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RESEARCH ARTICLE

IN VITRO CLONAL PROPAGATION OF THE ENDANGERED PLANT *ALTERNANTHERA HIRTULA* (AMARANTHACEAE)

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ABSTRACT

Alternanthera hirtula is a native plant from the Pampa biome in Brazil that is threatened with extinction due to loss of suitable habitats. Thus, the *in vitro* clonal propagation is a useful way to produce plants for the conservation of its germoplasm and to introduce it in nature. Shoots, obtained from wild plants were used as the source of nodal segments for *in vitro* culture.The effect of cytokinins (6-benzylaminopurine, kinetin or thidiazuron) and carbohydrates (sucrose, fructose or glucose) on plant production was tested. The best shoot multiplication was achieved on medium with 1.0 µM thidiazuron. Among carbohydrates tested, sucrose (30 or 40 g L^{-1}) and glucose (20 g L^{-1}) promoted better multiple shoot proliferation and plant development. Plants were successfully acclimatized and showed normal development in a greenhouse. Some plants were planted in botanical gardens and others were reintroduced into natural habitats. The proposed method could effectively be applied for clonal propagation and conservation of this important and endangered plant.

Abbreviations: BAP: 6-benzylaminopurine, DW: Dry Weight, MS: Murashige and Skoog media, TDZ: Thidiazuron.

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INTRODUCTION

The Amaranthaceae family comprises more than 175 genera and 2000 species, being considered the most species-rich lineage within the flowering plant order of Caryophyllales (Simpson, 2010). In Rio Grande do Sul State (RS, Brazil), there are 43 species and 23 of them are on the list of threatened species, including *Alternanthera hirtula* (Marchioretto, 2013). *A. hirtula* is an herbaceous plant native of natural grasslands of southern Brazil (Pampa biome, RS, Brazil), whose red-violet inflorescences are very showy and have high durability indicating its potential for ornamental purposes. Recent studies have shown a decline in the area of *A. hirtula* occurrence, and the few populations are restricted to regions with low anthropic interference (Marchioretto, 2013). This had led to its inclusion on the list of endangered species (DOE RS, 2014). Among the main threats to populations of *A. hirtula*, we highlight overgrazing, the expansion of areas for monocultures (such as soy and rice), the inappropriate use of pesticides, the presence of exotic species [such as *Eucalyptus* sp., and grass anonni (*Eragrostis plana* Ness)], which are factors that limit the expansion of *A. hirtula* populations in their habitat (Marchioretto, 2013). Thus, besides protecting the areas of occurrence, development of strategies of propagation and conservation of native and endangered species are urgently needed and essential to minimize the impacts on biodiversity. There has been no information available on the reproductive biology of *A. hirtula*, not even on the production of seeds in nature. Rooting from the fragile stems is difficult making propagation by cuttings unfeasible. Thus, *ex situ* conservation strategies may be employed as an alternative to natural cultivation, especially for these genotypes that have high risk of habitat loss. Moreover, tissue culture could provide an efficient technique for mass propagation and germoplasm conservation of threatened plant species (Sarasan *et al*., 2011), especially for those which vegetative propagation methods are not available or feasible. Several studies have reported the *in vitro* propagation of *Alternanthera* genera (Singh *et al*., 2009; Gao *et al*., 2011; Macedo *et al*., 2011; Reis *et al*., 2015), however there is no information about *in vitro* propagation of

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A. hirtula. Thus, the goal of this study was to develop effective protocols for the *in vitro* propagation of *A. hirtula* from stem segments.

MATERIALS AND METHODS

Plant material, *In vitro* establishment and conditions of culture

Stem segments (about 20 cm long) of *A. hirtula* (Mart.) R. E. FR. were harvested from a field-grown population in Santa Maria, in the State of Rio Grande do Sul (RS), Brazil. Adult plants in flowering phase were used (Fig. 1A). A voucher specimen (SMDB 11840) was placed in the Herbarium of the Department of Biology, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil. The young shoots were cut into sections of 5 cm long and surface-disinfected by: immersion in 70% (v/v) ethanol solution for 10s; followed by immersion in 1.0% sodium hypochlorite (v/v) plus two drops of commercial detergent per 100 mL solution for 10min; and then rinsed five times in sterile, distilled water. Then nodal segments (10 mm-long microcuttings) were inoculated on Murashige and Skoog (MS; Murashige and Skoog, 1962) medium supplemented with 100 mg L^{-1} myo-inositol, 30 g L^{-1} sucrose, and 6 g L^{-1} agar (all from Sigma-Aldrich Co, St. Louis, USA).The pH of the medium was adjusted to 5.8 with 1N HCl or NaOH prior to autoclaving at 121°C and 1.05 kg cm^{-2} for 20min. The cultures were maintained in culture jars (10 mL medium per vessel) and incubated in a growth room at 25±2°C under a 16-h photoperiod at a light intensity of 35 µmol photons m^{-2} s^{-1} provided by 40 W cool-white fluorescent lights (Philips, Tianjing, China). After 45 days in culture, the regenerated axillary shoots were excised and cut into single nodal segments and grown in the same medium and conditions of culture described above. After five subcultures (45 days each), these plants served as the source of explants for the trials.

Effects of cytokinins on micropropagationfrom nodal explants

Nodal segments (each 10 mm-long and two buds) from plants grown *in vitro* were inoculated into culture bottles containing 50 mL of cultured MS medium supplemented with 1.0 or 3.0 µM of 6-benzylaminopurine (BAP), kinetin or thidiazuron (TDZ) (all from Sigma-Aldrich Co, St. Louis, USA). The cytokinin-free medium was used as a control. The number of shoots and nodal segments per explant, and shoot height (cm) were recorded after 45 days of culture.

Effects of carbon sources on micropropagation

Nodal segments (each 10 mm-long and two buds) from regenerated plants with 1.0 µM TDZ, for 45 days, were inoculated in culture jars containing the same medium (50 mL medium and five explants per vessel), but supplemented with sucrose, fructose or glucose (20, 30, or 40 g L^{-1}) (all carbohydrates from Sigma-Aldrich Co, St. Louis, USA). The number of shoots and nodal segments, height of plants (cm), dry weight (DW; g $plant^{-1}$), total chlorophyll index (i.e., chlorophyll $a + b$) and chlorophyll a/b ratio were recorded after 45 days. Indirect readings of leaf chlorophyll (chlorophyll index) were performed in the first well of expanded leaves (one leaf per plant) with a portable chlorophyll meter (ClorofiLOG 1030®, FalkerAutomação Agricola, Porto Alegre, Brazil). Because TDZ inhibits plant rooting, nodal segments (each 10 mm-long and two buds) from plantlets grown on medium supplemented with different carbon sources were subcultured on hormone-free MS medium. The percentage of rooting of the plants was evaluated after 30, 45, 60 and 90 days of culture. For acclimatization, single plants with well-developed root and shoot systems were carefully removed from jars and washed in running tap water. Ten plants from each treatment were transplanted to pots (2000 cm^3) containing commercial substrate (1000 cm^3) (Carolina II, Carolina Soil Brazil Ltda, RS, Brazil). The pots were covered with polythene bags for 30 days and irrigated daily with water once a day to keep relative humidity at 50-99%. The plants were maintained in a growth room (at 25±2°C, intensity of 35 µmol photons m^{-2} $s^{-1}$with 16-h photoperiod). After 60 days, the percentage of plant survival and chlorophyll index (total and chlorophyll a/b ratio) were recorded. The total chlorophyll index in these plants were compared with values obtained from wild plants (n = 20) of *A. hirtula*. After this, plants were transferred to square plastic trays (36 cm x 22 cm x 7 cm) containing the same substrate and maintained in a greenhouse for hardening. Three months later, the plants (n = 25) were transferred to flowerbeds in the Botanic Garden of the Universidade Federal de Santa Maria (Santa Maria, RS, Brazil) for *ex situ* conservation. Also, some plants (n = 25) were reintroduced into natural habitats (i.e., Pampa biome), located on a private farm (São Pedro do Sul, RS, Brazil).

Experimental design and statistical analysis

The experiments were arranged in a completely randomized design with five replicates and each replicate consisted of six explants (*in vitro* trials) or two plants (during acclimatization). The data were subjected to analysis of variance (ANOVA; $P \leq 0.05$) followed by Tukey's multiple range test.

RESULTS AND DISCUSSION

Effects of cytokinins on multiple shoot propagation

In general, TDZ was more effective than other cytokinins for multiple proliferation of shoots and nodal segments, although the use of 1.0 µM BAP produced the tallest plants (Table1). Explants cultured with cytokinin, especially those in medium containing TDZ, showed initial callus formation from the cut ends and concomitantly induction of multiple shoots in both the explant buds (Fig. 1B). On the other hand, in the absence of cytokinins, only one axillary bud from nodes began to grow, produced the shortest shoots with the least number of nodal segments and without any detectable callus formation (Table1). Furthermore, these shoots plus those regenerated from the *in vitro* establishment phase (all formed on phythormone-free MS medium) had a slow growth rate (about 1.0 to 2.0 cm every 45 days) and a low frequency of rooting (mean 10%) (data not shown). The proliferation rate of plants grown is on medium with TDZremained stable after three subcultures (data not shown). Thus, the medium supplemented with 1.0 µM TDZ was found to be the best for shoot proliferation, and was therefore used for shoot proliferation from nodal segments of *A. hirtula* in further studies. TDZ had not yet been studied for *in vitro* propagation of *Alternanthera*, but in other Amaranthaceae genera (i.e., *Pfaffia* and *Achyranthes*) this cytokinin gave good results (Flores *et al*., 2010; Desai *et al.,* 2016).

Effects of carbon sources on micropropagation

A. hirtula shooting and elongation were only slightly affected by carbohydrate source and concentration (Table 2). The maximum number of shoots and nodal segments, and the greatest plant DW (0.08 g plant^{-1}) were obtained on 20 g L^{-1} glucose, but increasing the glucose concentration reduced the elongation of plants and, consequently, the plant DW (Table 2). The concentrations of sucrose did not affect the proliferation and elongation of aerial parts, however there was a slight increase in plant biomass with increased sucrose concentration (Table 2), although some studies have shown that high sucrose concentration propitiated increasing shoot DW (Stancato and Tucci, 2010). Similar results were observed in *Solanum tuberosum* by Mohamed and Alsadon (2010). The ability to utilize carbohydrates varies by species and different plant genotypes respond differently even with the same carbon source, as was observed in *Pfaffia glomerata*, which can be *in vitro* cultured using sucrose (Flores *et al*., 2010) or glucose (Vasconcelos *et al*., 2014).Until now, only sucrose has been a carbon source used for micropropagation of the *Alternanthera* genus (Macedo *et al*., 2011; Reis *et al*., 2015), but in *A. hirtula*, both sucrose (30 or 40 g L^{-1}), and glucose (20 g L^{-1}) showed similar effects and can be used for *in vitro* propagation of this species. According to Yaseen *et al*. (2013), the differential morphogenetic responses to carbons sources (type and/or concentration) among species are explicated by their differential enzymology system for metabolism, by variations and differences in the endogenous concentrations of reducing sugars in *in vitro* tissues, and also by differential sensitivity of the cells to the breakdown products.

In this study, fructose (20 at 40 g L^{-1}) induced shoot formation (Table2), but it failed to develop morphologically normal shoots (i.e., with small and stunted leaves). In other species, such as *Withania somnifera* (L.) Dunal, the fructose, glucose and maltose failed to induce development of shoots (Sivanandhan *et al*., 2015), demonstrating that the effect of carbohydrates on *in vitro* morphogenetic potential is genotype-specific (Yaseen *et al.,* 2013; Vasconcelos *et al.,* 2014). The carbohydrate used for *in vitro* cultures also affects the production of photosynthetic pigments (Hazarika *et al.,* 2000; Stancato and Tucci, 2010; Mohamed and Alsadon, 2010) and enzymes (Premkumar *et al*., 2001; Koch, 1996). Some studies reported that leaves developed during *in vitro* cultures have a low content of chlorophyll and/or display reduced activity of photosynthesis enzymes (Hdiderand Desjardins, 1994; Sivanesan *et al.,* 2008) and, this behavior has been related to the presence of carbohydrates as carbon sources. However, unlike in other plants, in *Citrus* and *Anthurium andraeanum*, linear increase in chlorophyll content has been observed with the addition of sucrose in the medium (Hazarika *et al*., 2000; Stancato and Tucci, 2010). In *A. hirtula*, carbon sources and concentrations did not influence the chlorophyll index or the chlorophyll chl*a*/chl*b* ratio (Table2), confirming that exogenous carbo-hydrates do not always affect the chlorophyll content of plants *in vitro*.

Rooting and acclimatization

After being cultured for 30 days on hormone-free MS medium, shoots showed a low rooting response (less than 40% of plants produced roots) (Fig. 2A-C), except for those plants previously cultivated with 30 g l^{-1} fructose, more than half of which, in this period, had already induced the root system (Fig. 2C). In general, there was an increase in the rooting rate over time (Fig. 2). In medium with sucrose, the percentage of rooting after 45 days of culture was stable (Fig. 2A). On the other hand, using glucose, there was a small increase in the percentage of rooting over the culture period, but in both carbon sources, rooting rates were lower than 75% (Fig. 2A, B). Similar results were recorded at 20 and 40 g L^{-1} fructose (Fig. 2C).

However, the use of 30 g L^{-1} of fructose in the multiplication phase favored the development of the root system. For example, after 30 days 60% of the plants showed visible root system, reaching 100% rooting at the end of culture (Fig. 2C). Rooting is a process that requires high energy and can only occur at the expense of available metabolic substrates (mainly carbohydrates), and the nature of carbon source and its concentration also affect *in vitro* rooting in many species (Yaseen *et al.,* 2013). In this study, although fructose was not effective in shoot multiplication, if the nodal segments of these shoots are transferred to a medium with only sucrose, fructose pre-treatment can facilitate the rooting of the shoots (Fig. 2C). This result is very important due to the fact that *A. hirtula* presents low *in vitro* rooting. Currently, there are no reports on long-term effects of explant exposure to fructose on rooting and further studies are needed to explain the mechanism by which fructose improves rooting development in *A. hirtula*. During the acclimatization phase, plants of *A. hirtula* showed a high survival rate (90-100%), except in those plants grown with 20 g L^{-1} fructose (30%) (Table 3). Plants did not differ in relation to shoot growth (average height of 2.4 cm) (Table 3).

There were no significant variations between treatments, although the highest chlorophyll index and lowest chlorophyll *a*/*b* ratio were observed in those previously cultivated plants with 30 g L^{-1} glucose (Table 3). In general, the success of subsequent acclimatization after a period of *in vitro* growth is mainly dependent on nutrients and macromolecules reserved in the leaves developed *in vitro,* such as chlorophylls. Adjustments in levels and the relationship between the pigments result in higher photosynthetic efficiency and can be used as plant adaptability markers to different environments (Souza *et al*., 2011), since photosynthetic adaptation is critical to the survival of plants (Chazdon, 1988). Indeed, in this study, compared to the content of chlorophyll from *in vitro* plants (Table 2), *ex vitro* plants (Table 3) showed a significant increase in total chlorophyll index, approximating the value obtained in wild plants (n = 20 plants) of *A. hirtula* (total chlorophyll index average of 37.7 ± 5.0).

Moreover, it was observed that during the acclimatization phase the extent of increase was higher in chlorophyll b than a; thus as a result of these changes, the chlorophyll *a/b* ratios were lower in *ex vitro* plantlets (Table 2; Table 3), which may be considered an important physiological response related to adaptation of the photosynthetic system to environmental conditions, being that in most species, chlorophyll *a* is 2-3 times more abundant than chlorophyll *b* (Kramer and Kozlowski, 1979). Similar results were observed by Premkumar *et al*., (2001), comparing the *in vitro* culture conditions and acclimatization on the content of photosynthetic pigments and enzymes. These authors also found a higher ratio of chlorophyll *a*/*b* in *in vitro* plants of strawberry, avocado, olive and oak when compared to those *ex vitro*.

Table 1. Comparison of cytokinins on shoot proliferation from nodal segments of *Alternanthera hirtula*, after of 45 days of *in vitro* culture on MS medium

Cytokinin	Concentration (µM)	Number of shoots per explant	Number of nodal segments per explant	Shoot height (cm)
BAP	1.0	1.4 ± 0.2b	2.6 ± 0.3b	4.2 ± 0.3a
	3.0	1.3 ± 0.1b	2.4 ± 0.2b	2.4 ± 0.3b
KINETIN	1.0	1.2 ± 0.1b	1.8 ± 0.2bc	2.0 ± 0.3bc
	3.0	1.1 ± 0.1b	1.2 ± 0.1c	1.5 ± 0.2bcd
TDZ	1.0	6.3 ± 0.5a	18.1 ± 1.3a	1.2 ± 0.2cd
	3.0	7.0 ± 0.6a	21.5 ± 1.5a	1.4 ± 0.3cd
Control	-	1.0 ± 0.1b	1.1 ± 0.1c	1.0 ± 0.2d

Mean values followed by the same lower-case letter in each column are not significantly different at $P \leq 0.05$ using Tukey`s multiple range test. Each value represents mean ± SE calculated from six replicates of five explants for each treatment ($n = 30$).

Table 2. Comparison of effect of carbohydrates on shoot proliferation and chlorophyll content from nodal segments of *Alternanthera hirtula*, after 45 days of *in vitro* culture on MS medium containing TDZ (1.0 µM).

Carbon sources	(g L^{-1})	Number of shoots	Number of nodal segment	Plant height (cm)	DW (g $plant^{-1}$)	Chlorophyll index	Chl*a*/*b* ratio
Sucrose	20	5.5ab	16.0ab	1.6ab	0.05c	23.2a	7.4a
	30	5.7ab	17.5ab	1.8a	0.04d	17.7a	7.0a
	40	4.0ab	12.4ab	1.3ab	0.05c	20.2a	8.3a
Glucose	20	6.2a	20.0a	1.8a	0.08a	18.9a	7.1a
	30	5.6ab	18.3ab	1.3ab	0.06b	17.1a	7.7a
	40	4.7ab	11.7ab	1.1b	0.04d	17.8a	7.0a
Fructose	20	2.7b	9.3b	1.7ab	0.02f	14.9a	6.8a
	30	4.3ab	12.4ab	1.3ab	0.03e	18.5a	8.0a
	40	3.6ab	9.9b	1.3ab	0.03e	22.3a	5.4a

Mean values followed by the same lower-case letter in each column are not significantly different at $P \leq 0.05$ using Tukey`s multiple range test. Each value represents mean calculated from six replicates of five explants for each treatment ($n = 30$).

Table 3. Effects of carbohydrates used during multiplication phase on *ex vitro* acclimatization of *Alternanthera hirtula* plants recorded 60 days after transplanting to substrate.

Carbon sources	(g L^{-1})	Survival (%)	Plant height of(cm)	Chlorophyll index	Chlorophyll *a*/*b* ratio
Sucrose	20	100a	2.5a	33.7ab	6.2a
	30	100a	2.8a	35.8ab	4.9ab
	40	100a	2.7a	35.6ab	4.7ab
Glucose	20	100a	2.1a	35.0ab	4.9ab
	30	100a	2.6a	42.9a	3.9b
	40	100a	1.6a	39.8ab	4.5ab
Fructose	20	30b	2.1a	32.4b	4.6ab
	30	90a	3.4a	35.5ab	4.9ab
	40	90a	1.7a	35.5ab	4.6ab

Mean values followed by the same lower-case letter in each column are not significantly different at $P \leq 0.05$ using Tukey`s multiple range test. Each value represents mean calculated from ten replicates of one plant for each treatment ($n = 10$).

Plants with higher chlorophyll content possibly have greater chances of survival in the acclimatization phase due to a higher photosynthetic competence (Premkumar *et al*., 2001; Cassana *et al*., 2010). But in this study, in general, *in vitro* and *ex vitro* conditions favored the production of chlorophyll and plant development, which indicates that the lower survival rate observed in those plants previously multiplied with 20 g L^{-1} fructose (30%) (Table 3) is probably due to some other factor (possibly anatomical and/or physiological alterations), but not related to the chlorophyll content of leaves. After transfer to the greenhouse, we achieved more than 97% survival of the plants that were acclimatized and most of plants showed normal growth and development (Fig. 1C), having a good root development during acclimatization (Fig. 1D), which may explain the high survival rate of the plants after transfer to the botanical garden (80.0%).

This indicates that *A. hirtula* has a high physiological plasticity (Gratani, 2014), which may provide advantages in development and facilitate its conservation, reintroduction into the wild, and its cultivation for ornamental purposes. The first plant reintroduction in nature was done in February in a sunny and well-drained soil location in the Pampa biome (RS, Brazil) located on a private farm, taking care so that the plants did not suffer anthropic influence. In this period, it was found that *A. hirtula* showed susceptibility to cold and frost, losing all leaves in the winter period, but sprouted from the radicular system in early spring. This behavior was also observed the in plants maintained in the greenhouse (Fig. 1E). According to Marchioretto (2013), the presence of a well-developed subterranean system, which allows vegetative reproduction, is an ecological adaptive strategy very common in Amaranthaceae species, ensuring survival in unfavorable situations (such as frost, fire, grazing or drought).

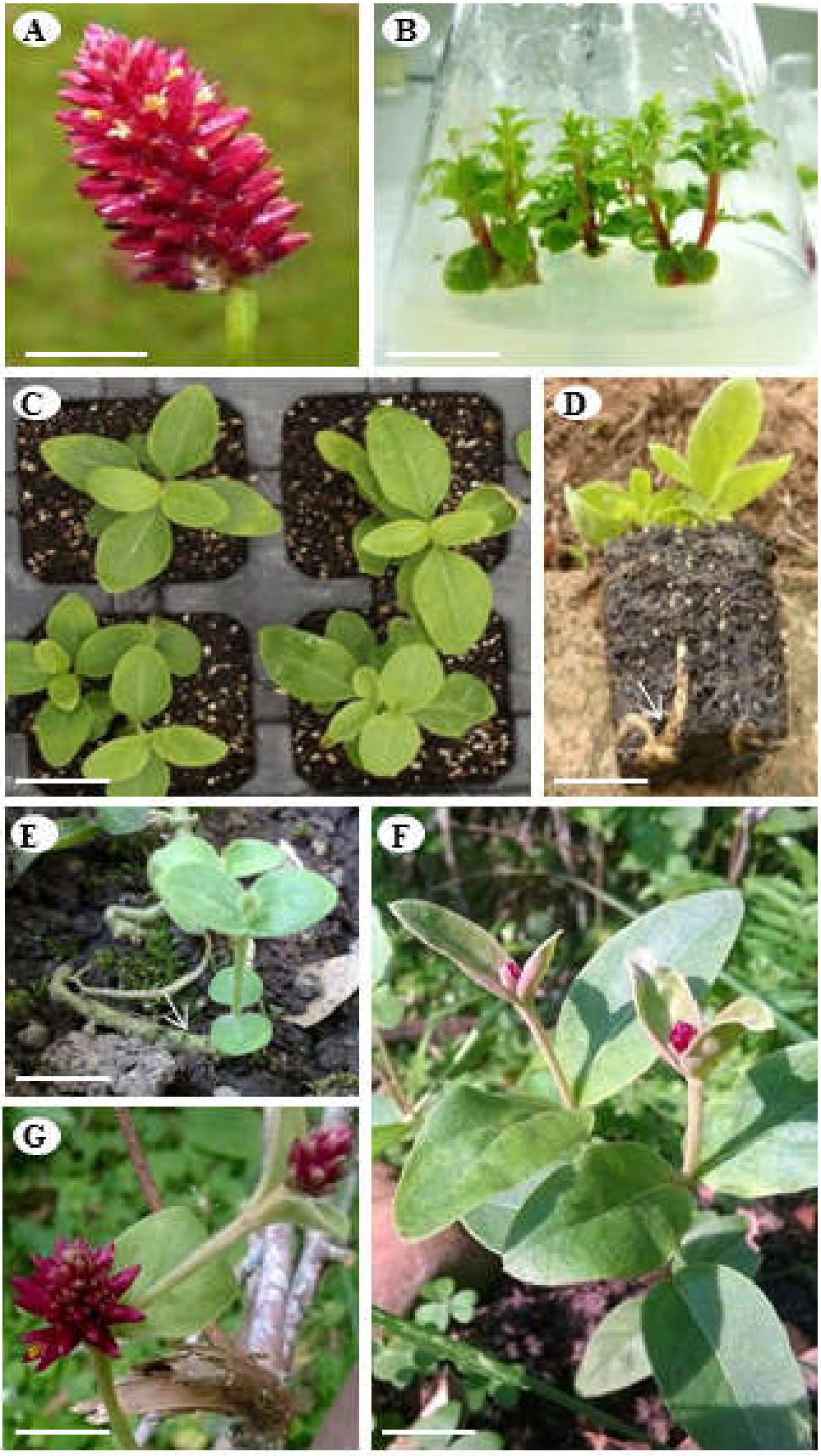

Fig. 1. Micropropagation from nodal segments of *Alternanthera hirtula* adult plants and reintroduction of the plants in nature. A: Inflorescence of adult plants. B: Multiple shoots formed on medium supplemented with 1.0 µM TDZ. C: Acclimatization of the *in vitro*-derived plants after transfer to greenhouse. D: Plants transferred to Botanical Garden, showing the presence of good root formation (*white arrow*). E: Shoots formed from roots (*white arrow*) observed in plants maintained in greenhouse. F: *In vitro*-derived plantsreintroduced in the field (Pampa biome, RS, Brazil), seven months after planting. G: Inflorescence formed from *In vitro*-derived plants reintroduced in the Pampa biome. Bars: A, B: 1 cm; C-E: 3 cm; F: 2 cm; G: 1.5 cm

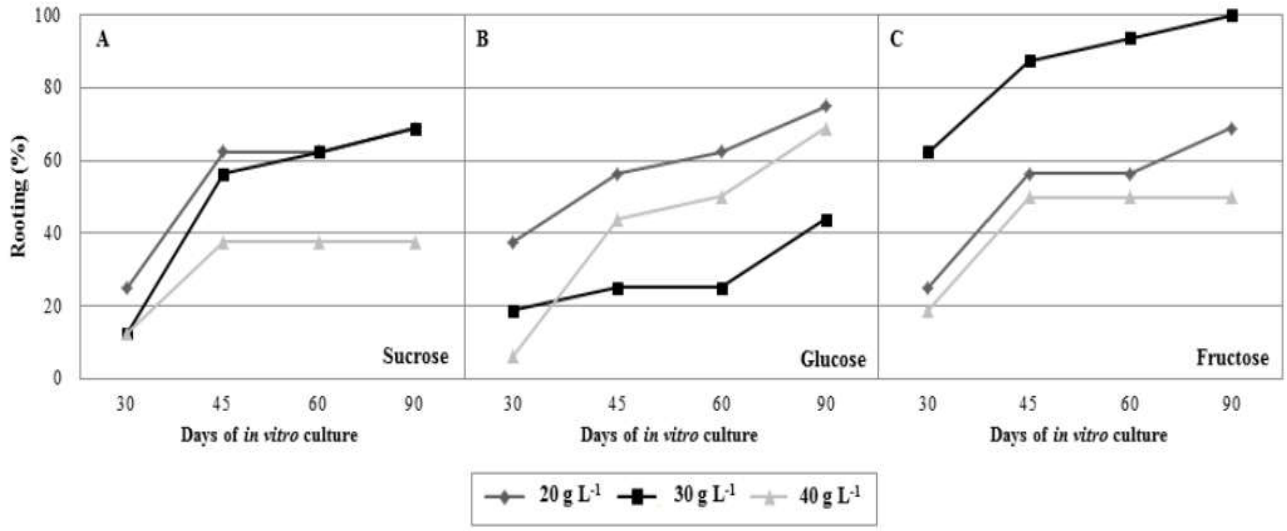

Fig. 2. Effect of concentrations of glucose, sucrose and fructose used in the multiplication phase on the frequency of rooting in the shoots of *Alternanthera hirtula* after 30, 45, 60 and 90 days of *in vitro* culture in medium containing 30 g L^{-1} sucrose

Generally, all the plants showed the developmental of several shoots, with average length of 8.0 cm and mean chlorophyll index of 35.2. Seven months after planting, vegetative growth was followed by flowering (Fig. 1F) and inflorescences showed a remarkable aroma and high durability (about three months). These inflorescences formed from *in vitro*-propagated plants (Fig. 1G) were morphologically similar to inflorescences of plants grown naturally in the field (Fig. 1A). Thus, *A. hirtula* shows great adaptability to environmental conditions and vegetative reproduction strategies, which indicates that the reduction of its population in nature results from environmental degradation imposed by anthropic actions.

Conclusions

Based on these observations, our protocols for the *in vitro* propagation of *A. hirtula* is viable. Optimal results for multiplication were obtained by using MS basal plus TDZ (1.0 µM) and supplemented with sucrose (30 or 40 g L^{-1}) or glucose (20 g L^{-1}) and rooting in MS basal with sucrose (30 g L^{-1}). In addition, these plants presented excellent adaptation when reintroduced into the environment. This study is the first investigation of clonal propagation, *ex situ* conservation and reintroduction of the *in vitro* derived-plants into a natural environment for the *Alternanthera* genus, and provides new perspectives for studies of vegetative propagation and conservation of *A. hirtula* germoplasm. Future studies may investigate selection of genotypes that have greater ornamental potential and ultimately lead to its commercial cultivation. The protocol is repeatable for *A. hirtula* and might also be applicable to others species of the genus.

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Conflict of Interest

The authors declare no financial or commercial conflict of interest.

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