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PLANT TISSUE CULTURE

In vitro propagation and genetic stability analysis of *Evolvulus* spp. Biotechnological tools for the exploration of native germplasm with ornamental potential

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Abstract The goal of the present work is to establish a protocol that allows the *in vitro* propagation of *Evolvulus glomeratus* and *Evolvulus arizonicus*. Nodal segments of both species were disinfected by the standard method (ethanol/NaClO/Tween 80) with and without the addition of an antibiotics–antifungal mixture to the Murashige and Skoog (MS) complete medium, growth regulator free. *E. arizonicus* did not survive to the *in vitro* culture starting procedure. To determine the nutritional requirements for *E. glomeratus*, the nodal segments were cultured on different dilutions of the MS macronutrients, being the complete MS the more adequate basal medium. Two types of explants were isolated from *E. glomeratus* for their *in vitro* propagation: nodal segments and leaves. The first ones were cultured on complete MS medium supplemented with increasing benzylaminopurine (BA) concentration from 0 to 4.4 μM, and the second ones onto the same basal medium but supplemented with the following naphthalene acetic acid and BA concentration (micromolars): 0.0, 1.3, 2.6, and 5.3 for naphthalene acetic acid and 0.0, 1.1, 2.2, and 4.4 for benzylaminopurine in all possible combinations. Under the conditions applied from the leaves, no shoot regenerations were detected. On the other hand, it was possible to recover an average close to four shoots per explant when the nodal segments were cultured on a medium supplemented with 2.2 μM benzylaminopurine. For the rooting and rustication step, *in vitro* and *ex vitro* (with Growing Mix #2 and Perlite as substrate) strategies were tested. The best result was 91.6% efficiency of acclimated plants obtained with the *ex vitro* procedure using Perlite. The use of intersimple sequence repeat showed no differences among the tested regenerated plants under the applied experiment conditions. The protocol developed here is the starting point for the application of biotechnological techniques for both the massive propagation and the improvement of *E. glomeratus* and other related species.

Keywords Micropropagation · Tissue culture · ISSR

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Introduction

The genus *Evolvulus* belongs to the Convolvulaceae family, and it includes close to 100 species, widely distributed from USA down to the north of the Patagonia (Pontiroli 1983). In Argentina, this genus is represented by seven species (Krapovickas 1999) showing an interesting diversity in flower shape and color including blue, white, and lilac. Among them, *Evolvulus glomeratus* Nees et Martius ssp. *grandiflorus* (D. Parodi) van Oostst and *Evolvulus arizonicus* A. Gray, are herbaceous perennial species with 7 to 25 cm height and highly branched from their base (Krapovickas 1999). These species have an ornamental potential due to the sky blue color of their flowers (Fig. 1).

In germplasm collection management, DNA analysis can be used to study the genetics stability of *in vitro* propagation. Among dominant multilocus DNA fingerprinting methods, intersimple sequence repeats (ISSRs; Zietkiewicz et al. 1994) are molecular markers widely used for genetic diversity assessment, genetic relationship estimation, and establishing patterns for genomic identification (Nybon 2004). The primer motif used in the ISSR markers is a microsatellite consisting of tandemly repeated short sequence motifs (1–6 bp) which are ubiquitous in eukary-

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Figure 1. (*a*) *E. glomeratus* Nees et Martius ssp. *grandiflorus* (D. Parodi) van Oostst. (*b*) Details of *E. glomeratus* ssp. *grandiflorus* flowers. (*c*) *E. arizonicus* A. Gray. (*d*) Details of *E. arizonicus* flowers.

otic genomes and have the potential to provide extremely polymorphic marker systems in plants (Jain et al. 1999). This technique was successfully applied for cultivar identification in crops species (Giancola et al. 2002), as well as in horticulture and ornamental plants (Weising et al. 2005).

The goal of the present work is to develop a reliable protocol for the *in vitro* culture establishment and propagation of *E. glomeratus* and *E. arizonicus* and the adjustment of the ISSR technique in the genus *Evolvulus* as a starting point of a breeding program.

Materials and Methods

Plant material. The sources of plant material were two accessions of *E. glomeratus* and *E. arizonicus* collected in September 2004 and maintained under standard greenhouse conditions at the Instituto de Floricultura, Instituto Nacional de Tecnología Agropecuaria (34°36′ S, 58°40′ W, 26 m over the sea).

In vitro culture establishment. Nodal segment from both species were used as explants. The mother plants were previously treated three times with a mixture of fungicides (Iprodine™ and Benomil™, 1.0 g/L each). Once excised, the explants were washed for 30 min under tap water. Two disinfection methods were tested: (1) the standard method: ethanol 70%, active chlorine (5.5%), and Tween 80 (0.01%), according to Alderete et al. (2006) and (2) the standard method plus the addition of the Sigma antibiotics–antifungal mixture (SAA), containing 10,000 U penicillin, 10 mg streptomycin, and 25 μg anfotericine B per milliliter (Sigma Code: A-5955; Sigma, St. Louis, MO) to the culture medium.

Nodal segments of both *Evolvulus* species, 19 explants per treatment, were placed onto Murashige and Skoog (MS) media without plant growth regulators (Murashige and Skoog 1962), supplemented with 20.0 g/L sucrose with pH adjusted to 5.7 with KOH and solidified with 0.7% Sigma™ agar; for *E. glomeratus*, the SAA 1× mixture was added to the medium the first 30 d of culture, while *E. arizonicus* was initiated without SAA.

Mineral requirement assay. To establish the mineral requirement for both species, microcuttings obtained from the *in vitro* developed plantlets of *E. glomeratus* and *E. arizonicus* were transferred to the following dilutions of the MS macronutrient: 1×, 0.5×, 0.25×, and 0.125×. The different media were supplemented as indicated above. The explant number was 60 per treatment. The explants were evaluated for browning, rooting, and the development indicator (DI). The DI was defined as the ratio between the final length and the initial length per nodal segment after 1 mo culture.

Growth regulator requirement assay. We studied the *in vitro* response of nodal segments and leaf cuttings of *E. glomeratus* to different plant growth regulator combinations. Nodal segments from plantlets cultured under *in vitro*

conditions were transferred to an MS medium supplemented with 20.0 g/L sucrose, pH=5.7, 0.7% agar Sigma™, and 0.0, 0.44, 1.1, 2.2, and 4.4 μM benzylaminopurine (BA). There were 15 explants per treatment. After 30 d of culture, the relative growth (RG) was calculated as the ratio of the average of the explants' height cultured on different BA concentrations in relation to those achieved by the control. The number of shoots per explant on the different BA treatments was recorded after 60 d of culture.

Leaf cuttings from *in vitro* plantlets were subcultured on Petri dishes containing MS medium supplemented as above and with 0.0, 1.3, 2.6, or 5.3 μM naphthalene acetic acid (NAA) and 0.0, 1.1, 2.2, or 4.4 μM BA in all possible combinations. Two Petri dishes were used per treatment, one of them with the leaves cultured with the abaxial side in contact with the medium and other with the explant adaxial side in contact with the medium. Each Petri dish contained ten explants.

In all cases, the medium was sterilized by autoclaving 121°C, 1 atm for 15 min. The SAA mixture was added to the medium after autoclaving under sterile conditions.

The culture conditions were 24±2°C temperature, with photoperiod of 16 h light with an irradiance of 52 μmol $m^{-2}s^{-1}$ for all experiments.

Rooting and acclimatizing assays. For the rooting step, both *in vitro* and *ex vitro* alternative protocols were applied. Shoots 1.5–2.0 cm in length obtained from the 2.2 μM BA treatment were used. For the *in vitro* rooting procedure, 150 shoots were subcultured on 0.5× MS medium without growth regulators. After one subculture, rooted plants measuring 3–4 cm in length were acclimatized according to Escandón et al. (2003); the rooted plants were transferred to a 10-cm-diameter pot containing Growing Mix # 2 Fafard™ as substrate, and they were maintained under humid conditions. The nylon bags used to make the humidity chamber were gently perforated once per day until no condensation was detected inside them. The plants were then considered acclimated.

For the *ex vitro* rooting alternative, four different procedures were tested: with and without the submersion of the basal portion of the shoots into a solution of 4,921.25 μM indole butyric acid (IBA) and using either Growing Mix #2 Fafard™ or Perlite as substrate. The shoots were transferred to transparent plastic containers 26×19×8 cm in size, covered, and cultured under room culture conditions (as mentioned above). Each treatment was applied to 24 shoots. Of those 24, 14 were treated with an auxin, and the remaining ten served as a control.

The *ex vitro* plantlets were acclimatized in the same manner as the *in vitro* described above. Afterward, the plants were grown under greenhouse conditions. Statistical analysis were performed using analysis of variance and the Duncan test ($P \leq 0.05$) supported by the software STATISTICA 6.0.

Evaluation of the genetic stability of the micropropagated recovered plants. The genetic stability of the recovered plants was studied by ISSRs. Genomic DNA was extracted from young leaves of *E. glomeratus*, following the cetyltrimethylammonium bromide procedure according to Saghai-Maroof et al. (1984). DNA samples were extracted and purified from four plants, chosen at random, of each different rooting condition, except for the *in vitro* rooting procedure in which three plants were used.

Qualitative and quantitative measures of DNA were determined by running in 0.8% agarose traditional Tris acetate–EDTA (TAE) 1× gel, using λ Hind III as molecular weight pattern.

The primers were 3′GGG: $GGG(TGGGG)_2TG$ and 3′AG: $(AG)_8C$ (University of British Columbia; http://www.michaelsmith.ubc.ca/services/NAPS/PrimersSets/) and 5′CA: $CCCGGATCC(CA)_9$ and 5′GT: $CCCGGATCC(GT)_9$ (Blair et al. 1999). The polymerase chain reaction (PCR) reactions were carried out in a final volume of 25 μL containing 30 ng of genomic DNA, 0.5 U Taq polymerase, 2.5 μL of 10× reaction buffer (Kit InBio-Highway, Tandil, Argentina), 0.2 mM of each dNTP (Promega Corporation, Madison, WI; local distributor: Biodynamics, S.R.L., Buenos Aires, Argentina), 0.8 μM primers (Operon, Alameda, CA, USA; local distributor: Tecnolab S. A., Buenos Aires, Argentina), and for the primers 3′GGG, 3′AG, and 5′GT 3.0 mM $MgCl_2$ and for the primer 5′CA 2.0 mM $MgCl_2$ (InBio-Highway).

DNA was amplified in a Thermal Cycler (Bio-Rad, Hercules, CA, USA; local distributor: Tecnolab S. A.) with a preliminary step of 90 s at 94°C, for the primers 3′GGG and 3′AG, followed by 40 cycles with the following conditions: 40 s at 94°C, 45 s at 57°C, 90 s at 72°C, and a final 10 min extension at 72°C. For the primer 5′CA and 5′GT, the annealing temperature was 62°C.

Samples of 10 μL PCR products were analyzed on 2.5% agarose gels in TAE buffer running at 60 V for 4 h. The gels were stained using ethidium bromide (0.3 mg/mL) and the bands obtained were sized (in base pairs) by comparison with standard marker (100 bp ladder, PB-L, UNQ, Quilmes, Argentina).

In order to assess the consistency of band profiles, DNA isolation and PCR reactions were carried out three times. Only well-defined and reproducible bands were scored. Bands with the same migration were considered to be homologous fragments, regardless of intensity. The analysis was made twice, manually and individually, with the support of the Gel-Pro Analyzer software.

Data analysis. Each amplification fragment was considered as independent *loci*. Accordingly, the presence of a band

Table 1. Results obtained with the different MS macronutrients dilutions

MS dilutions	*n*	DI	R (%)	B (%)
MS×1	56	6.575a	0	6.6
MS×0.5	51	7.333a	11.6	15
MS×0.25	33	4.909b	0	45
MS×0.125	40	7.608a	5	33.3

Means followed by different *letters* within *columns* are significantly different by Student test ($P<0.05$)

n number of explants, *DI* development indicator, *R* explants showing root development, *B* explants showing browning

was scored as "1", while the absence of the band was scored as "0". The degree of similarity between genotypes was estimated by applying the Dice similarity coefficient: $2a/(2a+b+c)$, where a represents double matching and b and c simple matching (Dice 1945).

We constructed a dendogram using cluster analysis on the basis of the similarity matrix by using the unweighted pair-group method with arithmetic averages. The cophenetic matrix derived from dendogram was compared to the similarity matrix using the Mantel test with the NTSYS PC version 2.02e software.

Results and Discussion

Tissue culture: disinfection and in vitro establishment. No differences were detected between the two disinfection procedures. The standard method of ethanol/NaClO and the pretreatment of mother plants seem to be the adequate procedure for establishing *in vitro* tissue cultures of these species. In fact, avoiding the SAA mixture reduces the costs of the experiment, and in both treatments, shoot progress was observed, with more vigorous development on the MS medium without SAA (data not shown).

Tissue culture: nodal segments tissue culture. Under the applied *in vitro* culture conditions, *E. arizonicus* explants quickly suffered browning, necrosis, and death. On the other hand, *E. glomeratus* responded well to *in vitro* culture.

Table 1 shows the different *E. glomeratus* nodal segments behavior observed under the experimental conditions used. Except for the treatment 0.25× MS, no significant differences were observed. Despite the DI showed by the 0.125× MS and 0.5× MS treatments, the percentages of nodal segments with browning symptoms in both treatments were higher than those observed when the explants were cultured on 1× MS medium. It was evident that 0.25× MS had poorer results than other concentrations.

When the *E. glomeratus* nodal segments were cultured on media containing different BA amounts, the explants progressed at different rates (Fig. 2*a*). Table 2 shows the measured height among the treatments. The nodal segments of *E. glomeratus* were very sensitive to increased amounts of BA. The height average shows a significant difference between the treatments with and without BA. Within the treatments with BA, there was an apparent inflexion point at 1.1 μM BA where the shoot height and RG were greatly enhanced, even if there are no significant differences among the treatments above 1.1 μM BA.

In the treatments from 1.1 to 4.4 μM BA, the development of a green calluses was observed at the basal portion of the explants. After 60 d culture, adventitious shoots were recovered from these portions of the explants (Fig. 2*b*). The shoot regeneration and the multiplication rate varied with the addition of BA to the culture media. Under these culture conditions, adventitious shoots could be recovered only from treatments with 1.1 μM BA or higher (Table 2).

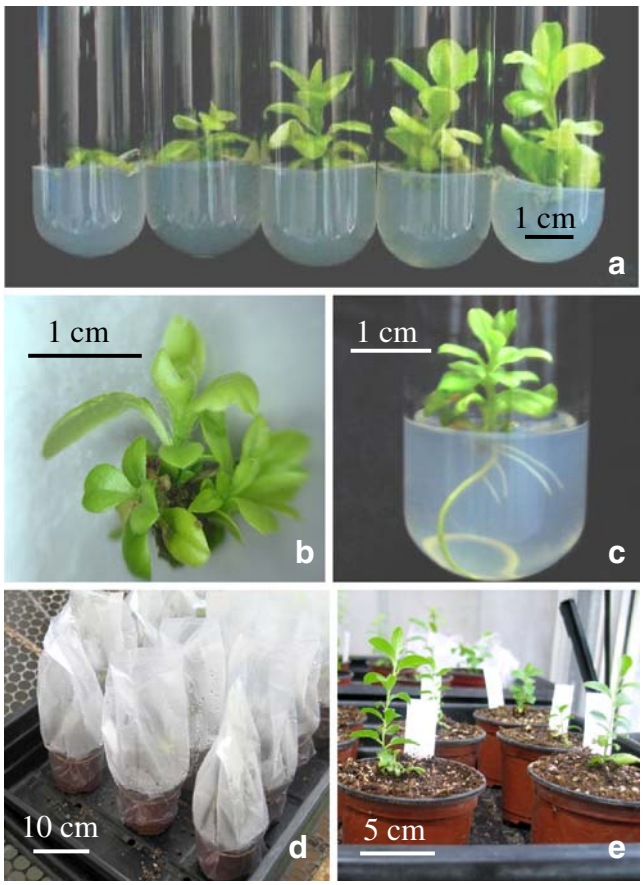

Figure 2. Response of *E. glomeratus* ssp. *grandiflorus* nodal segments toward increasing BA amounts and their *in vitro* tissue culture progress. (*a*) Explants after 30 d growing on a medium supplemented with the following BA concentrations, from *left* to *right* (micromolars): 0.0, 0.44, 1.1, 2.2, and 4.4. (*b*) Adventitious shoots regenerated from the callus developed at the basal portion of the explant. (*c*) Shoot rooted under *in vitro* condition. (*d*) *Ex vitro* plants during the rustication stage. (*e*) Rusticated plants growing under greenhouse conditions.

Table 2. Height differences measured after 30 d among the shoots cultured in different BA concentrations and the average of the number of adventitious shoot recovered per explant

BA (μM)	*n*	height average (mm)	SD	RG	S/E
0.0	15	5.800	3.098	1a	0
0.44	14	11.285	4.286	1.94b	0
1.1	15	22.066	10.046	3.80c	1.46
2.2	14	23.714	13.447	4.08c	3.66
4.4	14	27.642	11.881	4.76c	3.93

Means followed by different *letters* within *columns* are significantly different by Student test ($P<0.05$)

n number of explants per treatment, *SD* standard deviation, *RG* relative growth, *S/E* shoot recovered per explant

Likewise, the fact that the multiplication rate showed an increment from 1.46 shoots per explant for 1.1 μM BA to 3.66 and 3.93 for 2.2 and 4.4 μM, respectively, confirms the BA sensitivity observed when the RG was measured (Table 2).

Tissue culture of leaves. Table 3 summarizes the responses obtained when leaf cuttings were cultured under the proposed conditions. We detected different response types according to the growth regulator ratio in each treatment. In those treatments with only BA, no changes were observed.

When the medium was only supplemented with BA, a clear difference was evident in the *E. glomeratus* leaf cuttings' reactions to the different concentrations and the nodal segments' reactions. In contrast to the lineal response of the nodal segments to increasing BA concentrations, the leaf cuttings were recalcitrant to increasing BA concentrations in this experiment.

At intermediate levels of growth regulator ratio, the principal response observed was root development, mostly from the adaxial face of the leaves. The response observed when the explants were cultured at higher NAA/BA ratios was mostly callus development on the border of the section leaves.

Rooting and acclimatizing stage. Only 8% of the shoots rooted after 30 d of culture in 0.5× MS medium (Fig. 2*c*).

Table 3. Responses obtained when *E. glomeratus* portion leaves were cultured under different NAA/BA ratios

Growth regulators		BAP (μM)			
		0.0	1.1	2.2	4.4
NAA (μM)	0.0	nc	nc	nc	nc
	1.3	nc	r	r	nc
	2.6	nc	r	r	nc
	5.3	c	c	c	c

nc no changes, *c* calluses, *r* roots

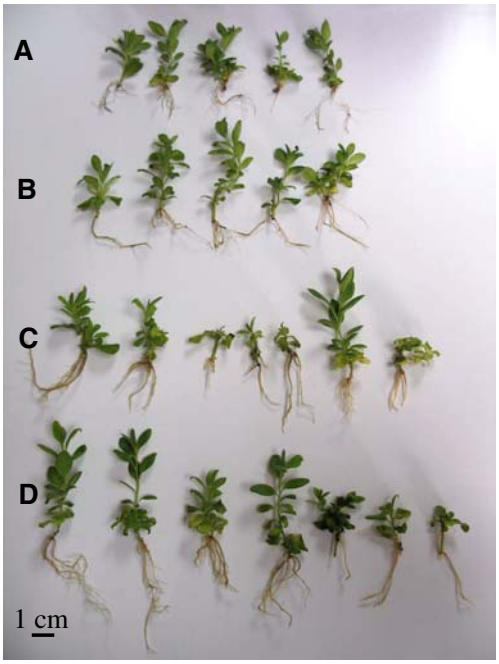

Figure 3. Plants rooted under *ex vitro* conditions after 4 wk. *Lane A*: Growing Mix with IBA. *Lane B*: Growing Mix without IBA. *Lane C*: Perlite with IBA. *Lane D*: Perlite without IBA.

Of the plants, 58.3% survived transplantation (Fig. 2*d*, *e*). Of the 150 transferred to 0.5× MS medium, only 12 rooted plants recovered, and only seven of those became fully acclimated.

During the rooting and acclimatizing step, 24 shoots were transferred to Growing Mix or Perlite. Of the Growing Mix group, 35.4% of shoots rooted with IBA and 50% without IBA. Of the Perlite group, 50% of the shoots rooted with IBA, and 70% rooted without IBA.

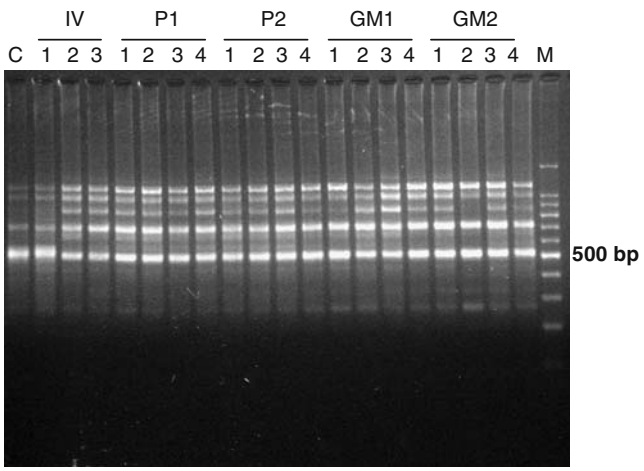

Figure 4. Band pattern obtained with the PCR amplification products using the primer 3′AG. *C* mother plant. The *numbers* indicate different individuals from: *IV in vitro* rooted plants, *P* plants rooted on Perlite, with IBA (*1*) without (*2*), *GM* plants rooted on Growing Mix, with IBA (*1*) without (*2*), and *M* weight molecular marker (100 bp ladder).

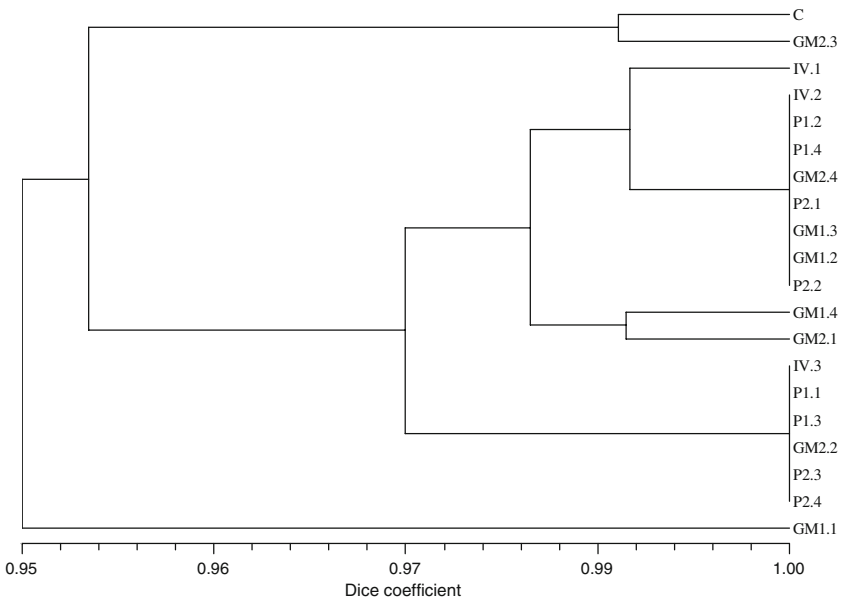

Figure 5. Dendrogram of 20 analyzed individuals. *C* mother plant. The *numbers* indicate different individuals from: *IV in vitro* rooted plants, *P* plants rooted on Perlite, with IBA (*1*) without (*2*), and *GM* plants rooted on Growing Mix, with IBA (*1*) without (*2*).

Figure 3 shows the rooted plants obtained when the *ex vitro* procedure was applied. It is possible to appreciate the quality and the number of adventitious roots developed per plant (Fig. 2*c*). A total of 22 plants (91.6%) overcame the acclimatizing step and were transferred to standard greenhouse conditions.

Because of the superior results and lower cost of the *ex vitro* strategy using Perlite, it is the preferred method for rooting and acclimatizing in future assays with this species

Genetic stability evaluation. Tissue culture is a possible source of genetic variability when plant regeneration occurs by indirect organogenesis (Cardone et al. 2004). Leroy et al. (2000) working on cauliflower (*Brassica olaracea*) tissue culture demonstrated that ISSRs are an adequate tool for the analysis of the genetic instability.

The four primers employed produced a total of 45 bands per individual (11.25 bands per primer) and 95.5% of them were monomorphic. The size of these amplified bands

Table 4. Comparison of different traits between the mother plants and 14 of the regenerated plants

Individual	Evaluated traits			
	Leaf area (mm^2)	Stem diameter (mm)	Internodal size (mm)	Flower diameter (mm)
Mother plant	190.71±39.63a	1.15±0.09a	19.96±5.28a	23.03±1.83a
# 1	152.05±41.64a	1.10±0.09a	20.57±2.86a	22.81±1.34a
# 2	144.76±48.28a	1.11±0.06a	15.56±5.78a	20.14±2.72a
# 3	125.51±32.85a	1.16±0.11a	14.31±3.53a	24.17±1.17a
# 4	148.22±20.60a	1.10±0.07a	13.78±0.95a	23.56±1.48a
# 5	137.81±29.93a	1.05±0.07a	13.61±2.36a	23.76±1.27a
# 6	109.86±27.02a	1.05±0.09a	14.23±2.83a	20.01±0.97a
# 7	137.49±31.00a	1.10±0.07a	21.78±5.42a	23.89±1.30a
# 8	113.34±36.94a	1.08±0.06a	13.60±2.84a	23.39±1.57a
# 9	96.58±15.37a	1.02±0.13a	13.68±5.60a	23.06±1.49a
# 10	152.15±28.20a	1.06±0.14a	15.75±3.05a	20.26±1.82a
# 11	114.00±26.91a	1.03±0.07a	12.22±3.01a	19.70±1.76a
# 12	170.90±38.36a	1.02±0.07a	15.16±4.37a	21.40±1.66a
# 13	161.77±50.12a	1.05±0.09a	18.12±2.14a	21.30±3.79a
# 14	119.92±38.27a	1.01±0.09a	13.03±2.51a	22.85±2.27a

Means followed by same *letters* within *columns* indicate no different significance by Student test ($P<0.05$)

oscillated between 220 and 1,790 bp. Figure 4 shows, as an example, the amplification products profiles obtained with the primer 3′AG.

The similarity coefficient between the analyzed individuals ranked from 0.95 to 1.0 (13 of them showed a coefficient superior to 0.98). The cophenetic correlation coefficient comparing the dendrogram and the similarity matrix was 0.90 (Fig. 5).

Giancola et al. (2002), working with soybean, proposed a coefficient major or equal to 0.80 to considerate two individuals as true-to-type plants. In the present work, the reported coefficient of 0.95 suggests that the recovered individuals were identical. Visual analysis of the plants did not reveal any morphological aberrations from the original plant (Table 4).

Likewise, the relevance of biotechnological approaches for the exploration and domestication of the wild ornamental germplasm was demonstrated by our group in previous reports (Alderete et al. 2006; Escandón et al. 2005, 2006, 2007).

Conclusion

A protocol for the *in vitro* establishment for *E. glomeratus* was developed.

For the *in vitro* establishment protocol, the species studied here showed different *in vitro* requirements.

Analogously, different BA sensitivity was observed among different explants of *E. glomeratus*. Under the experimental conditions applied, no shoot regeneration was observed from leaf portions.

The *ex vitro* rooting and rustication procedure using Perlite as substrate was the best strategy to close the *in vitro* cycle of *E. glomeratus*. The similarity coefficient obtained with ISSR markers indicates a high probability that the analyzed individuals were identical.

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