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MICROPROPAGATION

Micropropagation of *Mandevilla moricandiana* (A.DC.) Woodson

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Abstract A protocol was developed for micropropagation of *Mandevilla moricandiana* (A.DC.) Woodson, a native plant from Brazil. Shoots, obtained from *in vitro* plantlets were used as source of nodal segments for shoot production from axillary buds. The nodal segments were grown on Murashige and Skoog medium supplemented with different concentrations of 6-benzyladenine and/or indole-3-acetic acid to induce axillary bud elongation. After a 2-mo culture period, the medium supplemented with 1.0 mg L^{-1} 6-benzyladenine gave the largest number of nodal segments per explant. The nodal segments obtained from plants developed under these conditions were grown on medium supplemented with different concentrations indole-3-acetic acid, α-naphthaleneacetic acid, and indole-3-butyric acid. The use of the medium supplemented with indole-3-acetic acid and indole-3-buryric induced shoot elongation and shoot development, formation of basal callus, and/or indirect organogenesis of roots. Following transfer of shoots to soil, the plants with only basal callus showed 10% survival and developed roots from callus, while *in vitro*-rooted plants had a maximum 40% survival rate *ex vitro*. Regardless of the auxin added to the rooting medium, the acclimatization period allowed the plants rooted *in vitro* to develop their shoots fully. The protocol developed here is suitable for the production of shoots and rooted plantlets of *M. moricandiana*.

Keywords *Mandevilla* · Apocynaceae · Micropropagation · Morphogenesis · Tissue culture

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Introduction

Mandevilla Lindley (Apocynaceae, Apocynoideae) includes about 170 species, native to the Neotropical region. *Mandevilla* has racemose inflorescences, which may have large, brightly colored flowers and it has potential for use as an ornamental or landscape plant. Most of the species have a climbing habit, although they may occur as shrubs, subshrubs, herbs, or epiphytes (Metcalfe and Chalke 1950; Sales *et al.* 2006).

Mandevilla is among the most commonly grown ornamental plants in the world. *Mandevilla sanderi* and *Mandevilla splendens*, popularly known as "Brazilian jasmine," are among the 15 species most often sold by the largest online retailers (Wiersema and León 1999). *Mandevilla* is also recognized for decorative use (Alves and Oliveira 1992; Santos *et al.* 2009).

Mandevilla has been the subject of studies in taxonomy (Woodson 1933; Sales *et al.* 2006), anatomy and adaptive morphology (Appezzato-da-Glória and Estelita 2000; Martins and Alves 2008; Boutebtoub *et al.* 2009), ethnobotany (Adams *et al.* 2007), and *in vitro* culture (Handro *et al.* 1988; Biondo *et al.* 2004, 2007). In addition, some species show potential for production of pharmaceuticals (Calixto *et al.* 1985).

In vitro culture of *Mandevilla illustris* and *Mandevilla velutina* has received some attention because of the potential pharmacological use and the need for conservation of these threatened endemic species. The underground system of both species, composed of xylopodium or tuberous roots, is used in popular medicine, as infusions or alcoholic extracts, and has proven effective in treating snake bites and for inhibiting the edema-inducing activities of toxins such as the venom of *Bothrops* and *Crotalus* (Calixto *et al.* 1985; Biondo *et al.* 2004, 2007).

Handro *et al.* (1988) established a protocol for plant regeneration from explants of *M. velutina* and suggested the possibility of using *in vitro* techniques for the production of pharmaceuticals *in vitro*. Biondo *et al.* (2004) established a protocol for direct organogenesis of *M. illustris* from nodal segments, and Biondo *et al.* (2007) established a micropropagation protocol for *M. velutina*.

Micropropagation leads to the generation of plants in large quantities (Teixeira *et al.* 2001) with the production of homogeneous metabolites with reliable quality (Amaral and Silva 2003). Micropropagation techniques also enable genetic and epigenetic manipulations (Rao and Ravishankar 2002), the establishment of germplasm banks (Rout *et al.* 2000), and the patent protection of drug production methods, controlling illegal extraction and preventing the decline of ecosystems where plants occur naturally (Medeiros 2003).

In Brazil, at least 70 species of *Mandevilla* have been identified, and new species are continually being described, with high ornamental and pharmacological potential. *Mandevilla* are distributed along the Amazon region and the southeast (Sales *et al.* 2006). *Mandevilla moricandiana* (A.DC.) Woodson is found in several states in northeastern and southeastern Brazil, where it grows in sandy coastal dune forests and scrub ("restinga") and rocky grasslands, which are ecosystems with great diversity and high endemism. *M. moricandiana* is a vine with a trailing habit; it has twining and latescent branches and nodal appendages around the nodal region. The inflorescence has three to seven flowers with a pink and white funnel-shaped corolla, and the corolline tube may have a white or yellow interior. It flowers prolifically in November and December, waning slowly until April (Woodson 1933; Sales *et al.* 2006; Pioker *et al.* 2010). This species has an underground system composed by tuberous roots.

The objective of this study was to establish an efficient protocol for micropropagation of *M. moricandiana*, to maintain its genetic variability to promote *in vitro* conservation of germplasm, and to produce seedlings for ornamental purposes.

Materials and Methods

Plant material. M. moricandiana fruits and branches were collected in the Restinga de Jurubatiba National Park, located between 22° and 22°23′S and 41°15′ and 41°45′W in the municipalities of Macaé, Carapebus, and Quissamã, Rio de Janeiro. Voucher is deposited at the Herbarium Bradeanum (HB), under accession number HB 93029.

Culture media and conditions. The basal medium consisted of Murashige and Skoog (MS) (Murashige and Skoog 1962) salts, supplemented with MS vitamins and 3% sucrose (*w*/*v*), and solidified with 0.75% agar (*w*/*v*). Different concentrations (1.0, 2.0, or 5.0 mgL^{-1}) of 6-benzyladenine (BA), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and α-naphthaleneacetic acid (NAA) were evaluated. The pH of the medium was adjusted to 5.8, and the molten medium was dispensed in glass tubes (2.0×15.0 cm) for culture establishment or glass bottles (7.5×13.5 cm) for shoot multiplication and rooting. Media were autoclaved for 15 min at 121°C. All cultures were maintained in a growth room at 25±1°C, under a 16-h photoperiod at a photosynthetic flux of 23 μmol $m^{-2}s^{-1}$ provided by cool daylight fluorescent lamps.

Culture establishment. Seeds were obtained after the dehiscence of the fruits. They were surface-sterilized, under agitation, with 10% (*v*/*v*) commercial detergent for 15 min, 70% (*v*/*v*) ethanol for 10 min, and 30% (*v*/*v*) commercial bleach for 5 min, and then washed three times with sterile distilled water. The disinfected seeds were inoculated into glass tubes containing 10 mL of MS medium and were

maintained in a growth room for 60 d. After this period, shoot apices and nodal segments were excised from the seedlings and cultured on MS medium for 6 mo, with one subculture after 3 mo. *In vitro* plants were used as sources of nodal segments for the shoot production experiments.

Shoot multiplication. Isolated nodal segments (1.0 cm long) were inoculated into glass bottles with 50 mL of MS medium supplemented with 0.0, 1.0, 2.0, or 5.0 mgL^{-1} BA combined with 0.0, 1.0, 2.0, or 5.0 mgL^{-1} IAA. The experimental design was fully randomized in a 2×4 factorial scheme, consisting of two plant growth regulators (BA and IAA) in four different concentrations, with six replicates per treatment. Each replicate was a bottle of five nodal segments (n=30/treatment). After 2 mo, shoot multiplication was evaluated using the following parameters: shoot height, shoots per explant, nodal segments per explant (multiplication rate), and root or callus formation.

Rooting. Shoots produced in the culture medium that was most efficient in inducing bud nodal segments were excised and placed in glass bottles with 50 mL of MS supplemented with different concentrations (1.0, 2.0, or 5.0 mgL^{-1}) of IAA, NAA, or IBA. The experimental design was fully randomized in a 3×4 factorial scheme, consisting of three auxins (IAA, NAA, and IBA) in four different concentrations, with six replicates per treatment; each replicate was a bottle containing five nodal shoots (n=30/treatment). Plant development was evaluated using the following parameters: plant height, nodal segments per explant (multiplication rate), and roots or callus formation after a 3-mo culture period.

Acclimatization. In vitro shoots of more than 5.0 cm height were washed in tap water to remove excess medium and carefully transferred to plastic tubes (3.0×10.0 cm) containing autoclaved vermiculite. The tubes were placed in a plastic box covered with plastic film to preserve the high humidity. The box was maintained in a greenhouse at 28±2° C for acclimatization. During the course of 1 mo, the plastic film cover was removed gradually. The acclimatized plants were evaluated for *ex vitro* survival and the number of nodal segments, each month for 3 mo.

Statistics. Data were subjected to analysis of variance, and means were compared with the Tukey–Kramer test at 0.05% significance level, using the software *GraphPad InStat*, version 3.01.

Results and Discussion

The disinfection method applied to seeds of *M. moricandiana* was 90% successful in eliminating seed contamination. After 1 mo, 61% of the seeds germinated, producing seedlings with fully developed roots and shoots (Fig. 1*a*). After 3 mo of culture in hormone-free MS medium, the shoot developed but roots did not form.

After 1 mo of culture in the cytokinin-containing shoot multiplication media, all explants cultured in medium supplemented with BA showed axillary shoot development from the nodes (Fig. 1*b*) and shoot production *via* the development of preexisting meristems (Fig. 1*c*). After 2 mo, there were no differences between the shoot number per explant in plants cultivated with BA alone (1.0, 2.0, or 5.0 mgL^{-1}) or in combination with IAA (1.0, 2.0, or 5.0 mgL^{-1}) (Table 1). The IAA-BA combination in the shoot multiplication was used in an attempt to increase the multiplication rate, as observed in other studies with Apocynaceae (Handro *et al.* 1988; Pereira-Netto 1996; Sudha *et al.* 2005; Nishitha *et al.* 2006). However, for *M. moricandiana*, the combination of BA and IAA resulted in fewer shoots on average.

Use of media supplemented with 1.0 or 2.0 mgL^{-1} BA gave the highest numbers of shoots (Fig. 1*b*, *c*; Table 1). After 2 mo, the medium supplemented with 1.0 mgL^{-1} BA yielded a shoot multiplication rate of 1:21. Other studies with *Mandevilla* reported shoot multiplication using MS medium supplemented with BA within the range of 0.1–1.0 mgL^{-1}, with multiplication rates between 1:3 and 1:6.7 (Biondo *et al.* 2004, 2007). The multiplication rate obtained here for *M. moricandiana* with 1.0 mgL^{-1} BA was seven times more productive than the best results previously obtained for *Mandevilla*.

The explants cultured in MS supplemented only with IAA showed shoot production only from the apical meristems. In the presence of IAA, plants were more elongated (Fig. 1*d*), as the shoot height was the highest (Table 1). After 2 mo, the maximum multiplication rate was 1:6.

After 2 mo of culture, all explants placed on the cytokinin-containing shoot multiplication media, except those cultivated on MS without growth regulators, showed callus formation on the base of the explants with only 6.6% rooting (Table 1). Previous studies on the micropropagation of Apocynaceae species did not provide data on callus or root formation during the shoot multiplication, and therefore the data obtained here cannot be compared with these studies.

The use of auxins in the media resulted in the formation of roots and basal friable calli on nodal segments of *M. moricandiana*. Treatments containing IBA (Fig. 2*a*) or IAA (Fig. 2*b*) at concentrations of 2.0 and 5.0 mgL^{-1} were the most effective in promoting root formation. Optimum rooting response using IBA has been reported for several species of Apocynaceae (Pereira-Netto 1996; Raha and Roy 2001; Nishitha *et al.* 2006), including *M. illustris* (Biondo *et al.* 2004).

During the rooting phase, plants developed roots when cultivated for 3 mo on MS supplemented with IAA alone

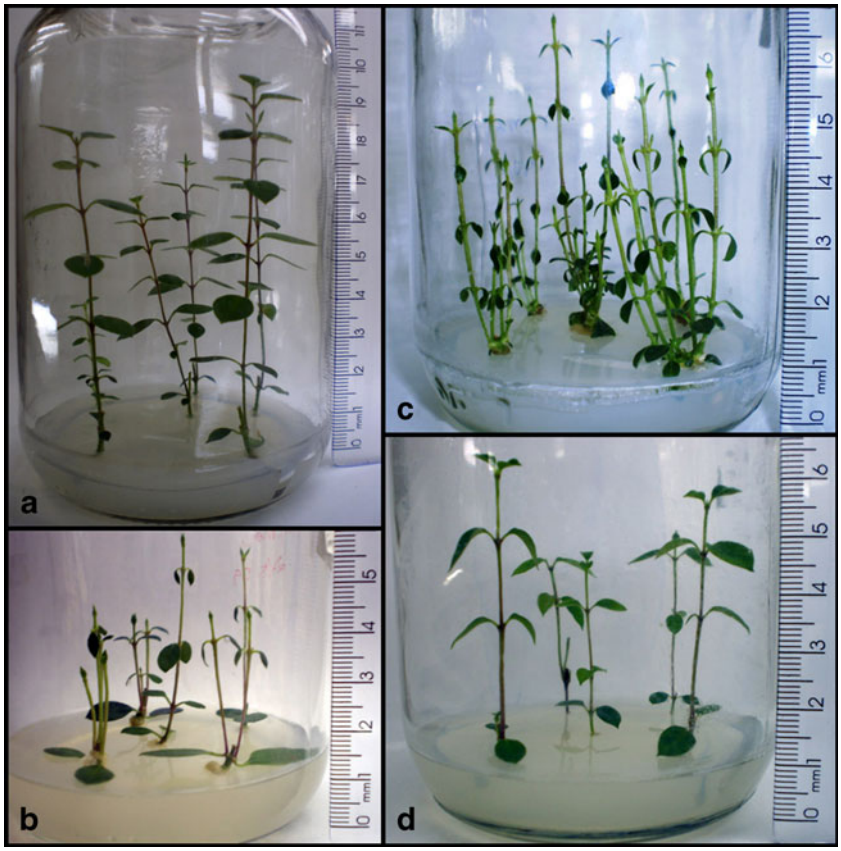

Figure 1. *In vitro* cultures of *M. moricandiana*: (*a*) shoot culture after 3 mo of culture; (*b*) early proliferation of shoots on MS medium with 1.0 mgL^{-1} BA after 1 mo of culture; (*c*) shoots on MS medium with 1.0 mgL^{-1} µM BA after 2 mo; (*d*) tissue on MS medium with 2.0 mgL^{-1} IAA after 2 mo of culture.

(Table 2), but no roots were formed when cultivated for 2 mo on the same medium during the shoot development phase (Table 1). The length of the culture period seems to be important for the production of roots. Biondo *et al.* (2007)

Table 1. Effect of growth regulators BA and IAA on *in vitro* development of *M. moricandiana*, after 2 mo of culture

Media	Shoot height (cm)	Numbers of shoots per plant	Nodal segment per shoot (multiplication rate)	Root and callus formation (%)	
				Roots	Callus
MS	6.15±2.477 bcd	1.23±0.430 c	5.53±1.634 efg	0	0
MS + 1.0 mgL^{-1} BA	6.29±2.367 bcd	3.40±1.380 a	21.43±8.549 a	0	100
MS + 2.0 mgL^{-1} BA	5.11±2.866 cde	2.63±1.129 a	21.2±9.693 a	0	100
MS + 5.0 mgL^{-1} BA	3.33±2.108 e	2.33±1.422 ab	13.4±9.357 b	0	100
MS + 1.0 mgL^{-1} IAA	8.23±2.887 ab	1.33±0.607 bc	6.65±1.778 defg	0	100
MS + 1.0 mgL^{-1} IAA + 1.0 mgL^{-1} BA	8.63±2.745 a	2.56±1.251 a	12.26±4.076 bc	0	100
MS + 1.0 mgL^{-1} IAA + 2.0 mgL^{-1} BA	7.30±1.561 abc	2.96±1.033 a	10.6±4.938 bcd	0	100
MS + 1.0 mgL^{-1} IAA + 5.0 mgL^{-1} BA	6.56±1.962 abcd	2.90±1.583 a	12.12±4.790 bc	0	100
MS + 2.0 mgL^{-1} IAA	6.10±4.028 bcd	1.10±0.607 c	4.22±2.063 g	6.67	100
MS + 2.0 mgL^{-1} IAA + 1.0 mgL^{-1} BA	7.18±2.289 abc	2.73±1.143 a	9.46±4.614 bcde	0	100
MS + 2.0 mgL^{-1} IAA + 2.0 mgL^{-1} BA	6.89±1.202 abcd	2.66±1.093 a	10.36±5.288 bcd	0	100
MS + 2.0 mgL^{-1} IAA + 5.0 mgL^{-1} BA	5.42±1.469 cde	3.23±0.935 a	8.86±3.340 bcdefg	0	100
MS + 5.0 mgL^{-1} IAA	6.93±4.259 abcd	1.06±0.640 c	4.46±2.631 fg	0	100
MS + 5.0 mgL^{-1} IAA + 1.0 mgL^{-1} BA	6.69±2.176 abcd	2.63±1.189 a	8.1±4.366 cdefg	0	100
MS + 5.0 mgL^{-1} IAA + 2.0 mgL^{-1} BA	6.14±2.150 bcd	2.73±1.048 a	8.43±3.048 cdefg	0	100
MS + 5.0 mgL^{-1} IAA + 5.0 mgL^{-1} BA	4.76±1.794 de	3.33±0.959 a	8.3±2.654 cdefg	0	100

Different *letters*, in each *column*, indicate statistical differences by the Tukey–Kramer test ($p<0.05$)

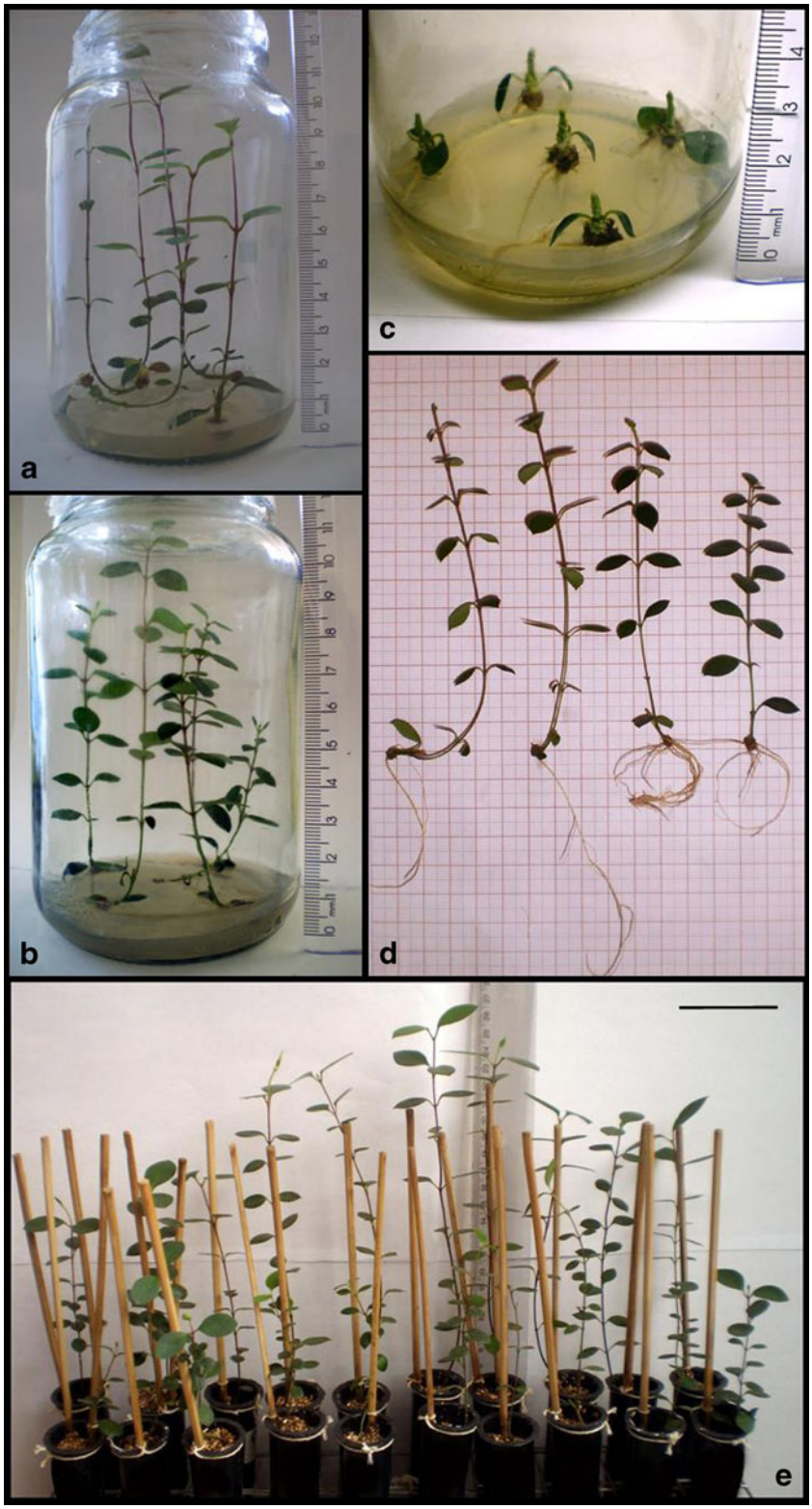

Figure 2. *In vitro* rooting and acclimatization of *M. moricandiana*: (*a*) shoots on MS medium containing 5.0 mg L^{-1} IBA after 3 mo of culture; (*b*) shoots on MS medium with 5.0 mg L^{-1} IAA after 3 mo of culture; (*c*) shoots on MS medium with 2.0 mg L^{-1} NAA after 3 mo of culture; (*d*) rooted plants after 3 mo of acclimation; (*e*) acclimatized plants in plastic tubes containing vermiculite, after 5 mo of acclimation; the *bar* indicates 5.0 cm.

observed that the culture period is directly proportional to rooting rates caused by auxins in *M. velutina*.

Although use of NAA-containing media promoted rooting by indirect organogenesis, NAA seemed to prevent shoot development (Fig. 2*c*), which is contrary to data obtained with *M. velutina* (Handro *et al.* 1988; Biondo *et al.* 2007) and another Apocynaceae (Sudha *et al.* 2005), where roots and calli were formed in cultures supplemented with NAA without compromising the development of shoots.

Table 2. Effect of growth regulators IAA, IBA and NAA on *in vitro* development of *Mandevilla moricandiana*, after 3 mo of culture

Rooting media	Plants height (cm)	Nodal segment per plant	Root and callus formation (%)	
			Roots	Callus
MS	8.00±2.741 abc	6.26±1.522 ab	0	50
MS + 1.0 mgL^{-1} IAA	6.38±1.982 abc	5.20±1.031 bc	17	83
MS + 2.0 mgL^{-1} IAA	6.60±2.221 abc	5.56±1.888 ab	53	100
MS + 5.0 mgL^{-1} IAA	9.47±3.151 ab	7.44±1.917 ab	68	100
MS + 1.0 mgL^{-1} NAA	4.25±4.844 cd	2.96±2.810 cd	23	100
MS + 2.0 mgL^{-1} NAA	1.26±1.143 d	1.26±0.785 d	27	100
MS + 5.0 mgL^{-1} NAA	1.00±0 d	1.00±0 d	100	100
MS + 1.0 mgL^{-1} IBA	6.30±2.307 bc	5.13±1.814 bc	27	100
MS + 2.0 mgL^{-1} IBA	9.12±4.521 ab	5.52±2.257 ab	48	100
MS + 5.0 mgL^{-1} IBA	9.76±3.224 a	6.70±2.588 ab	30	100

Different *letters*, in each *column*, indicate statistical differences by the Tukey–Kramer test ($p<0.05$)

All plants of *M. moricandiana*, which developed using MS medium supplemented with IAA or IBA, regardless of the presence or absence of roots and/or callus, were evaluated for acclimatization. After 3 mo in soil, the plants that were unrooted at the end of the rooting phase showed only 10% *ex vitro* survival. The surviving plants developed a root system from the callus at the base of the stem. This reduction of the number of plants in the sample prevented a statistical analysis of the number of nodal segments at the end of acclimatization *versus* the rooting medium. Similar results were obtained for *M. velutina*: *in vitro* unrooted plants showed only 10% survival after transfer to soil (Handro *et al.* 1988).

Of the plants rooted in IAA, the plants grown on MS medium supplemented with 1.0 mgL^{-1} showed a lower percentage of rooting (Table 2), but performed better in the *ex vitro* survival (Table 3). Of the plants rooted in IBA, those grown on MS medium supplemented with 2.0 and 5.0 mgL^{-1} showed the highest survival rate, reaching 40% (Table 3). Although *in vitro*-rooted plants showed different numbers of nodal segments at the beginning of acclimatization, after 3 mo, irrespective of their original medium, all plants showed a similar number of nodal segments and full development of roots (Fig. 2*d*). The survival rate of plants rooted *in vitro*, over 3 mo after transfer to soil, and the number of nodal segments developed in the same period in relation to the original media are showed in Table 3.

Approximately 40% of the acclimatized *M. moricandiana* plants that had formed roots at the end of the rooting phase survived *ex vitro* and 90% of acclimated plants without roots died. Although in a micropropagation protocol it is desirable to produce roots directly from the shoots, rather than from an intermediate callus, Handro *et al.* (1988) reported, for *M. velutina*, the formation of basal calluses in 100% of the plants at the end of the rooting phase. Studies on the micropropagation of *Mandevilla* (Handro *et al.* 1988; Biondo *et al.* 2004, 2007) have shown that plants with underground systems with a xylopodium or tuberous roots need to be rooted *in vitro* for successful acclimatization. For *M. moricandiana*, at the end of the rooting phase, all plants showed callus production and the roots emerged from this callus.

In conclusion, although further optimization is needed to increase the survival and rooting rates during acclimatization in soil, the micropropagation protocol developed here

Table 3. Percentage of survival and number of nodal segments of *in vitro*-rooted plants of *M. moricandiana* during 3 mo of acclimatization *ex vitro*

Origin media	1 mo		2 mo		3 mo	
	Survival (%)	Number of nodal segments	Survival (%)	Number of nodal segments	Survival (%)	Number of nodal segments
MS + 1.0 mgL^{-1} IAA	69	5.42±0.756 cd	46	6.44±0.882 b	35	8.00±1.155 a
MS + 2.0 mgL^{-1} IAA	80	5.93±1.611 bcd	53	7.27±1.104 ab	20	8.75±0.957 a
MS + 5.0 mgL^{-1} IAA	73	7.57±0.646 a	20	8.75±0.500 a	12	10.33±0.577 a
MS + 1.0 mgL^{-1} IBA	50	5.20±0.788 d	33	7.00±0.816 a	24	8.20±0.836 a
MS + 2.0 mgL^{-1} IBA	40	7.00±1.309 ab	40	8.31±0.991 a	40	9.65±1.061 a
MS + 5.0 mgL^{-1} IBA	84	6.75±1.545 abc	40	8.00±1.773 a	40	9.00±2.070 a

Different *letters*, in each *column*, indicate statistical differences by the Tukey–Kramer test ($p<0.05$)

provides an effective means for the production of *M. moricandiana* plantlets (Fig. 2*e*).

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