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Early selection of elite clones of an ornamental bromeliad *in vitro*

Seleção precoce *in vitro* de clones elite de uma bromélia ornamental

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ABSTRACT

Orthophytum grossiorum *is a typical bromeliad from Atlantic forestry threatened of extinction. The objectives of this research were to select* ***O. grossiorum*** *clones with ornamental values easy to propagate* in vitro*, and establish* in vitro *propagation protocols for these clones. The project was developed in three steps: germination and in vitro selection of seedlings responsive to BAP (6-benzylaminopurine), selection of clones with ornamental values, and establishment of protocol for* in vitro *propagation of the selected clones. In the first step only 18.33% of plantlets germinated* in vitro *were responsive to BAP. These plantlets were selected and replicated* in vitro *several times, each replicated plantlet constituting a clone. In the second step these clones were established* ex vitro *and surveyed for ornamental attributes. Five out of 11 clones were selected in this step. These clones presented distinct phenotypic traits and were considered of high ornamental quality. In the third step a protocol for* in vitro *propagation was developed for each selected clone.*

Key words: *micro-propagation, tissue culture, breeding.*

RESUMO

Orthophytum grossiorum *é uma bromélia ameaçada de extinção típica de Mata Atlântica. Os objetivos deste trabalho foram selecionar clones de* ***O. grossiorum*** *com potencial ornamental e de fácil propagação* in vitro *e estabelecer protocolo de propagação in vitro para esses clones. O trabalho foi desenvolvido em três etapas: germinação e em seleção* in vitro *de plântulas responsivas a BAP (6-benzylaminopurine), seleção de clones com valores ornamentais e estabelecimento de protocolo para propagação* in vitro *dos clones selecionados. Na primeira etapa, foi observado que apenas 18.33% das plântulas germinadas* in vitro *eram responsivas a BAP. Essas plântulas foram selecionadas e reproduzidas em* in vitro*, e cada plântula selecionada e reproduzida constituiu um clone. Na segunda etapa, esses clones foram estabelecidos* ex vitro *e selecionados em relação aos atributos ornamentais. Nessa etapa, foram selecionados cinco entre 11 clones. Esses apresentaram características fenotípicas distintas, sendo considerados de alta qualidade ornamental. Na terceira etapa, o protocolo para em propagação* in vitro *foi desenvolvido para cada clone selecionado.*

Palavras-chave: *micropropragação, cultura de tecido, melhoramento.*

INTRODUCTION

Considering the current growing concern with the environment, and Brazil being one of the largest centers of biological diversity in the world, emphasis has been given to stimulating the search for alternatives for preservation and conservation of certain species. One of the alternatives is to provide plants grown in nurseries to the market, to reduce the pressure of exploitation of the wild ornamental specimens.

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Orthophytum grossiorum is a new species of Bromeliaceae from Brazil described and illustrated by LEME & PAULA (2003). ***O. grossiorum*** is a bromeliad at risk of extinction (MMA, 2009) and present several ornamental characteristics. The species present distinct basal rhizomes, lepidote leaves, smaller spines on leaf margin, inflorescence sometimes compound, yellow floral bracts with smaller marginal spines, and smaller flowers. It also displays intense contrasting colors with brownish red leaves and its yellow floral bracts (LEME & PAULA, 2003). These characteristics combined with rusticity, small size, easy handling and adaptation as well as long-lasting inflorescence, makes it a perfect ornamental plant to be commercially explored (ANDERSON, 2007). However, it is not cultivated commercially, since no agronomically-defined techniques are currently available at a commercial scale. To transform this species into a commercial ornamental plant ***O. grossiorum*** has to be tailored in two aspects: easy propagation and uniform clones.

The first step in the breeding of a wild plant species is to define its propagation methods. The propagation of bromeliads can occur both through the seminiferous via and by natural vegetative propagation using axillary shoots (BENZING, 2000). The vegetative process is the most used method of propagation for commercial purposes.

Vegetative propagation *in vitro*, also known as micro-propagation, is a practical form of tissue culture that has great impact on the propagation of new clones. This system allows rapid clone multiplication with high genetic fidelity. In cultivation *in vitro*, the addition of growth regulators to the cultivation medium, (specifically, cytokinins) is indispensable for breaking apical dominance and axillary bud induction (PAEK & HAHN, 2000). Of the cytokinins commercially available, 6-benzylaminopurine (BAP) is the most commonly used, because it is cheap and highly efficient in promoting multiplication in several bromeliad species (GUERRA et al., 1999; MERCIER & NIEVOLA, 2003; PASQUAL et al., 2008).

For a successful *in vitro* vegetative propagation, it is fundamental that the selected clones react to the stimuli applied to the growth environment, especially to growth regulators. However, the response of different plant species that are cultured *in vitro*, varies as a function of the genotype and the cultivation conditions (MERCIE & NIEVOLA, 2003). One genotype-dependent particularity is that many genotypes are recalcitrant to cultivation *in vitro*.

For some bromeliads the *in vitro* cultivation can be a easy and simple method that provides a high rate of multiplication and production of healthy plants, and representes a wide variety of genotypes in space and a shorter time (RECH FILHO et al., 2005; POMPELLI & GUERRA, 2006; SILVA et al., 2007).

Therefore, this research aimed to test a precocious selection of genotypes *in vitro* of ***O. grossiorum*** to obtain clones that are easy to propagate *in vitro* and with ornamental potential.

MATERIAL AND METHODS

This research was taken at the Laboratory of Cell and Plant Tissue Culture and at the Bromaliaceae Conservation and Research Unit (UPCB). The research was carried out in three stages: germination and selection of plantlets responsive to BAP (6-benzylaminopurine), selection of high ornamental quality clones and protocol adjustment for the *in vitro* propagation of each of the selected clones.

For the germination and selection of BAP responsive plantlets, one ripe fruit (berries) of ***O. grossiorum***, obtained by open pollination, were collected from one matrix plants established at the Bromeliaceae Conservation and Research Unit(UPCB), in Viçosa-MG, and cleaned by washing in running water for 30 minutes. The sixty-three seeds extracted from these fruit were then disinfected as follows: immersion in alcohol 70% (v/v) for one minute, rinsing in sterile deionized water, followed by immersion in sodium hypochlorite (2.0% of active chlorine) and Tween 20 (0,01%) for 15 minutes. Throughout the disinfection process, the seeds were under agitation; afterwards, they were rinsed three times in sterile deionized water, followed by inoculation in culture medium containing 50% MS (MURASHIGE & SKOOG, 1962) (macro and micro salts) and supplemented with 30g l^{-1} MS sucrose, vitamins and 100mg l^{-1} myo-inositol. Medium pH was adjusted to 5.7±0.01 and the medium was solidified with 8.0g l^{-1} agar (Grupo Química®); 10mL of the medium was placed in assay tubes measuring 25x150mm, and autoclaved at 121°C and 1.5atm for 20 minutes. The tubes were sealed with plastic lids and transparent PVC film. After inoculation, the explants were transferred to the growth chamber at 25±2°C, with a photoperiod of 16 hours and irradiance of 40μmol m^{-2} s^{-1}, supplied by 40W white fluorescent bulbs (Philips®).

Based in our preliminary studies, performed by the same authors, the plantlets were transferred 60 days after germination into MS stationary liquid containing 10μM of BAP. After 60 days, only the

plantlets that responded to the treatment were selected, using as reference the emission of adventitious shoots. These plantlets were replicated then expanded in the conditions described above and clones were obtained in order to continue the research.

Five plantlets of 11 clones selected to BAP were transferred to a greenhouse in the UCPB. Plantlets measuring 20 to 30mm height possessing roots were acclimatized in plastic vases 100mm in diameter x 80mm in height with a 500ml capacity, filled with Bioplant®, a commercial growth substrate. These vases were arranged in a completely randomized fashion, and five repetitions of 11 treatments (clones) were given; a vase containing one plant was considered to be an experimental unit. The plants were maintained in a greenhouse under shade screem sombrite® 70%, receiving daily irrigation and fertilized weekly with 50ml of NPK (20-20-20) at a dose of $2gl^{-1}$.

After 190 days of acclimatization and development at the adult phase, before floral emission, a panel of five experts in bromeliads and ornamental plants evaluated the 11 clones. The experts evaluated five replica plants of each clone. For the selection of superior clones, the panel of experts assigned a subjective score ranging from zero to 10 in five important traits responsible for the commercial success of bromeliads. These included phytosanitary conditions, uniformity, plant color and shine, as well as espinescence expression. Together, these traits define the agronomic quality and ornamental potential of these plants. The sum of these scores produced an ornamental quality variable that was used to guide the selection of clones with high commercial production potential. Plant precocity was also considered for clone selection. Plants that emitted floral stem before 200 days of vase planting were considered to be precocious.

To the adjustment of the protocol for propagation *in vitro*, five clones were selected which presented the best means for produced score an ornamental variable quality. Then, the five clones selected were retrieved from stocks maintained *in vitro* at the Laboratory of Cell and Plant Tissue Culture. The experiment used shoots from these plantlets that were uniform in size and lacking roots. Five BAP levels (0, 10, 20 30 and 40μM) were tested on shoots arranged in a completely randomized design, with 3 replications, in stationary MS liquid medium, and a flask containing two plants was considered to be an experimental unit. The culture medium pH was adjusted to 5.7±0.01 before sterilization at 121°C and 1.5atm for 20 minutes. Flasks measuring 50x140mm with a 350ml volume were filled with 20ml of culture medium. The explants were incubated in a temperature-controlled room at 25±2°C, with a 16-hour photoperiod and irradiance of 40μmol m^{-2} s^{-1} supplied by 40W white fluorescent bulbs (Philips®).

This procedure was carried out individually for each clone selected. The effects of auxin were not tested at this stage as preliminary studies revealed that this class of growth regulator had no effect on the *in vitro* multiplication and growth of this species being in some bromeliads prejudicial to the growth (KIMBERLY et al., 2003). After 60 days of incubation, the traits evaluated were: mean length of the shoots and number of shoots per plantlet.

The results obtained by evaluating all of the parameters to score produced an ornamental quality variable were submitted to variance analysis and their means tested by the Scott-Knott test (P>0,01). The five clones presenting the best means at this stage were selected for further micro-propagation protocol adjustment.

The results obtained from the five clones micro-propagation were analyzed separately, submitted to variance analysis and their means were fitted to regression in order to adjust the protocol for each clone. Statistical analyses were performed using the computer program "GENES" (CRUZ, 2006). After multiplication and before acclimatization shoots shorter than 5.0mm were elongated in MS liquid medium devoided of BAP.

RESULTS AND DISCUSSION

Seeds of ***O. grossiorum*** germinated after 15 days of incubation *in vitro*, reaching a 100% germination rate. In stationary MS liquid medium supplemented with 10μM of BAP, only 18.33% (11 plantlets) responded to the treatment by emitting adventitious shoots, thus showing the great influence of the genotype on the response to growth regulators *in vitro*.

Shoot formation occurred through the development of adventitious buds that formed in the foliar axil region. Bromeliads in general are highly efficient at *in vitro* germination, with a rate above 90% being common for ***Vriesea hieroglyphica*** (MERCIER & KERBAUY, 1995), ***Cryptanthus sinuosus*** (CARNEIRO et al., 1998), ***Tillandsia Eizii*** (KIMBERLY et al., 2003), ***Alcantarea imperialis*** (NAVES et al., 2003), ***Vriesea gigantea*** (DROSTE et al., 2005) and ***Neoregelia mucugensis*** (BELLINTANI et al., 2007).

The influence of the genotype on the success of propagation *in vitro* is well-known in the literature. For instance, LONE et al. (2008) similarly

observed in orchids that only 18.7% of the genotypes selected from ***Dendrobium phalaenopsis*** responded to *in vitro* cultivation in MS medium.

Shoot formation occurred through the development of adventitious buds that were formed in the foliar axil region. The development of these adventitious buds made viable the mass multiplication of a large number of uniform plants.

The advantage of first selecting genotypes responsive to multiplication *in vitro* is to guarantee the availability of the selected clones in the market, since not all the genotypes selected in a standard breeding program can be multiplied in this fashion. This process minimizes the breeding program cost by reducing the size of breeding trials for the selection of ornamental plants. In this case, for instance, the 82% of the genotypes that were non-responsive to the *in vitro* stimuli applied were quickly eliminated.

This process is the inverse of the methods practiced by most ornamental plant breeders, as they usually first select plants of interest and only then try to introduce and multiply the genotypes *in vitro* sometimes unsuccessfully due to genotypic recalcitrance.

After 30 days of being planted in vases, the ***O. grossiorum*** clones were acclimatized to the *ex vitro* environment and started growing and there were emission of new leaves. All of the transferred clones presented distinct traits from one another, variations which intensified as they developed. These differences included color, length, width, and leaf thickness, the presence of scales on the leaf, thorn size, spacing between thorns, rosette diameter, and height of the plant's rosette, color of the inflorescence, presence of bunches, and floral tassel length (Table 1). The differences obtained are due to the rising of the pollination process. Plans were originate from a single fruit by open pollination. In open pollination has only the maternal parental control, not knowing paternal origin.

At 190 days after vase planting, at the imminence of floral emission and when leaf coloration is more intense in ***O. grossiorum***, an expert panel evaluated the plants for ornamental characteristics values. Five clones had an outstanding performance: 01, 04, 05, 08 and 09 (Table 2), displaying values above 50 out of 60 for the variable ornamental quality. The selection of clones with excellent agronomic and ornamental quality is important for the commercial success of the clone.

The clones selected were characterized by diversity in color and leaf size, scale presence, and size and spacing between thorns when compared to one another (Figure 1).

Selection of breeding processes can lead to narrowing of the genetic variability, and thus plants with ornamental value might not be selected. In this research, in vitro preselection was not detrimental to the point to discard all useful variability as the selected clones presented aggregating traits of ornamental interest (Figure 1).

Table 1 - Morphological description of the clones of the bromeliad ***Orthophytum grossiorum***.

Clone	Original	01	04	05	08	09
Leaf color of the abaxial and adaxial face	Dark red	Reddish green	Greenish red	Rust red	Opaque rust red	Dark red
Presence of scales on the adaxial face	Yes [1]	Yes [3]	Yes [1]	Yes [1]	Yes [1]	Yes [1]
Presence of scales on the abaxial face	Yes [2]	Yes [5]	Yes [3]	Yes [3]	Yes [1]	Yes [4]
Leaf length (mm)	100-150	110-130	90-110	120-160	90-108	85-100
Leaf width (mm)	19-22	22-25	20-25	18-21	16-19	18-20
Leaf thickness (mm)	2-3	3-4	2-3	2-3	3	3-4
Thorn size (mm)	2	1.5-2	1-2	1.5-2	2	1-1.5
Spacing between thorns (mm)	5	5-6	4-6	5-8	5	3-4
Rosette diameter(mm)	190-230	180-220	160-200	200-240	200-210	150-170
Rosette height (mm)	70-80	70-80	70-90	70-80	70-80	60-80
Number of leaves	12	13	11	12	14	10
Color of inflorescence	Yellowish green	Yellowish green	Yellowish green	Yellowish green	Reddish green	Yellowish green
Presence of bunches on the inflorescence	Yes	Yes	No	Yes	No	No
Floral tassel length (mm)	220	250	210	270	230	200

Intensity:[1] very low, [2] low, [3] intermediary, [4] high, [5] very high

Table 2 - Phytosanitary scores average (FtS), size uniformity (SU), color uniformity (CU), espinescence expression (EE), shine (S), ornamental potential (OP), and ornamental quality variable (OQV) of ***Orthophytum grossiorum***.

Clone	FtS	SU	CU	EE	S	OP	OQV
01	9,03 [a]	9,03 [a]	8,97 [a]	8,93 [b]	8,90 [b]	9,07 [a]	53,93*
02	7,74 [e]	7,67 [f]	7,57 [f]	7,60 [g]	7,64 [e]	7,74 [e]	45,96
03	7,48 [f]	7,38 [g]	7,52 [f]	7,41 [g]	7,48 [f]	7,41 [f]	44,68
04	8,59 [b]	8,62 [b]	8,66 [b]	8,62 [c]	8,59 [c]	8,66 [b]	51,74*
05	8,53 [b]	8,40 [c]	8,40 [c]	8,40 [d]	8,43 [c]	8,50 [c]	50,66*
06	8,00 [d]	7,90 [e]	7,79 [e]	7,83 [f]	7,79 [e]	7,76 [e]	47,07
07	8,26 [c]	8,15 [d]	8,19 [d]	8,15 [e]	8,19 [d]	8,12 [d]	49,06
08	9,07 [a]	9,10 [a]	9,14 [a]	9,17 [a]	9,14 [a]	9,21 [a]	54,83*
09	8,45 [b]	8,41 [c]	8,41 [c]	8,38 [d]	8,35 [d]	8,38 [c]	50,38*
10	7,69 [e]	7,59 [f]	7,59 [f]	7,57 [g]	7,48 [f]	7,42 [f]	45,34
11	7,11 [g]	6,97 [h]	6,97 [g]	7,07 [h]	7,07 [g]	7,10 [g]	42,29

In each column, means followed by same letter do not differ by the Scott-Knott test (P>0.01).
* selected clones

Size uniformity and the shape of thorns on the leaf margins, as well as the presence of scales associated with the rosette, enhance the beauty of the ***O. grossiorum*** plants and allow them to be characterized as ornamental even when they are not flowering. The presence of scales on the leaves allows bromeliads to adapt to environments extremely unfavorable to other plants and also confers the different shades of color to the leaves of ***O. grossiorum***.

Inflorescence is one of the characteristics of the plant that attracts most of attention and has a great impact on its commercial value. While the flower, by itself, is very small (5-mm on an average), the bract set surrounding the inflorescence highlights the contrast between the color of the rosette and the color of the flower, enhancing the commercial value of ***O. grosssiorum*** plants . This contrast is observed mainly between the color of the inflorescence rosette and the color of the plant rosette in the clones selected for this research. The height and beauty of the floral tassel make this flower ideal for the production of floral arrangements (Figure 1), similar to the ornamental pineapple (BORGES et al., 2003).

However, in order to meet the needs of the consumer market, the plants selected must also be rustic and precocious, since distribution needs to be uniform and continuous without a drop in quality. The five clones selected in this research showed a superior performance in precocity and their resistance to damage caused by pests and diseases is reflection of rusticity.

The colorful, lush foliage and inflorescence of the bromeliads underlies their widespread use in landscaping and interior design (SOUZA et al., 2007). The plant under study in this research is special in the floriculture sector since it can be used as a vase flower, cut flower and garden flower (Figure 1). It is recommended as a vase flower for its aesthetic harmony and small size; as a cut flower for its height and beautiful floral tassel; and, as a garden flower in landscaping for the striking contrast of color between its rosette and inflorescence as well as its adaptability and rusticity.

In this study, it was determined that clones 01, 04, and 08 multiply better at lower BAP concentrations (10µM) than clones 05 and 09 (20µM). It was also observed that clones 05 and 08 were the most prolific, presenting, on average, 13.8 and 12.6 shoots per plantlet, respectively. The number of shoots observed in the other clones was fewer than six per plantlet, indicating that the response to BAP varies between the genotypes of ***O. grossiorum***.

The use of BAP in the culture medium to induce *in vitro* multiplication of ***O. grossiorum*** reduced the length of the shoots produced, with the shortest lengths, in general, being observed at the highest BAP concentrations.

Despite the addition of this extra stage, shoot quality was not affected, as all of the shoots had roots and possessed no deformities or morphological alterations, and displayed a high acclimatization capacity.

The use of BAP in the culture medium to induce *in vitro* multiplication of ***O. grossiorum*** reduced the length of the shoots produced, with the shortest lengths, in general, being observed at the highest BAP concentrations. This reduction in shoot length may be attributed to a decrease in the auxin/cytokinin ratio of the plantlets *in vitro* (TAIZ & ZEIGER, 2008) and competition between emerging shoots, as the number of shoots increased in culture medium containing BAP. A similar phenomenon was observed by NAVES et al (2003), for ***A. imperialis***, by MERCIER & KERBAUY (1995) for ***Vriesea hieroglyphica*** and ***Vriesea forsteriana***, and by PASQUAL et al. (2008) for ***Ananas comosus var. erectifolius***. Reduction in shoot length led to addition of a subsequent shoot-elongation stage before the transfer of plantlets to the vases, conducted in the same culture medium but without BAP addition (PASQUAL et al., 2008). This new stage adds 30 days to the overall process.

Despite the addition of this extra stage, shoot quality was not affected, as all shoots had roots and possessed no deformities or morphological

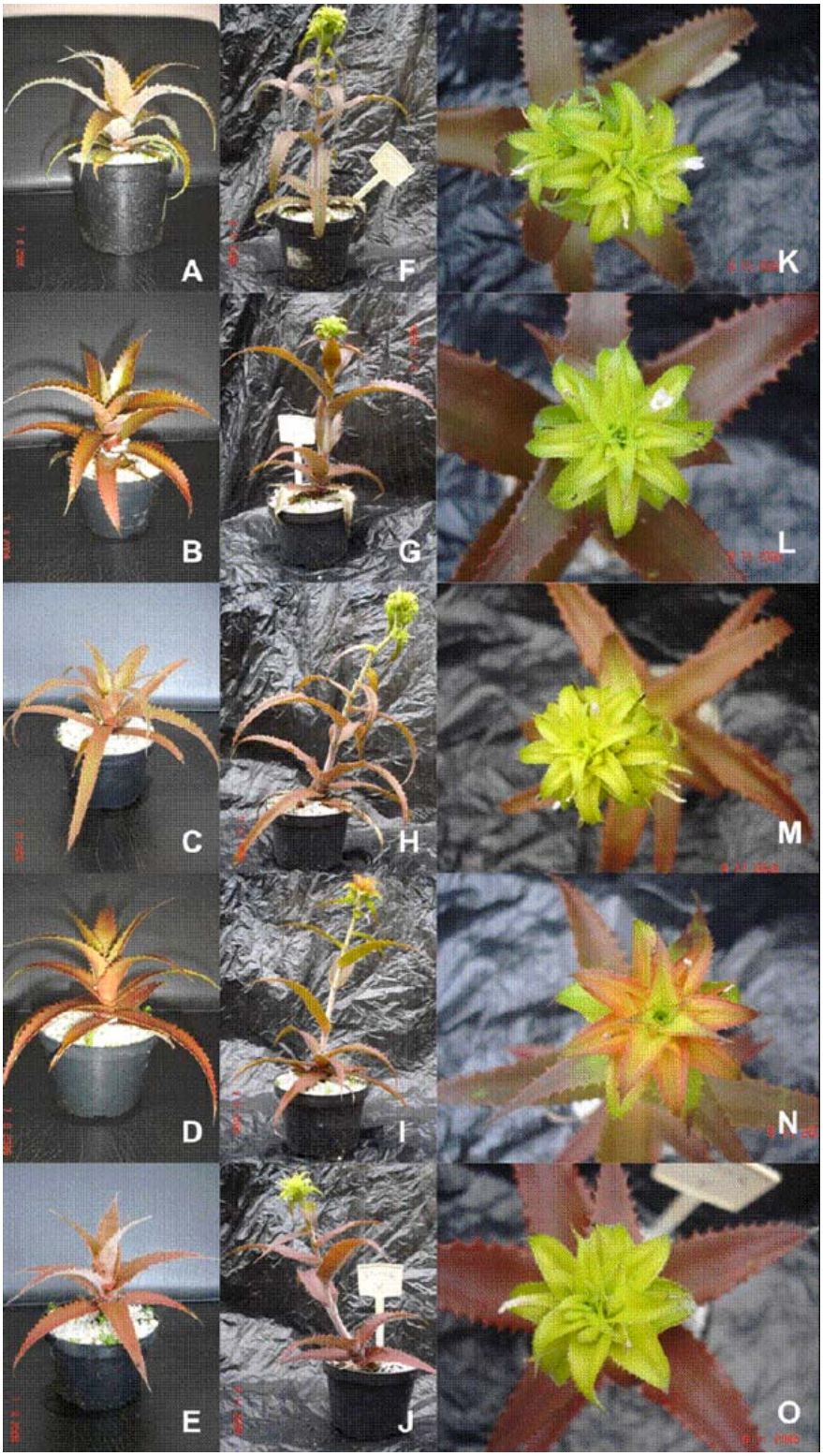

Figure 1 - Clones Selected of the bromeliad ***Orthophytum grossiorum***. A-F-K) clone 01, B-G-L) clone 04; C-H-M) clone 05; D-I-N) clone 08; E-J-O) clone 09; A to E) plant at 200 days after transfer to the vase; F to O) floral set of clone plants at 360 days after transfer to the vase.

alterations, and displayed a high acclimatization capacity.

CONCLUSION

The process of pre-selection *in vitro* is interesting from the ornamental horticulture viewpoints, as it does not exclude plants with potential ornamental traits and reduces the cost of breeding ornamental bromeliads. The selection method proposed in this research was efficient for preselecting plants of ornamental value and ease of propagation, facilitating their domestication and commercial exploration when eventual multiplication in vitro is intended.

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