Micropropagation of *Dyckia agudensis* Irgang & Sobral – an extinction threatened bromeliad

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ABSTRACT – *Dyckia agudensis* is an endemic species, that occurs on the Agudo hill, Agudo County (RS), it is considered threatened of extinction. The aim of this study was to develop a micropropagation protocol for this species to aid its conservation. The seeds were disinfected and germinated *in vitro*. Seedlings were submitted to the treatments of 0; 0.5; 1.0 or 2.0 mg.L^{-1} of BAP and IBA (same concentration) at 15 days of germination. The MS medium was supplemented with 2 ml.L^{-1} of Fuji vitamins, 30 g.L^{-1} of sucrose, and solidified with 6 g.L^{-1} of agar. After 60 days of cultivation, plantlets were transferred to a multiplication and regeneration medium. The medium was MS with 2 ml.L^{-1} of Fuji vitamins, 30 g.L^{-1} of sucrose, 0.5 mg.L^{-1} of BAP, and 7 g.L^{-1} of agar. The rooting medium was MS without growth regulators. The disinfection process obtained 85% of success. The same concentrations of BAP and IBA on the seedlings followed a quadratic regression, increasing shoot and bud numbers and swelling percentage, but reduced the percentage of rooting. The maximum efficacy was obtained close to 1.0 mg.L^{-1} of BAP and IBA in the culture medium. The multiplication phase proceeded together with the regeneration. The isolated shoots rooted without growth regulators. The acclimatization process was carried out in a hydroponic system for 15 days before transferring to the substrate.

Key words: *in vitro* culture, bromeliaceae, direct organogenesis, hydroponics.

RESUMO – **Micropropagação de *Dyckia agudensis* Irgang & Sobral – uma bromélia ameaçada de extinção.** *Dyckia agudensis* é uma espécie endêmica, que ocorre no morro Agudo, município de Agudo (RS), considerada ameaçada de extinção. O objetivo deste trabalho foi desenvolver um protocolo de micropropagação para esta espécie a fim de auxiliar na sua conservação. As sementes foram desinfestadas e germinadas *in vitro*. Após 15 dias da semeadura, as plântulas foram submetidas aos tratamentos de 0; 0,5; 1,0 ou 2,0 mg.L^{-1} de BAP e AIB (concentrações iguais), suplementados ao meio MS, adicionado de 2 ml.L^{-1} de vitaminas Fuji, 30 g.L^{-1} de sacarose e solidificado com 6 g.L^{-1} de agar. Após 60 dias, as plântulas foram colocados em meio para a multiplicação e regeneração, constituído do meio MS com 2 ml.L^{-1} de vitamínas Fuji, 30 g.L^{-1} de sacarose, 0,5 mg.L^{-1} de BAP e solidificado com 7 g.L^{-1} de agar. Para o enraizamento foi usado o meio MS sem reguladores de crescimento. No processo de assepsia obteve-se 85% de desinfestação. Os efeitos das concentrações iguais de BAP e AIB sobre as plântulas seguiram regressões quadráticas, sendo positivas para as características número de brotos e de gemas e percentagem de entumescimento e negativa para a percentagem de enraizamento. A máxima eficiência obtida para estas características permaneceu próxima de 1,0 mg.L^{-1} de BAP e AIB. A fase de multiplicação seguiu juntamente com a regeneração. Os brotos posteriormente isolados enraizaram sem a presença de reguladores de crescimento. Os clones foram aclimatizados em um sistema hidropônico durante 15 dias e posteriormente transferidos para substrato.

Palavras-chave: cultivo *in vitro*, bromeliaceae, organogênese direta, hidroponia.

INTRODUCTION

Dyckia agudensis Irgang & Sobral is an endemic species found only on the Agudo hill, Agudo County, Rio Grande do Sul State (RS), Brazil. It was considered as threatened of extinction by the RS' red list (Sema, 2005). This species shows ornamental characteristics, it had an inflorescence with a bolting of 0.9 m tall and flowers of yellow corolla (Irgang & Sobral, 1987). This species belongs to Bromeliaceae family.

Bromeliads species are relatively difficult to propagate, because of their slow growth (Hosoki & Asahira, 1980). Therefore, no many individuals are produced from one plant. In this scenery, the micropropagation technique is the most efficient methodology available. The micropropagation technique was already described with success for the species *Dyckia sulphurea* Koch (Murashige, 1974) with shoot tip explants; *Dyckia macedoi* L. B. Sm. (Mercier & Kerbauy, 1993) with leaf explants; *Dyckia distachya* Hassler (Pompelli, 2002; Pompelli & Guerra, 2005) with seed explants; and *Dyckia maritima* Baker (Silva, 2005) with plantlet explants.

Germplasm conservation is important to conserve genetic diversity. The *in situ* conservation keeps diversity as it occurs in nature and preserves the ecosystem against external pressure (Ferreira, 1988), consequently it facilitates the coevolution of species in their natural habitat. The micropropagation technique facilitates the *in vitro* conservation, which is an auxiliary tool for the species conservation.

This paper describes an efficient method for *in vitro* propagation of *D. agudensis*.

MATERIAL AND METHODS

The experiments were carried out at Plant Tissue Culture of the Department of Plant Production of the Federal University of Santa Maria (UFSM). All the experiments were maintained in a growth room with temperature of 25ºC ± 2ºC and 16 hours of photoperiod under a light intensity of 14.3 $\mu E.m^{-2}.S^{-1}$ obtained with white fluorescent lamps, with exception of the acclimatization experiment that was conducted in a greenhouse.

D. agudensis fruits were collected on the Agudo hill, Agudo County, Rio Grande do Sul State, Brazil. Seeds were mechanically removed from the fruits and used with the presence of the winged region. Disinfection was done through seed immersion in alcohol solution of 70% during five minutes and rinsed twice in sterilized distilled water, immersion in a commercial bleach (5% NaOCl) for 60 min and rinsed three times in sterilized distilled water. The germination medium consisted of distilled water and 30 $g.L^{-1}$ sucrose and solidified with 6 $g.L^{-1}$ agar. The pH was adjusted to 5.7 prior to autoclaving.

Plantlets with 15 days were transplanted to a medium with growth regulators, containing the same concentration of BAP (6-benzylaminopurine) and IBA (indole-3-butyric acid) of 0; 0.5; 1.0 or 2.0 $mg.L^{-1}$. The basal medium was MS (Murashige & Skoog, 1962) supplemented with 30 $g.L^{-1}$ of sucrose and 2 $mL.L^{-1}$ of Fuji vitamins (2.5 $g.L^{-1}$ nicotinic acid, 2.5 $g.L^{-1}$ piridoxine chloridrate, 0.5 $g.L^{-1}$ thyamine chloridrate, 50 $g.L^{-1}$ inositol e 1 $g.L^{-1}$ glycine). The medium was solidified with 6 $g.L^{-1}$ of agar and the pH was adjusted to 5.7 prior to autoclaving. The evaluations were done after 60 days of cultivation based upon swelling and rooting percentage and bud and lateral shoot numbers.

The multiplication medium for the lateral buds and shoot clusters was the MS supplemented with 30 $g.L^{-1}$ of sucrose, 2 $mL.L^{-1}$ of Fuji vitamins, 0,5 $mg.L^{-1}$ of BAP and solidified with 7 $g.L^{-1}$ of agar. After 40 days of cultivation, the final shoot mean number and shoot increment (quotient between the final shoot number and the shoot initial number) were evaluated.

Lateral shoots from clusters were isolated and cultivated in a MS medium supplemented with 30 $g.L^{-1}$ sucrose, without growth regulators, and solidified with 7 $g.L^{-1}$ of agar and pH adjusted to 5,7 prior to autoclaving. The evaluations of rooting percentage and lateral shoot percentage and number were evaluated after 40 and 60 days of cultivation.

Shoots with roots were removed from *in vitro* conditions and roots were washed off to remove culture medium residues. A hydroponic system was used for the acclimatization in alveolated trays containing coconut fiber substrate and a nutritive solution of complete MS medium without sucrose. The tray containing the plantlets was placed in a plastic basin with nutritive solution. The nutritive solution was completed with one liter of distilled water and the pH was adjusted to 5.7 every four days. Plantlets were evaluated after 15 days and transferred into a commercial substrate (Terra vegetal, Vitaplan®). After 15 days of cultivation, the plantlets were evaluated again. The evaluated characteristics were shoot length (cm), leaf and root numbers, fresh mass (g) and survival percentage.

The experimental design was a complete randomized with five replicates of five explants. The data was submitted in a normality analysis for the Bartlett´s method and, followed by regression analysis at the level of 5% likelihood error. All regression analysis were done following the procedures of the software GENES (Cruz, 2001).

RESULTS

The seed disinfection process resulted in 85% of success and 83% of germination. The same concentrations of BAP and IBA followed a positive quadratic regression for swelling percentage, lateral shoot and bud numbers. For the rooting percentage, the data followed a negative quadratic regression. The maximum efficacy obtained for the swelling percentage was 1.1 mg.L^{-1} BAP and IBA; for the rooting percentage was 1.2 mg.L^{-1}, for the lateral shoot number reached 1.1 mg.L^{-1} and 1.2 mg.L^{-1} for the lateral bud number (Fig. 1 and 2). The multiplication and regeneration of lateral buds and shoots in clusters occurred simultaneously (Fig. 3). The shoot final mean number was 4,6 ± 2.5 and the increment of shoot number was 1,6 ± 1. Rooting occurred without growth regulators in the medium. The rooting percentage after 40 and 60 days was 48 ± 9 and 64 ± 11%, respectively. Isolated shoots produced sprouts in their base that resulted in a percentage of 48 ± 11% sprouts for isolated shoot. During plantlet acclimatization, there was a continuous growth of the shoot in length, during the hydroponic and substrate period. There was an increase in root number during the acclimatization, and in the number of leaves during the hydroponic period, but a decrease after transferring to the substrate. There were no changes in fresh mass value in the hydroponic period, even so, there was an increase in this characteristic after the transplanting to the substrate. The percentage of survival was stable in 100% during the first 30 days of acclimatization (Tab. 1), after 60 days it was reduced to 90% and stabilized in the 120 days. Some lateral sprouts were formed in the base of some plantlets during the acclimatization.

TABLE 1 – *Dyckia agudensis* acclimatization after 15 days of culture on hydroponic system and after 15 days of culture in soil.

Characteristics	Initials values	After 15 days (Hydroponics)	After 30 days (Soil)
Shoot length (cm)	2.4 ± 1.0*	2.7 ± 1.0	2.8 ± 1.0
Leaves number	13.2 ± 2.7	13.6 ± 2.2	13.3 ± 2.8
Roots number	1.7 ± 2.0	1.9 ± 1.9	2.3 ± 2.0
Fresh mass (g)	0.4 ± 0.3	0.4 ± 0.4	0.5 ± 0.4
Survival	100 ± 0.0	100 ± 0.0	100 ± 0.0

* Values corresponding to the standard deviation of the mean.

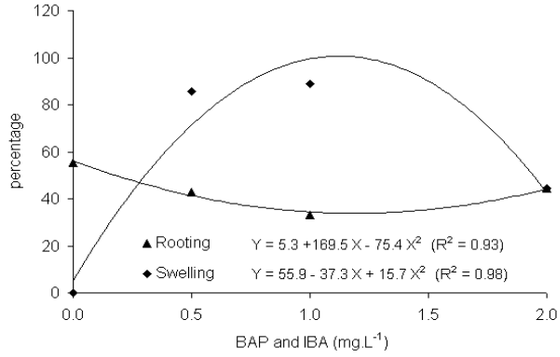

Fig. 1. Effects of BAP and IBA concentration over swelling percentage and rooting percentage in plantlets of *Dyckia agudensis*, after 60 days of *in vitro* culture.

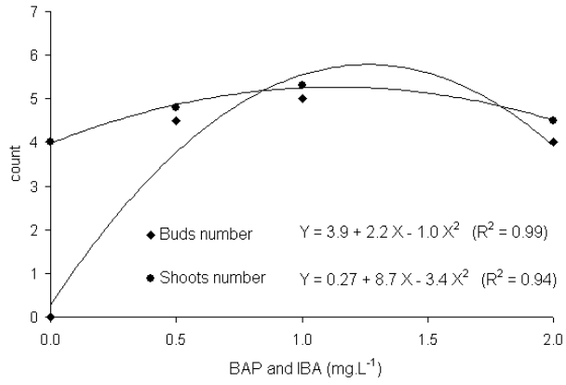

Fig. 2. Effects of BAP and IBA concentrations over the lateral buds and shoots number in plantlets of *Dyckia agudensis*, after 60 days of *in vitro* culture.

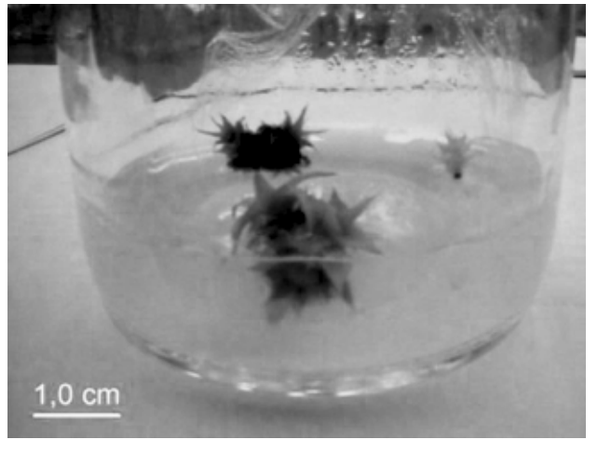

Fig.3. Multiplication and regeneration in *Dyckia agudensis* in MS medium supplemented with 0.5 mg.L^{-1} of BAP and Fuji vitamins after 40 days of *in vitro* culture.

DISCUSSION

The time of seed immersion in a NaOCl solution was considered high, however the time of exposition used for the *D. maritima* species was higher (Silva, 2005), hence, the cause of this high value is because there was no removal of seed winged region, as it was made in *D. distachya*, where the time of exposition in NaOCl was 30 min (Pompelli, 2002). The value obtained for germination percentage can be related to the disinfection process, or to the seed physiological quality.

The concentration 0 $mg.L^{-1}$ of BAP and IBA (control) produced lateral shoots, differing from the ones found in *D. maritima*, where these concentrations do not produced lateral shoots. It is possible that it can be promoted by the presence of Fuji vitamins in the culture medium, whereas in *D. maritima* there was no supplementation of these vitamins in the culture medium. The different age of explant can be the cause of this difference, whereas in *D. maritima* the explants was a 25 days, while in *D. agudensis* was 15 days (Silva, 2005). BAP and IBA concentrations produced lateral shoots. Similar results were obtained in *Tillandsia fasciculata* Swartz var. *fasciculata* with the concentrations of BAP and IBA varying from 0,5 to 10 µM, where all the seedlings produced multiple shoots (Koh & Davies, 2001). Similar results were found *in Vriesea reitzii* Leme & Costa, where structures similar to lateral buds were produced from the basal region of seedling explants after 10 weeks, cultured on liquid medium supplemented with 4 µM of BAP and 2 µM of NAA (a-naphthaleneacetic acid) (Rech-Filho *et al.*, 2005).

There was probably an ideal concentration for the induction of these competent morphogenics structures, because the maximum efficacy obtained for swelling and rooting percentage and bud and shoot numbers was around 1.0 $mg.L^{-1}$ BAP and IBA. The results obtained in the multiplication and regeneration phase showed similar results with BAP in *D. distachya* (Pompelli & Guerra, 2005), because there was buds regeneration. However, this result disagrees with the results showed in *D. maritima* with similar concentrations of BAP promoted buds regeneration inhibition (Silva, 2005). However, in many bromeliad species protocols consider to use BAP with lower NAA concentration. In *Vriesea fosteriana* L.B. Smith the best result were 8.9 µM of BAP and 2.7 µM of NAA for bud proliferation (Mercier & Kerbauy, 1992). In *D. macedoi* the best results were with 5.0 $mg.L^{-1}$ of BAP and 0.1 $mg.L^{-1}$ of NAA where buds were produced in the basal leaf explants (Mercier & Kerbauy, 1993). In *Vriesea reitzii*, the highest rate of shoot multiplication was with 2-4 µM of BAP and 1-2 µM of NAA (Rech-Filho *et al.*, 2005).

The roots formation in isolated shoots without the presence of growth regulators in *D. agudensis* was similar to the observations found in *D. macedoi* (Mercier & Kerbauy, 1993) and *D. distachya* (Pompelli, 2002), even so, the rooting percentage can be increased with the addition lower auxins concentration, especially NAA, in the concentrations 1.1 µM and 0.1 $mg.L^{-1}$, respectively (Mercier & Kerbauy, 1992; 1993). The lateral shoot formation in isolated shoots during the acclimatization was also observed in *D. maritima* (Silva, 2005), indeed, possibly this fact occurred because of the permanence of a BAP endogenous level, originated from the prior multiplication phase. For the *D. maritima* species there was suggested a prior phase of shoot elongation to submit them to a rooting phase (Silva *et al.*, 2004). However, in *Vriesea fosteriana* the addition of 0.54 µM of NAA was necessary to stop these lateral shoots proliferation as well as to re-stabilize apical growth of the shoots, in this way, rooting was easily induced (Mercier & Kerbauy, 1992).

The acclimatization made in a hydroponics for 15 days promoted fast acclimatization, on the contrary, in *D. distachya* this phase needed four months (Pompelli & Guerra, 2005). The acclimatization results obtained in *D. agudensis* are similar to those obtained with *D. maritima* species, which also was acclimatized in this same hydroponic system (Silva, 2005). The fact that there was no decrease in plantlets fresh mass after 15 days in the hydroponic period, and that occurred an increase in this value after transplanting to substrate, it suggests that the plantlets do not suffer hydric stress. Moreover, there was no decrease in the root number, what suggest that the roots produced *in vitro* were functional, as found in *D. distachya* (Pompelli, 2002). Similar results also were found in *Colocasia esculenta* (L.) Schott. (Araceae) where it was compared normal acclimatization with acclimatization in a hydroponic system, after 30 days, the survival rate, number of leaves and plant height were higher under the hydroponic system (Nhut *et al.*, 2004).

The results of these experiments demonstrated the viability of the micropropagation technique for

the massal reproduction and it can be useful as a tool for *in vitro* germplasm conservation of the *D. agudensis* species. The fact of this protocol uses plantlets is very interesting, because it allows to maintain the genetic diversity in the original populations, with positive impacts on their conservation (Rogers, 1984; Carneiro & Mansur, 2004; Pompelli & Guerra, 2005).

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