d at 4°C. Well-developed plantlets regenerated from encapsulated, stored nodal segments were rooted and acclimatized successfully, with 100% survival.

Keywords Alginate encapsulation · Germplasm exchange · *In vitro* culture · Regeneration · Synthetic seeds

This study is part of doctoral thesis of the first author

S. Z. Cordeiro (✉)
Federal University of Rio de Janeiro, Av. Carlos Chagas Filho, 373 K2-032, Cidade Universitária, Rio de Janeiro, RJ 21941-902, Brazil
e-mail: sandrazorat@hotmail.com

N. K. Simas
Department of Natural Products and Food, College of Pharmacy, Federal University of Rio de Janeiro, Av. Carlos Chagas Filho, 373, Cidade Universitária, Rio de Janeiro, RJ 21941-902, Brazil
e-mail: naomisimas@yahoo.com

A. B. Henriques
Laboratory of Plant Development Physiology, Federal University of Rio de Janeiro, Av. Carlos Chagas Filho, 373, Cidade Universitária, Rio de Janeiro, RJ 21941-902, Brazil
e-mail: abh@biologia.ufrj.br

A. Sato
Laboratory of Plant Tissue Culture, Botany Department, Federal University of the State of Rio de Janeiro, Av. Pasteur 458, 414, Rio de Janeiro, RJ 22290-040, Brazil
e-mail: alicesato@unirio.br

Introduction

Mandevilla Lindl. (Apocynaceae, Apocynoideae) includes approximately 150 species in the Neotropical region, and new species are continually being described. Approximately 70 species of *Mandevilla* are found mainly in the Amazon and southeastern regions of Brazil (Rapini *et al.* 2010). This genus has several ethnobotanical citations related to uses in traditional folk medicine (Schultes 1979), but its pharmacological potential originates from the presence of an underground system consisting of a xylopodium with tuberous roots. This xylopodium has an extremely stiff consistency and the ability to produce buds, which have medicinal properties (Metcalfe and Chalke 1950; Appezzato-da-Glória and Estelita 2000; Sales *et al.* 2006).

Interest in *Mandevilla* was spurred by pharmacological studies reporting the use of infusions or alcoholic extracts of xylopodium, either from *Mandevilla velutina* (Mart. ex Stadelm.) Woodson or *Mandevilla illustris* (Vell.) Woodson, as a snake

antivenom. However, unsustainable harvesting practices have led to reduced natural populations of these species. Therefore, strategies for *in vitro* conservation have been applied in order to maintain the genetic diversity of these populations and allow research on their pharmacological properties, including the production of secondary metabolites with pharmacological activities (Calixto *et al.* 1985; Handro *et al.* 1988; Maraschin *et al.* 2000, 2002; Biondo *et al.* 2004, 2007; Bertoni *et al.* 2010). *Mandevilla* has endemic species in impacted areas by predatory extraction and irregular occupation in southeastern Brazil (Wiersema and León 1999; Cordeiro *et al.* 2012). Although these species are promising from both pharmacological and ornamental perspectives, the genus is poorly studied; hence, little is known about the best conditions for seed storage and preservation of collections. *Mandevilla moricandiana* (A.DC.) Woodson has been reported in the *restinga* and rocky grassland areas of some northeastern Brazilian states, as well as in the southeastern region. It is a woody vine with twining and latescent branches. The ornamental potential originates from its inflorescence, which has very showy flowers with a pink and white funnel-shaped corolla and a yellow coralline tube (Woodson 1933; Cordeiro *et al.* 2012), while the pharmacological potential originates from its very well-developed xylopodium.

Germplasm conservation of native species with high ornamental or pharmacological potential has been used to preserve their genetic variability and allow the study of their properties in a controlled environment and is supported by *in vitro* culture techniques (Ford-Lloyd and Jackson 1991; Vasil 1991; Villalobos *et al.* 1991; Bertoni *et al.* 2010). The establishment of an *in vitro* protocol for the conservation of a wild species allows for the creation of reference databases for future studies of other species (Cordeiro *et al.* 2012).

Basically, *in vitro* plant conservation involves changing the culture environment in order to slow or suppress the growth of cells, tissues, and organs. This practice, in turn, maximizes the interval between subcultures, thus reducing manpower, storage space, expense, and possible contamination. The conservation method should allow immediate access to the germplasm of interest and maintain genetic diversity without compromising genetic stability (Engelmann 2004; Shibli *et al.* 2006; Rai *et al.* 2009). *In vitro* conservation methods depend upon the type of culture employed and the laboratory conditions available. Accordingly, several alternative protocols have been developed to adapt to the species conserved, type of disposable explant, and storage time in order to maintain the recovery capacity of the culture (Engelmann 2004; Shibli *et al.* 2006).

The aim of this study was to establish and compare strategies for *in vitro* conservation of *M. moricandiana* to maintain cultivars and germplasm for ornamental and pharmacological uses. To accomplish this, the ideal culture medium for slow-growth germplasm storage was determined, and, as an alternative to cryopreservation, encapsulation–dehydration techniques were tested to establish encapsulation, preculture, and dehydration conditions for storing nodal segments of *M. moricandiana* at low temperatures. Both methods were used in an attempt to expand the options for storage of *M. moricandiana*, since they permitted the full recovery of explant viability.

Materials and Methods

Plant material. *In vitro*-grown plantlets of *M. moricandiana* were used as sources of nodal segments in all experiments. The *in vitro* cultures were obtained from seeds collected in the Restinga de Jurubatiba National Park (Rio de Janeiro, Brazil). The disinfestation of seeds and establishment of *in vitro* cultures, as well as the collection authorization and species identification, were performed as in Cordeiro *et al.* (2012).

Culture media and conditions. Slow-growth storage. The semisolid basal media consisted of Murashige and Skoog (MS) (Murashige and Skoog 1962) salts at full or half strength (MS and ½MS, respectively), supplemented with MS vitamins and 3% sucrose (*w*/*v*), and Woody Plant Medium (Lloyd and McCown 1980) salts at full or half strength (WPM and ½WPM, respectively), supplemented with WPM vitamins and 2% sucrose (*w*/*v*). All culture media were prepared from stock solutions of macro- and micronutrients according to Chawla (2002) and were solidified with 0.75% (*w*/*v*) agar. For slow-growth storage, the osmotic agents glucose, mannitol, and sorbitol were added individually or in pairwise combinations at a concentration of 2% (*w*/*v*) each. The salts, vitamins, sucrose, osmotic agents, and agar used in culture media composition were from Sigma-Aldrich (Saint Louis, MO). The pH of each medium was adjusted to 5.8, and the molten media were dispensed into glass bottles (7.5×13.5 cm) and autoclaved for 15 min at 121°C. The medium for growth recovery consisted of semisolid MS basal medium. All cultures were maintained in a growth room at 25±1°C, under a 16/8 h light/dark photoperiod, with irradiance of 23 μmol m^{-2} s^{-1} provided by daylight fluorescent lamps.

Encapsulation and preculture. The gelling medium was prepared with liquid MS basal medium without calcium and supplemented with 3% (*w*/*v*) sodium alginate (Sigma-Aldrich A-2033, medium viscosity). The polymerizing medium was prepared with liquid MS basal medium supplemented with 0.1 M calcium chloride. The preculture of capsules was performed in liquid MS basal medium supplemented with 0.40 or 0.75 M sucrose. The pH of each medium was adjusted to 5.8. All media were autoclaved for 15 min at 121°C. The medium for growth recovery consisted of semisolid MS basal medium. All cultures were maintained in a growth room under the same conditions as described above.

Rooting. The rooting medium consisted of MS salts, supplemented with MS vitamins, 3% sucrose (*w*/*v*), and 2.0 mg L^{-1} indoleacetic acid (IAA) (Cordeiro *et al.* 2012). The medium was solidified with 0.75% agar (*w*/*v*). The pH of the medium was adjusted to 5.8, and the molten medium was dispensed into glass bottles (7.5×13.5 cm) and autoclaved for 15 min at 121°C. All cultures for rooting were maintained in a growth room under the same conditions as described above.

Effect of basal medium and carbon source on slow-growth storage and growth recovery. Nodal segments (1.0 cm) from *in vitro* cultures of *M. moricandiana* were excised and placed in glass flasks containing 100 mL of semisolid MS, ½MS, WPM, or ½WPM medium, with or without glucose, mannitol, or sorbitol. The experimental design was fully randomized in a 4×7 factorial arrangement, consisting of 4 culture media (MS, ½MS, WPM, or ½WPM) with 7 different osmotic treatments: no osmotic agent, one osmotic agent [2% (*w*/*v*) glucose, mannitol, or sorbitol], or a combination of two osmotic agents [sorbitol+mannitol, sorbitol + glucose, or mannitol+glucose] at 2% (*w*/*v*) each. Each treatment consisted of five replicates, where each replicate was a flask with six nodal segments (n=30). After 6 mo in the growth room, plant development was evaluated by measuring survival, plant height, and nodal segments per explant (multiplication rate). At the end of the 6-mo period, plants from the media that allowed >50% survival were selected for the recovery phase. Nodal segments were excised from these plants and placed in semisolid MS basal medium. After 3 mo in the growth room, recovery capacity was evaluated by measuring survival and multiplication rate. The requirement that plant survival under slow-growth conditions be greater than or equal to 50% to pass to the recovery phase was chosen assuming that plants grown under conditions giving ≥50% survival would provide better results in the recovery phase. Low survival in the slow-growth phase might indicate some problem in explant maintenance and, consequently, more difficulty in explant recovery.

Effect of alginate matrix, storage temperature, and storage time on plantlet conversion. Approximately 420 nodal segments (0.5 cm) from *in vitro* cultures of *M. moricandiana* were excised and divided into two groups: nonencapsulated and encapsulated. In the nonencapsulated group, one lot consisting of 30 nodal segments was immediately placed in semisolid MS basal medium after excision. In the encapsulated group, nodal segments were placed in a glass flask with 50 mL MS medium without calcium and supplemented with 3% sodium alginate. Aliquots of the alginate solution, each of which contained one nodal segment, were taken up with a micropipette, dropped into 100 mL MS medium supplemented with 0.1 M calcium chloride, and maintained under agitation for 30 min to polymerize the capsules. After hardening, the alginate capsules were washed in distilled water and dried on filter paper. Thirty capsules were placed in semisolid MS basal medium. The remaining nonencapsulated nodal segments and capsules were placed into sterile cryogenic tubes and stored in darkness for 30 or 60 d at 4, 15, or 25°C. After storage, the nonencapsulated nodal segments and capsules were placed in semisolid MS basal medium and evaluated to determine the percentage of plant conversion after 1 mo in the growth room. The experimental design was fully randomized, consisting of both encapsulated and nonencapsulated nodal segments stored at three different temperatures (4, 15, and 25°C) and then sampled after 30 and 60 d, with three replicates per treatment. Each replicate was a cryogenic tube with 10 nodal segments (n=30).

Effect of different moisture contents on plantlet conversion from nonprecultured capsules. The determination of moisture content (MC%) of capsules was made as described by Subaih *et al.* (2007) and Melo *et al.* (2011), with modifications. One hundred capsules, prepared as described above, were divided into lots of 10 capsules each, placed in paper muffin cups with a blue silica gel indicator, and allowed to gradually dehydrate for 8 h at 25°C. After each 1-h period, the lots were weighed and returned to the paper muffin cup. After 8 h, all lots were dried in an oven at 70°C and then reweighed. The MC% obtained after each hour of dehydration was calculated using the following formula:

$$MC\% = \left[\frac{(fresh\ \ weight - dry\ \ weight)}{fresh\ \ weight}\right] \times 100.$$

To check the effect of moisture content on plantlet conversion, 30 capsules with nodal segments were prepared and placed in semisolid MS basal medium; these were identified as nonprecultured capsules with 100% MC. The remaining capsules were maintained for 1, 4, or 7 h in paper muffin cups with a blue silica gel indicator and allowed to gradually dehydrate, respectively reaching 80, 40, and 20% MC, as established above. After each period, capsules in lots of 30 each were placed in semisolid MS basal medium. After 1 mo in the growth room, the capsules were evaluated to determine the percentage of plant conversion under each condition.

Effect of sucrose concentration during preculture and moisture content on plantlet conversion from capsules. Capsules were placed in liquid MS basal medium with 0.40 or 0.75 M sucrose and then incubated for 2 d on a rotary shaker (100 rpm) in the growth room. After incubation, the capsules were washed in distilled water, and 30 capsules were placed in semisolid MS medium; these were identified as precultured capsules with 100% MC. The remaining capsules were subjected to dehydration, reaching 80, 40, and 20% MC as

described in the previous section, and for each MC%, one lot consisting of 30 capsules was placed in semisolid MS basal medium. The experimental design was fully randomized, consisting of two different sucrose concentrations of liquid MS basal medium at four different MC%, with three replicates per treatment. Each replicate consisted of 10 nodal segments (n=30) in glass flasks. All capsules placed in MS medium were evaluated after 1 mo to determine the percentage of plant conversion under each condition.

Effect of preculture time, storage temperature, and storage time on plantlet conversion from capsules. This experiment was carried out under the conditions that produced the highest percentage of plantlet conversion in the previous experiment using both 2 and 7 d of preculture. After preculture in liquid MS with 0.40 M sucrose and dehydration until reaching 40% MC, capsules in lots of 30 each were stored in sterile cryogenic tubes and subjected to different storage conditions: −80°C for 24 h; 0°C for 30, 60, and 90 d; and 4°C for 30, 60, and 90 d. The experimental design was fully randomized, with three replicates per treatment, where each replicate was a cryogenic tube with 10 capsules (n=30). At the end of each storage time, the cryogenic tubes were soaked in warm water for 90 s, and the capsules were rehydrated with 1 mL of liquid MS basal medium. The rehydrated capsules were placed in semisolid MS basal medium and maintained in the growth room for 1 mo. At the end of this time, the percentage of plant conversion was evaluated.

Rooting and acclimatization. After 6 mo, all plantlets (3–5 cm) with well-developed shoots that had regenerated from encapsulated, stored nodal segments were transferred to glass flasks containing 50 mL semisolid MS medium supplemented with 2 mg L^{-1} IAA for rooting and maintained for 3 mo in a growth room under the same conditions described above. After rooting, the plants were washed in tap water to remove excess medium and carefully transferred to plastic tubes (3× 10 cm) containing autoclaved vermiculite. The tubes were placed in a plastic box covered with plastic film to maintain high humidity. The box was maintained in a greenhouse at 28 ±2°C for acclimatization. Over 1-mo time, the plastic film cover was removed gradually. After 3–4 mo, plants were transferred to garden soil under field conditions.

Statistical analysis. Slow-growth storage and recovery data were subjected to two-way analysis of variance (ANOVA; P<0.05). Carbon source data were subjected to one-way analysis of variance (ANOVA), and comparisons of means were carried out with the Tukey–Kramer test (P<0.05). The significance of differences between means of percentage of plant conversion from alginate capsules was evaluated by the Student's t test or Duncan's multiple range test (P<0.05). All statistical analysis was performed using the SigmaPlot™ software for Windows, version 11.0.

Results and Discussion

Conservation under slow-growth conditions. Under slow-growth culture conditions, plants developed within 6 mo. Recovery of nodal segments from these plants was made in semisolid MS basal medium within 3 mo. Control plants developed in semisolid MS basal medium displayed a height of 5.23 cm and a multiplication rate of 6; however, it was considered desirable that plants developed under slow-growth conditions should display height and multiplication rates lower than those of the control (Table 1).

Two-way ANOVA (Table 2) showed that basal medium and carbon source did not interact to improve any of the three evaluated parameters (height and multiplication rate in slow-growth and multiplication rate in recovery). The basal medium (MS, ½MS, WPM, and ½WPM) did not affect either the slow-growth storage or the recovery of *M. moricandiana* under the tested conditions. The utilization of media with salt concentration reduced to half the usual level has been recommended for the conservation of germplasm as a means of decelerating plant growth (Collin and Edward 1998). However, in a unique study reporting on *Mandevilla* under slow-growth maintained on media with reduced salt concentration and supplemented with osmotic agents (Biondo *et al.* 2007), these conditions contributed to accelerated plantlet growth.

According to Bekheet (2011), the addition of osmotic agents to *in vitro* culture was an efficient method of growth inhibition, causing reduction of the plant's metabolic activities without affecting viability and also increasing the storage life of many *in vitro*-grown tissues of different plant species. In the present study, carbon source had a significant effect on slow-growth storage and recovery. Sucrose, the original carbon source of all tested media, promoted the highest values of height and multiplication rate under slow-growth storage, so it was considered unsuitable for this purpose. Sorbitol and glucose, separately or combined, promoted development of plants with low multiplication rate during the slow-growth period and with high multiplication rate during recovery (Fig. 1), demonstrating their suitability for use in culture medium for slow-growth storage of *M. moricandiana*.

All media that gave survival rates lower than 50% had mannitol as a supplement. The other media supplemented with mannitol gave more than 50% survival under slow-growth conditions, but the plants were short and had low multiplication rates. These plants were tested in the recovery phase because of the possibility that they would display high multiplication rates, but this did not occur (Table 1). Mannitol as a carbon source promoted high mortality in slow-growth storage and low height and multiplication rates in the recovery phase, demonstrating its ineffectiveness for use in conservation medium (Fig. 1). Although high mannitol concentration may be harmful and cause plant death (Silva and Scherwinski-Pereira 2011), this carbon source can enhance survival of plant germplasm

Table 1. Survival, height, and multiplication rate of *M. moricandiana* after 6 mo of culture on slow-growth storage medium, and survival and multiplication rate after 3 mo of recovery in MS medium

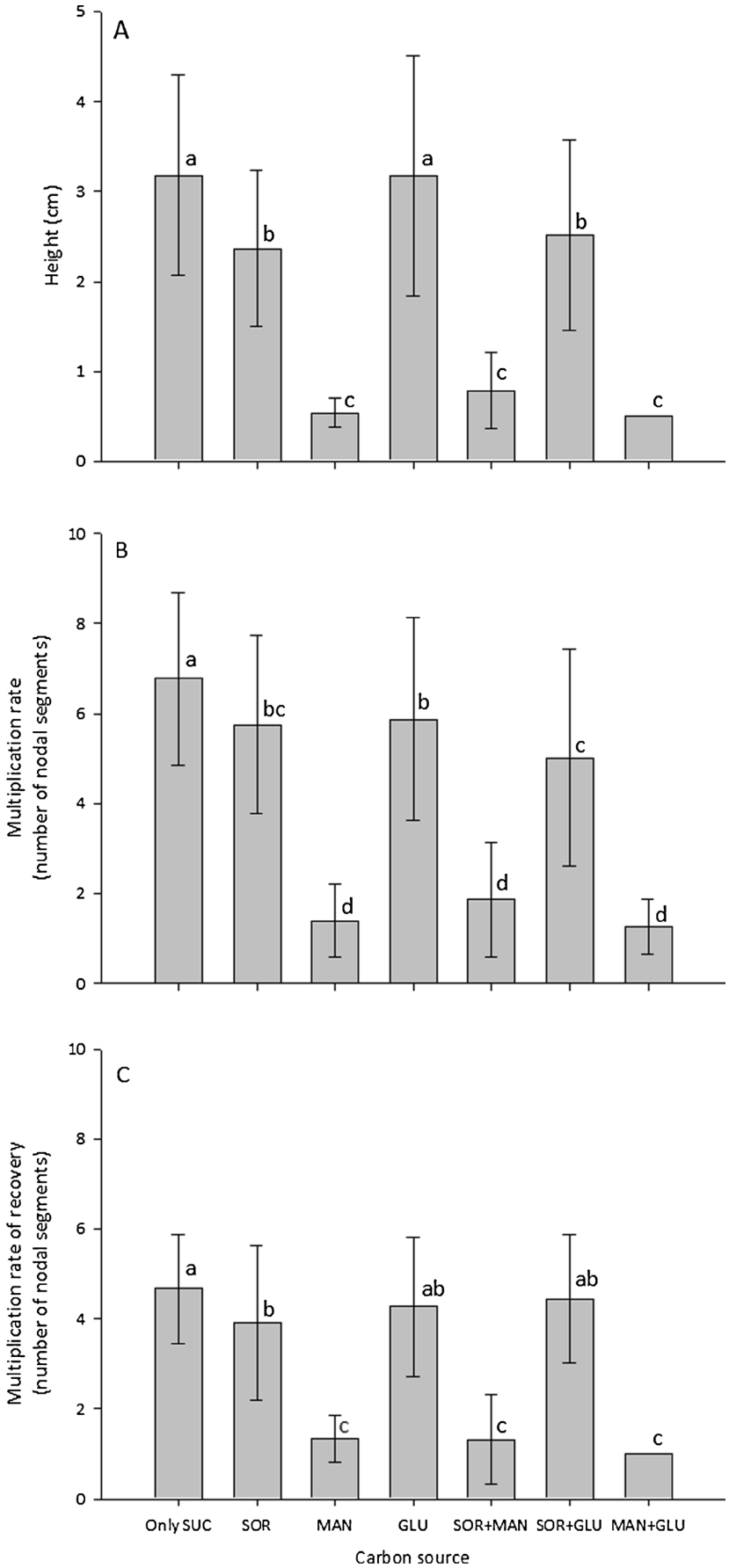
Medium	Slow-growth on indicated medium			Recovery in MS medium	
	Survival (%)	Height (cm)±SD	Multiplication rate	Survival (%)	Multiplication rate±SD
MS	100	5.23±1.66	6.00±1.43	100	4.29±0.75
MS_{SOR}	100	4.93±1.72	6.83±1.87	100	4.03±2.12
MS_{MAN}	83.3	1.14±0.41	1.68±0.98	100	1.33±0.51
MS_{GLU}	83.3	4.49±2.50	4.60±2.61	100	3.16±1.40
$MS_{SOR+MAN}$	76.6	1.37±0.82	2.13±1.60	100	1.00±0
$MS_{SOR+GLU}$	83.3	4.52±2.24	3.84±2.13	100	3.16±2.32
$MS_{MAN+GLU}$	63.3	1.00±0	1.47±0.84	100	1.00±0
½MS	100	6.46±1.96	7.13±1.77	100	5.90±1.18
$½MS_{SOR}$	100	4.58±1.28	5.46±1.73	100	3.66±1.84
$½MS_{MAN}$	46.6	1.00±0	1.00±0	–	– –
$½MS_{GLU}$	100	5.77±1.41	5.33±1.16	100	6.03±0.89
$½MS_{SOR+MAN}$	83.3	2.06±0.82	1.95±0.94	100	1.50±1.23
$½MS_{SOR+GLU}$	100	4.99±1.96	6.08±3.28	100	4.56±1.33
$½MS_{MAN+GLU}$	70.8	1.00±0	1.17±0.39	100	1.00±0
WPM	100	7.66±2.52	8.00±1.62	100	4.23±1.00
WPM_{SOR}	96.6	4.25±2.21	5.24±2.34	100	3.20±0.76
WPM_{MAN}	8.3	1.00±0	1.00±0	–	– –
WPM_{GLU}	100	7.11±2.58	6.50±2.04	100	3.20±1.03
$WPM_{SOR+MAN}$	12.5	1.00±0	1.00±0	–	– –
$WPM_{SOR+GLU}$	100	5.80±2.27	5.73±2.16	100	4.26±1.11
$WPM_{MAN+GLU}$	13.3	1.00±0	1.00±0	–	– –
½WPM	100	6.06±2.06	6.00±2.06	100	4.16±0.83
$½WPM_{SOR}$	100	5.16±1.52	5.46±1.59	100	4.76±1.54
$½WPM_{MAN}$	6.6	1.00±0	1.00±0	–	– –
$½WPM_{GLU}$	100	7.54±2.79	6.76±2.25	100	4.46±0.62
$½WPM_{SOR+MAN}$	20	1.00±0	1.00±0	–	– –
$½WPM_{SOR+GLU}$	96.6	4.67±1.79	4.41±1.18	100	5.03±0.92
$½WPM_{MAN+GLU}$	16.6	1.00±0	1.00±0	–	– –

SOR sorbitol, *MAN* mannitol, *GLU* glucose, *SD* standard deviation

Table 2. Two-way ANOVAs for effects of slow-growth storage medium on growth characteristics of *M. moricandiana* after 6 mo of slow-growth storage and after 3 mo of recovery in MS medium

Source of variation	Slow-growth storage										Recovery in MS medium				
	Height					Multiplication rate					Multiplication rate				
	df	SS	MS	*F*	*P* value	*df*	SS	MS	*F*	*P* value	*df*	SS	MS	*F*	*P* value
Basal medium	3	2.037	0.679	1.358	0.287	3	0.831	0.277	0.433	0.732	3	4.370	1.457	2.514	0.091
Carbon source	6	144.321	24.053	48.096	<0.001	6	149.526	24.921	38.981	<0.001	6	98.837	16.473	28.426	<0.001
Basal medium × carbon source	18	9.002	0.500			18	11.507	0.639			18	10.431	0.579		
Total	27	155.360	5.754			27	161.864	5.995			27	113.638	4.209		

Basal medium did not have a statistically significant effect on any of the parameters measured. Carbon source had a statistically significant effect on all three parameters measured ($P<0.001$)


A
Height (cm)
a
b
c
a
c
b
c
B
Multiplication rate
(number of nodal segments)
a
bc
d
b
d
c
d
C
Multiplication rate of recovery
(number of nodal segments)
a
b
c
ab
c
ab
c
Only SUC
SOR
MAN
GLU
SOR+MAN
SOR+GLU
MAN+GLU
Carbon source

◀ **Figure 1.** Average values of height (*A*) and multiplication rate (*B*) of *M. moricandiana* after 6 mo of culture in slow-growth storage and multiplication rate (*C*) after 3 mo of recovery in MS medium, according the carbon source used in the slow-growth storage medium. In each graph, *different letters* indicate statistical differences by the Tukey–Kramer test ($P<0.05$). *GLU* glucose, *MAN* mannitol, *SUC* sucrose, *SOR* sorbitol.

conserved *in vitro* (Sarkar and Naik 1998). Other studies with Apocynaceae have also utilized mannitol. The deleterious effects of mannitol in slow-growth storage were shown in *Hancornia speciosa* (Sá *et al.* 2011) and *Macrosyphonia velame* (Martins *et al.* 2011). The use of mannitol resulted in low multiplication rates in *M. velutina*, even though the plants had not senesced (Biondo *et al.* 2007).

In vitro conservation of *M. moricandiana* under slow-growth permits storage of at least 6 mo and rapid recovery of cultures without developmental difficulties. Unexpectedly, high survival rates of the explants during the slow-growth phase did not ensure their development during the recovery phase. The present results indicate that in addition to high survival rates, the explants require a minimum multiplication rate for recovery; indicating that time in slow-growth did not affect their regenerative capacity (Fig. 2).

Conservation by encapsulation. After nodal segment excision and before storage, the nonencapsulated nodal segments showed 100% plantlet conversion after 2 wk of culture, while the encapsulated nodal segments showed only 83.3% plantlet conversion after 1 mo of culture. The encapsulated nodal segments showed conversion into plantlets after storage for 30 d at 15°C, reaching 33.3% survival, but did not show conversion at either 4 or 25°C. After 60 d, no encapsulated nodal segments at any storage temperature showed conversion. On the other hand, storage at 4 and 15°C was lethal to the nonencapsulated nodal segments of *M. moricandiana*, which showed high resistance and gave 66.6% conversion into plantlets after 30 d of storage at 25°C, decreasing to 16.6% after 60 d of storage at the same temperature (Table 3).

Figure 2. *M. moricandiana* after 3 mo of recovery in semisolid MS basal medium, following 6 mo in slow-growth storage on ½WPM supplemented with 2% sorbitol and 2% glucose.

Dehydration of encapsulated nodal segments from 100 to 20% MC without preculture decreased the plant conversion percentage in semisolid MS basal medium from 86.6 to 56.6% after 30 d (Table 4). For precultured encapsulated segments, all MC levels and both sucrose concentrations in the preculture medium gave >40% conversion. Plantlet conversion rates above 80% were obtained from nonprecultured encapsulated nodal segments at 100% MC and from encapsulated nodal segments precultured in liquid MS medium with 0.40 M sucrose and at 40–100% MC. Encapsulated nodal segments precultured in medium containing 0.40 M sucrose and dried to 40% MC gave the best conversion rate (93.3%), so these conditions were chosen for a test of different storage times and temperatures. Although the nodal segments precultured in liquid MS medium with 0.40 M sucrose and at 100% MC had an 86.6% conversion rate, low moisture content is recommended for storage at low temperatures (Engelmann 2004; Melo *et al.* 2011) so this condition was not tested further.

When encapsulated nodal segments were precultured for 2 or 7 d in liquid MS medium with 0.40 M sucrose (Table 5), encapsulated nodal segments before storage showed ca. 90% plantlet conversion, with no significant difference between the two preculture periods. After 30 or 60 d of storage, only the encapsulated nodal segments stored at 4°C showed plantlet conversion. The encapsulated nodal segments precultured for 2 d resulted in 26.6% conversion after storage for 30 d, decreasing to 3.33% after 60 d, while for encapsulated nodal segments precultured for 7 d, the survival rate after 30 d was 33.3%, decreasing to 6.66% after 60 d. The conversion rates for each storage time showed no significant difference between the two preculture periods. After 90 d, no encapsulated nodal segments survived. Regardless of preculture time, none of the encapsulated nodal segments stored for any length of time at −80 or 0°C were recovered, indicating that preculture did not enable encapsulated nodal segments to convert into plants after storage at temperatures lower than 4°C.

Nodal segments encapsulated in sodium alginate matrix are shown in Fig. 3*a*. After emergence of the shoot from the capsule, plantlet conversion and development was easy and rapid (Fig. 3*b*). The sequence of development of plantlets

Table 3. Percentage of plantlet conversion from encapsulated and nonencapsulated nodal segments of *M. moricandiana* before storage and after storage at 4, 15, and 25°C for 30 and 60 d

Nodal segment type	Percentage of plantlet conversion from nodal segments						
	Storage time and temperature						
	Before storage	Storage for 30 d			Storage for 60 d		
		4°C	15°C	25°C	4°C	15°C	25°C
Encapsulated	83.3 b	0	33.3 a	0 b	0	0	0 b
Nonencapsulated	100 a	0	0 b	66.6 a	0	0	16.6 a

Different letters within a column indicate statistically significant differences by Student's *t* test ($P<0.05$)

Table 4. Plantlet conversion after 30 d from encapsulated nodal segments of *M. moricandiana* without preculture and after 2 d of preculture in MS with different sucrose concentrations and moisture content (MC) levels

MC% of encapsulated nodal segments	Plantlet conversion (%)		
	Encapsulated nodal segments not precultured	Encapsulated nodal segments precultured 2 d	
		Sucrose concentration	
		0.40 M	0.75 M
100	86.6 a	86.6 ab	76.6 a
80	76.6 ab	83.3 b	70 a
40	70 b	93.3 a	50 b
20	56.6 c	43.3 c	50 b

Different *letters* within a *column* indicate statistically significant differences by Duncan's multiple range test ($P<0.05$)

converted from encapsulated nodal segments 4 wk after placement in semisolid MS basal medium is illustrated in Fig. 3*c*.

Before storage, the recovery rate of nonencapsulated nodal segments was higher than that of encapsulated nodal segments. On the other hand, reports in literature have indicated that the sodium alginate matrix offers protection against dehydration and supplies nutrients to explants, resulting in a conversion rate of encapsulated nodal segments equal to or greater than that of nonencapsulated nodal segments (Singh *et al.* 2009, 2010). The before-storage results show that the sodium alginate matrix consistency may have impeded shoot emergence of *M. moricandiana*. Difficulties and delay in shoot or root emergence were reported for alginate beads with sodium alginate concentrations higher than 4–5% (Singh *et al.* 2010). Sodium alginate concentrations of 2.5–4% and 0.1 M calcium chloride, as used in this study, have been considered most suitable for the formation of beads by allowing optimally sized,

Table 5. Percentage of plantlet conversion from encapsulated nodal segments of *M. moricandiana* precultured in MS medium with 0.40 M sucrose, dehydrated to 40% moisture content, and stored under various conditions

Time of preculture (d)	Percentage of plantlet conversion from capsules							
	Storage time and temperature							
	Before storage	5 d	30 d		60 d		90 d	
		−80°C	0°C	4°C	0°C	4°C	0°C	4°C
2	90 a	0	0	26.6 b	0	3.33 c	0	0
7	93.3 a	0	0	33.3 b	0	6.66 c	0	0

Different *letters* within a *column* indicate statistically significant differences by Student's *t* test ($P<0.05$)

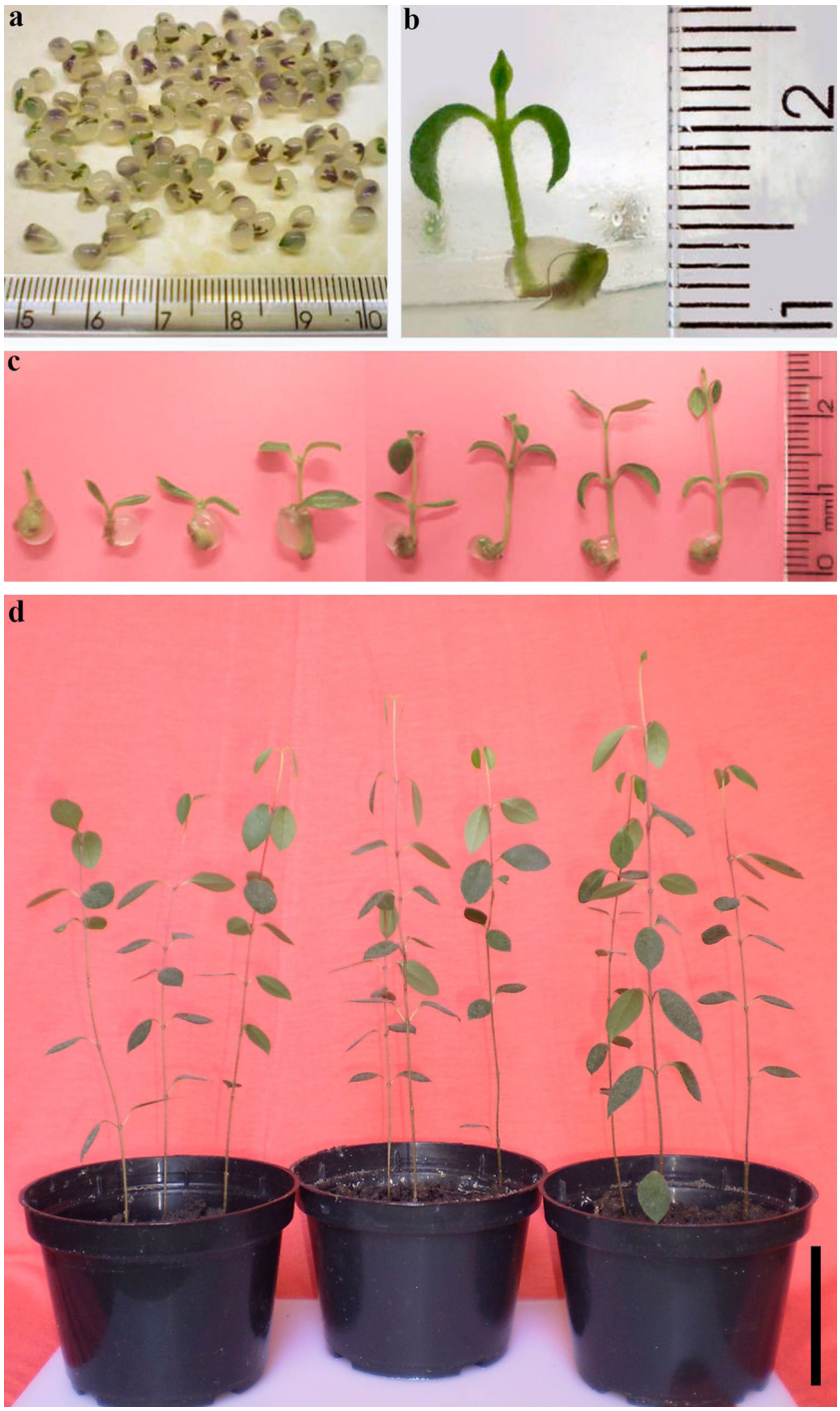


Figure 3. Plantlet regeneration from encapsulated nodal segments of *M. moricandiana*. *a*, Nodal segments encapsulated in sodium alginate capsules. *b*, Shoot emergence in semisolid MS basal medium. *c*, Sequence development of plantlets converted from encapsulated nodal segments during a period of 4 wk. *d*, Well-hardened plants of *M. moricandiana* in pots after 1 yr of acclimatization. *Bar*=5.0 cm.

uniformly spherical, firm capsules (Kumar *et al.* 2010; Sundararaj *et al.* 2010; Sharma and Shahzad 2012), thus reducing the effects of salt stress resulting from exposure of the nodal segments to the sodium alginate solution (Faisal et al. 2006; Singh *et al.* 2009; West and Preece 2009). In the absence of preculture, the sodium alginate matrix only protected nodal segments under storage at 15°C, showing negative effects at 4 and 25°C. In a study with *Rauvolfia tetraphylla*, encapsulated nodal segments stored at 4°C showed conversion rates close to 90% after 1 mo and close 30% after 2 mo (Faisal *et al.* 2006).

The encapsulation–dehydration method used in the present study is based on the method of producing synthetic seeds reported by Engelmann (2004). In this protocol, explants are encapsulated in a sodium alginate matrix, precultured on medium supplemented with sucrose from 1 to 7 d, dehydrated

under airflow or by exposure to silica gel to reduce the moisture content, and quickly frozen. According to Shibli *et al.* (2006), this procedure has been successfully used for conservation of large structures, such as apical and lateral buds, and for a variety of species in which other methods have been ineffective. The methodology used for *M. moricandiana* was the same as suggested by Engelmann (2004), except that the quick-freezing step was omitted. Storage at −80, 0, and 4°C was tested to enable alternative conservation methods, because cryopreservation requires more sophisticated conditions than those available in a typical laboratory.

The test of encapsulated nodal segments precultured in liquid MS medium with two different sucrose concentrations was aimed at selecting the most suitable concentration to ensure survival under subsequent storage at different temperatures. Since the accumulation of sugar by cells increases the stability of the membrane when exposed to dehydration, the preculture step was intended to induce resistance to dehydration and freezing (Sakai 2000).

Encapsulated nodal segments precultured for 2 d in liquid MS medium with 0.40 M sucrose reached the highest conversion rates after gradual drying to 40% MC on silica gel, but the conversion rate of the nonprecultured encapsulated nodal segments decreased in proportion to the decrease in MC%. Similar results were presented by Melo *et al.* (2011) in studies with cane sugar. These authors concluded that high concentrations of sucrose may result in osmotic shock, which would explain the decrease in conversion rates of the encapsulated nodal segments precultured in liquid MS medium with 0.75 M sucrose. According to Wang *et al.* (2002), progressive increase in sucrose concentrations can reduce the sensitivity of explants to high concentrations of sugar, but that procedure was not tested here.

After establishing the optimal concentration of sucrose in the preculture medium and MC% of the encapsulated nodal segments, the preculture time was extended in an attempt to increase the resistance of explants to dehydration and freezing. Nonstored encapsulated nodal segments reached the highest conversion rate when precultured for 7 d; however, increasing the preculture time did not significantly affect the conversion rates of encapsulated nodal segments. Irrespective of preculture time, only encapsulated nodal segments stored at 4°C survived, with ca. 30% survival after 30 d and ca. 5% after 60 d. According to Melo *et al.* (2011), the optimal time of preculture seems to be closely related to the concentration of sucrose used and the species studied.

All plantlets regenerated from encapsulated nodal segments stored for different periods at different temperatures had well-developed shoots and were placed in a rooting medium. After rooting, plants were successfully acclimatized in plastic pots and transplanted into field conditions, with 100% survival (Fig. 3*d*).

Although encapsulation–dehydration techniques are commonly applied to cryopreservation, the results reported here show the possibility of storing *M. moricandiana* encapsulated nodal segments at alternative temperatures for 1 mo. Adjustments in sucrose concentration, duration of preculture, and MC% can provide higher conversion rates and increased storage time. This is the first report of using encapsulation–dehydration techniques for *Mandevilla*.

Acknowledgments The authors thank CAPES (Conselho de Administração de Pessoal de Ensino Superior) for a doctoral scholarship for the first author; PBV-UFRJ (Programa de Pós-graduação em Biotecnologia Vegetal, Universidade Federal do Rio de Janeiro) and FAPERJ (Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro) for their financial support; Prof. Dr. Tatiana Ungaretti Paleo Konno of UFRJ-Macaé for providing seeds of *M. moricandiana*; taxonomists Prof. Dr. Jorge Fontella Pereira of Museu Nacional (UFRJ), MSc. Marcelo Fraga Castilhiori, and Inaldo do Espírito Santo of Herbarium Bradeanum for species identification; Prof. Dr. Aline Vieira Santos (PBV-UFRJ) for valuable assistance with encapsulation–dehydration protocols; Universidade Federal do Estado do Rio de Janeiro (UNIRIO) for providing transport to the collection areas; the Brazilian Institute for Environment and Natural Renewable Resources (IBAMA) for authorization to collect (*Scientific Research Activities* no. 18498–1); and specially to Dr. Randall P. Niedz (US Horticultural Research Laboratory) and to the editor of this Journal, Dr. John J. Finer, for their valuable contributions and suggestions to improve this manuscript.

References

Appezzato-da-Glória B, Estelita MEM (2000) The developmental anatomy of the subterranean system in *Mandevilla illustris* (Vell.) Woodson and *M. velutina* (Mart. Ex Stadelm) Woodson (Apocynaceae). Braz J Bot 23:27–35

Bekheet SA (2011) *In vitro* conservation of date palm germplasm. In: Jain SM, Al-Khayri JM, Johnson DM (eds) Date palm biotechnology. Springer, Dordrecht, pp 337–360

Bertoni BW, Souza AV, Biondo R, França SC, Telles MPC, Pereira AMS (2010) Genetic diversity among natural populations of *Mandevilla velutina*. Hortic Bras 28:209–213

Biondo R, Soares AM, Bertoni BW, França SC, Pereira MAS (2004) Direct organogenesis of *Mandevilla illustris* (Vell.) Woodson and effects of its aqueous extract on the enzymatic and toxic activities of *Crotalus durissus terrificus* snake venom. Plant Cell Rep 22:549–552

Biondo R, Souza AV, Bertoni BW, Soares AM, França SC, Pereira MAS (2007) Micropropagation, seed propagation and germplasm bank of *Mandevilla velutina* (Mart.) Woodson. Sci Agri (Piracicaba, Braz) 64:263–268

Calixto JB, Nicolau M, Yunes RA (1985) The selective antagonism of bradykinin action on rat isolated uterus by crude *Mandevilla velutina* extract. Brit J Pharmacol 85:729–731

Chawla HS (2002) Introduction to plant biotechnology. Science Publishers Inc., Enfield

Collin HA, Edward S (1998) Plant cell culture. BIOS Scientific Publishers, Oxford

Cordeiro SZ, Simas NK, Henriques AB, Lage CLS, Sato A (2012) Micropropagation of *Mandevilla moricandiana* (A.DC.) Woodson. In Vitro Cell Dev Biol Plant 48:620–626

Engelmann F (2004) Plant cryopreservation: progress and perspectives. In Vitro Cell Dev Biol Plant 40:427–433

Faisal M, Ahmad N, Anis M (2006) *In vitro* plant regeneration from alginate-encapsulated microcuttings of *Rauvolfia tetraphylla* L. Am Eurasian J Agric Environ Sci 1:1–6

Ford-Lloyd BV, Jackson MT (1991) Biotechnology and methods of conservation of plant genetic resources. J Biotechnol 17:247–256

Handro W, Floh EIS, Ferreira CM, Guerra MP (1988) Tissue, cell culture and micropropagation of *Mandevilla velutina*, a natural source of a bradykinin antagonist. Plant Cell Rep 7:564–566

Kumar S, Rai MK, Singh N, Mangal M (2010) Alginate-encapsulation of shoot tips of jojoba [*Simmondsia chinensis* (Link) Schneider] for germplasm exchange and distribution. Physiol Mol Biol Plants 16:376–382

Lloyd GB, McCown BH (1980) Commercially feasible micropropagation of mountain laurel (*Kalmia latifolia*) by use of shoot tip culture. Proc Int Plant Propag Soc 30:421–427

Maraschin M, Carobrez SG, Persike D, Peixoto ML, Ferreira AG, Ferracin R, Verpoorte R, Fontana JD (2000) Cell wall polysaccharides from *Mandevilla velutina* (Apocynaceae) cultured cells: extraction and chemical structure. Carbohydr Polym 41:55–60

Maraschin M, Sugui JA, Wood KV, Boham C, Buchi DF, Cantao MP, Carobrez SG, Araujo PS, Peixoto ML, Verpoorte R, Fontana JD (2002) Somaclonal variations: a morphogenetic and biochemical analysis of *Mandevilla velutina* cultured cells. Braz J Med Biol Res 35:633–643

Martins LM, Pereira MAS, França SC, Bertoni BW (2011) Micropropagação e conservação de *Macrosyphonia velame* (St. Hil.) Muell. Arg. em banco de germoplasma *in vitro*. Cienc Rural 41:454–458

Melo CG, Barbosa MHP, Motoike SY, Sabino MV, Ventrella MC, Peternelli LA, Oliveira MAR (2011) Preculture sugarcane tissue in sucrose supplemented culture medium to induce desiccation tolerance. Crop Breed Appl Biotechnol 11:320–329

Metcalfe CR, Chalke L (1950) Anatomy of the dicotyledons: leaves, stem and wood in relation to taxonomy with notes on economic uses. Clarendon, Oxford

Murashige T, Skoog FA (1962) Revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Rai MK, Asthana P, Singh SK, Jaiswal VS, Jaiswal U (2009) The encapsulation technology in fruit plants—a review. Biotechnol Adv 27:671–679

Rapini A, Koch I, Kinoshita LS, Simões AO, Spina AP (2010) Apocynaceae *in* Lista de Espécies da Flora do Brasil. Jardim Botânico do Rio de Janeiro. http://floradobrasil.jbrj.gov.br/2010/FB033698. Accessed 07 Oct 2011

Sá AJ, Lédo AS, Lédo CAS (2011) Conservação *in vitro* de mangabeira da região nordeste do Brasil. Cienc Rural 41:57–62

Sakai A (2000) Development of cryopreservation techniques. In: Engelmann F, Takagi H (eds) Cryopreservation of tropical plant germplasm. IPGRI, Tsukuba, pp 1–7

Sales MF, Kinoshita LS, Simões AO (2006) Eight new species of *Mandevilla* Lindley (Apocynaceae, Apocynoideae) from Brazil. Novon 16:113–129

Sarkar D, Naik PS (1998) Factors affecting minimal growth conservation of potato microplants *in vitro*. Euphytica 102:275–280

Schultes RE (1979) De Plantis Toxicariis e Mundo Novo Tropicale Comentationes. XIX. Biodynamic apocynaceous plants of the northwest Amazon. J Ethnopharmacol 1:165–192

Sharma S, Shahzad A (2012) Encapsulation technology for short-term storage and conservation of a woody climber, *Decalepis hamiltonii* Wight and Arn. Plant Cell Tissue Organ Cult 111:191–198

Shibli RA, Shatnawi MA, Subaih WS, Ajlouni MM (2006) *In vitro* conservation and cryopreservation of plant genetic resources: a review. World J Agric Sci 2:372–382

Silva TL, Scherwinski-Pereira JE (2011) *In vitro* conservation of *Piper aduncum* and *Piper hispidinervum* under slow-growth conditions. Pesq Agropec Bras 46:384–389

Singh SK, Rai MK, Asthana P, Pandey S, Jaiswal VS, Jaiswal U (2009) Plant regeneration from alginate-encapsulated shoot tips of *Spilanthes acmella* (L.) Murr., a medicinally important and herbal pesticidal plant species. Acta Physiol Plant 31:649–653

Singh SK, Rai MK, Asthana P, Sahoo L (2010) Alginate-encapsulation of nodal segments for propagation, short-term conservation and germplasm exchange and distribution of *Eclipta alba* (L.). Acta Physiol Plant 32:607–610

Subaih WS, Shatnawi MA, Shibli RA (2007) Cryopreservation of date palm (*Phoenix dactylifera*) embryogenic callus by encapsulation-dehydration, vitrification, and encapsulation-vitrification. Jordan J Agric Sci 3:156–171

Sundararaj SC, Agrawal A, Tyagi RK (2010) Encapsulation for *in vitro* short-term storage and exchange of ginger (*Zingiber officinale* Rosc.) germplasm. Sci Hortic 125:761–766

Vasil IK (1991) Plant tissue culture and molecular biology as tools in understanding plant development and in plant improvement. Curr Opin Biotechnol 2:158–163

Villalobos VM, Ferreira P, Mora A (1991) The use of biotechnology in the conservation of tropical germplasm. Biotechnol Adv 9:197–215

Wang QC, Batuman O, Li P, Bar-Joseph M, Gafny R (2002) A simple and efficient cryopreservation of *in vitro*-grown shoot tips of 'Troyer' citrange [*Poncirus trifoliata* Raf. × *Citrus sinensis* (L.) Osbeck.] by encapsulation-dehydration. Plant Cell Rep 20:901–906

West TP, Preece JE (2009) Bulk alginate encapsulation of *Hibiscus moscheutos* nodal segments. Plant Cell Tissue Organ Cult 97:345–351

Wiersema JH, León B (1999) World economic plants: a standard reference. CRC, Boca Raton

Woodson RE Jr (1933) Studies in the Apocynaceae. IV. The American genera of the Echitoideae. Ann Missouri Bot Gard 20:605–790