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Micropropagation and *in Vitro* Conservation of *Neoglaziovia variegata* (Arr. Cam.) Mez, a Fiber Producing Bromeliad from Brazil

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ABSTRACT

Neoglaziovia variegata (Arr. Cam.) Mez *is a Bromeliaceae native to the Caatinga, used for fiber extraction in the Northeast Region of Brazil. The antropic activity has place this species among the threatened ones. The objective of the work was to establish an in vitro propagation and conservation of caroá. Seeds were cultivated in MS medium in the presence or absence of light. In vitro germinated seedlings were multiplied in MS medium supplemented with the combinations 0.05 and 0.50 µM NAA and 2.2 and 4.4 µM BAP and KIN. The best percentages of germination were obtained with the seeds incubated in the presence of light. The highest multiplication ratio was obtained for the NAA (0,5 µM) + BAP (4,4 µM) treatment and the number of roots, with NAA (0.5 µM) + KIN (2.2 µM). Plant acclimatization presented differentiated results regarding the substrates tested. The conservation was established.*

Key words: Acclimatization, Bromeliaceae, Fiber extraction, *In vitro* preservation, Seedling production

INTRODUCTION

The Bromeliaceae family is composed by 51 genus and more than 3500 species (Coffani Nunes 2002), all native from the tropical and subtropical zones of the American continent, except for the *Pitcairnia feliciana* species, found in Africa (Padilha 1978; Reitz 1983). Brazil detains the largest genetic variability of this family, which vegetates in very humid places, such as the Mata Atlântica ecosystem (Reitz 1983) and even in very arid regions such as the Caatinga (Andrade-Lima 1981; Xavier 1982).

*Neoglaziovia variegat*a (Arr. Cam.) Mez. is a species which belongs to this family and is native to the lower stratum of the Brazilian Caatinga. It has striped leaves, flowers protected by bracts with bright coloration and fruits as juicy berries (Smith and Downs 1979; Plantas do Nordeste 2004). This species is known in the Northeast Region of Brazil as caroá and constitutes one of the most used raw material for use in craftsmanship in the region,

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generating jobs and income for many families. Its leaves are used in fiber extraction which is used for manufacturing string, hats, purses, rugs, hammocks, fishing nets and fabrics.

The caroá plant, however, has been collected directly in the Caatinga in extrativism manner, without any systematization of cultivation, having practically disappeared in some regions. This can be explained by the cutting system of the leaves adopted by the craftswomen and especially by the devastation of the Caatinga for the development of agriculture and cattle raising activities in the region, where caroá is considered a weed and without any commercial value.

Although this species reproduces by seeds, its propagation is mainly asexual by the development of buds and lateral rhizomes. The caroá seeds are hard to be found because animals and birds feed on the green and especially mature berries (Xavier 1982), hindering the collection of the fruits at the ideal maturation period. In addition, the sexual propagation of bromeliads, generally present limitations such as the long period of seed maturation and the low germination rates of some species (Fráguas et al. 2002).

Seed germination and *in vitro* multiplication of bromeliads from tissue culture techniques are in general, much superior, when compared to conventional methods (Pierik et al. 1984; Mercier and Kerbauy 1995). The use of seeds as explants is an interesting strategy for the conservation purposes, maintaining therefore, the variability of natural populations, whereas each will constitute a mother plant (Rech Filho et al. 2005). In species where a cultivation system does not yet exist, maintaining the natural genetic variability, avoiding the intensification of genetic erosion caused by accelerated extrativism or by the selection of few genotypes, is very important. For such, the development of micropropagation protocols from seeds is an alternative for conservation of these species *in vitro* germplasm banks. Additionally, it can be used for the production at commercial level, contributing for the production of a high number of plantlets with better agronomic performance due to the quality of the plants obtained by this technique. The development of an efficient propagation method, which enables the obtainment of healthy plantlets to be planted, constitutes the first step towards the establishment of a cultivation and production system avoiding predatory extrativism. The objectives of the present work were to evaluate the *in vitro* germination of caroá seeds and establish a micropropagation protocol aiming large scale production of plants and *in vitro* conservation.

MATERIAL AND METHODS

The work was carried out at the Plant Tissue Culture Laboratory and greenhouse at *Embrapa Cassava and Tropical Fruits,* located in Cruz das Almas, Bahia, Brazil.

In vitro germination

The seeds used in this study were taken from the mature and immature fruits from inflorescences collected in Valente county, a semi-arid region of Caatinga in Bahia state, where caroá plants occurs naturally. The seeds were washed in a water and detergent solution and rinsed three times in distilled water. The desinfestation procedure was carried out under aseptic conditions, in sterile laminar flow hood, with a solution of 70 % ethanol for five minutes and later in a 1:1 solution of commercial bleach (2% of active chlorine) with deionized water for 20 minutes. After each treatment, three successive washes with autoclaved distilled water were carried out.

For the seed inoculation, test tubes (25 x 150 mm) containing 15 mL of MS (Murashige and Skoog, 1962) medium supplemented with 30 g/L of sucrose, solidified with 2 g/L Phytagel®, pH adjusted at 5.8 and autoclaved at 121°C for 20 minutes, were used.

The seeds remained for 60 days under a photoperiod of 16 h with photon flow density of 22 $\mu E.m^{-2}.s^{-1}$ and in the absence of light, both in growth chamber with controlled temperature (27 ± 1°C). During this period, the beginning of seed germination from the rootlet was observed and after 60 days the cumulative germination and the ratio of germination (%) of all treatments were evaluated.

The experimental design was in random blocks in factorial scheme 2 (presence and absence of light) x 2 (stages of maturation of fruits), with 25 repetitions per treatment.

Micropropagation of adventitious shoots and plant development

Plantlets obtained with medium average of 4 cm in the previous experiment were used as explants for the establishment of this experiment and

distributed in a complete random design, in factorial scheme 2 [α-naphthaleneacetic acid (NAA) concentrations] x 4 [cytokinins: 6-benzylaminopurine (BAP) and kinetin (KIN) in two concentrations] + 1 (additional treatment – control), with eight repetitions per treatment.

All the combinations between the concentrations of NAA (0.05 µM and 0.5) with 2.2 and 4.4 µM of BAP and KIN in MS culture medium supplemented with 30 g/L of sucrose, solidified with 2 g/L of Phytagel®, pH 5,8 and sterilized at 121°C, were analyzed. The additional treatment served as the control group, since the plantlets were cultivated in the same medium in the absence of growth regulators.

The multiplication was carried out in five sub cultivations of buds and shoots, and the longitudinal subdivision of shoots was conducted whenever possible. Sub cultivations were carried out in intervals of 35 days and the plants maintained under temperature conditions of 27 ± 1ºC, 16 h of photoperiod and light intensity of 22 $\mu E.m^{-2}.s^{-1}$.

For each sub cultivation, the number of shoots and roots of the nine treatments were evaluated. In order to correct the deviations in the normal distribution, log (x + 10) was applied for the data obtained from the variables evaluated in the five sub cultivations.

Acclimatization of micropropagated plants

Shoots and caroá plants originated from the fifth sub cultivation in MS medium with NAA (0.5 µM) + BAP (4.4 µM) and NAA (0.5 µM) + KIN (2.2 µM) were used as explants for the acclimatization trials. Material originated from the BAP treatment were considered as shoots, since there was no root formation, and those originated from the KIN treatment, which developed a root system, were considered as plants.

The number of leaves (NL) and height of plant surface (PS), before transferring to test tubes (5 x 20 cm) (initial evaluation) and after 150 days of acclimatization in greenhouse (final evaluation), and the survival ratio (SR), were evaluated. For the NL variable, in order to correct the deviations in the normal distribution, the transformation log (x + 10), was applied. The experimental design was in complete blocks in subdivided plot scheme with 20 repetitions. The plots contained the following treatments: shoots originated from the NAA (0.5 µM) + BAP (4.4 µM) treatment and plants from the NAA (0.5 µM) + KIN (2.2 µM) treatment and by the substrates [washed sand (T1); Ecoterra® (T2); coconut fiber (T3); coconut fiber + Plantmax® (T4); Plantmax® (T5); vermiculite (T6); vermiculite + Plantmax® (T7)]. The sub plots were formed by both periods of evaluation.

The substrate averages were compared by the Scott-Knott test at 5% probability and the explants averages and evaluations were calculated by the Tukey test at 5% probability. The geometric growth ratio (r) between both periods of evaluation was calculated for both variables given by the expression $r = \left(\sqrt[t]{V_f / V_i} - 1\right) \times 100$, in percentage; whereas V_f refers to the evaluation in the final period, V_i refers to the evaluation in the initial period and t refers to the period, in months, between the evaluations.

***In vitro* conservation**

In order to establish an *in vitro* conservation protocol for this species, inflorescences from three different places in Valente county (BA) were collected. For the seed inoculation, test tubes (25 x 150 mm) containing 15 mL of MS medium supplemented with 30 g/L of sucrose, solidified with 2 g/L of Phytagel®, pH adjusted at 5.8 and autoclaved at 120°C for 20 minutes, were used.

The seeds were maintained in this medium for 60 days under a photoperiod of 16 h and photon flow density of 22 $\mu E.m^{-2}.s^{-1}$ and temperature of 27 ± 1ºC. After this period, ten plantlets originated from each inflorescence from the three locations were sub cultivated in fresh medium and inoculated in test tubes (25 x 150 mm) containing 20 mL of culture medium. Each 10 plants of one location made up the repetitions of one access in the Pineapple and Bromeliad *in vitro* Germplasm Bank at Embrapa Cassava and Tropical Fruits, following all the basic premises for the introduction of new access in the collections (Norms SIBRAGEN).

RESULTS AND DISCUSSION

***In vitro* germination**

The seeds from the mature fruits began to emit the first rootlets between the 10th and 17th day and finalized between the 30th and 40th day in the presence and absence of light, respectively (Fig. 1). Seeds originated from immature fruits required a larger period of time in order to germinate, which

occurred between the 15th and 40th days after seed inoculation in the presence of light. However, in the absence of light, the germination began on the 20th day, extending until the 45th day. These results indicated that the presence of light accelerated the beginning of seed germination, regardless of the stage of seed maturation. In a work reported for *Neoregelia bahiana* (Ule) L. B. Sm. (Bromeliaceae) seeds, the germination was obtained between the sixth day of establishment, extending until the 22nd day in the presence of light with a photoperiod of 16 h (Bellintani et al. 2005). Droste et al. (2005) working with *Vriesea gigantea* Gaudich. and *V. philippocoburgii* Wawra seeds, obtained germination responses after eight days after inoculation under the same photoperiod. In fact, seed germination of Bromeliaceae took into consideration not only the environamental conditions but also the species.

The interaction of both the factors (luminosity and stage of fruit maturation) in the *in vitro* germination capacity of caroá seeds can be observed in Figure 1. The best germination percentages were obtained with incubated seeds in the presence of light resulting in germination rates of 100 and 80% for the seeds originated from mature and immature fruits, respectively. In the absence of light, the germination of seeds was reduced, reaching a ratio of 60 and 48%, for the seeds originated from mature and immature fruits, respectively.

Regardless of the stage of seed maturation, light requirement for germination stimuliation in caroá seeds seemed to be a preponderant factor for this species. The phytochrome present in the seed is responsible for the absorption of light. In the presence of the light treatment, it rapidly reacts from the inactive to the active form; however, this reaction is slower in the dark, decreasing seed germination (Zaidan and Barbedo 2004). According to Socolowski and Takaki (2004) seeds with phytochrome A can germinate under light and darkness conditions and if just phytochrome B is present the seeds germinate only in presence of light.

Some native bromeliads that occur in the Restinga ecosystems produce seeds that require light for germination (Pinheiro and Borghetti 2003). The percentage of germination obtained in caroá was, however, similar to the results obtained with *Ananas ananassoides* (Baker L. B. Sm) (91.2%), in the presence of light with a 12 h photoperiod (Figueiredo et al. 2003) and with *V. gigantea* (93,9%), with a 16 h photoperiod (Droste et al. 2005).

The germination of caroá seeds demonstrated to be efficient since it generated whole plantlets in a period of two months, resulting a large number of independent plants in the fruit maturation stage (Fig. 3a and 3b).

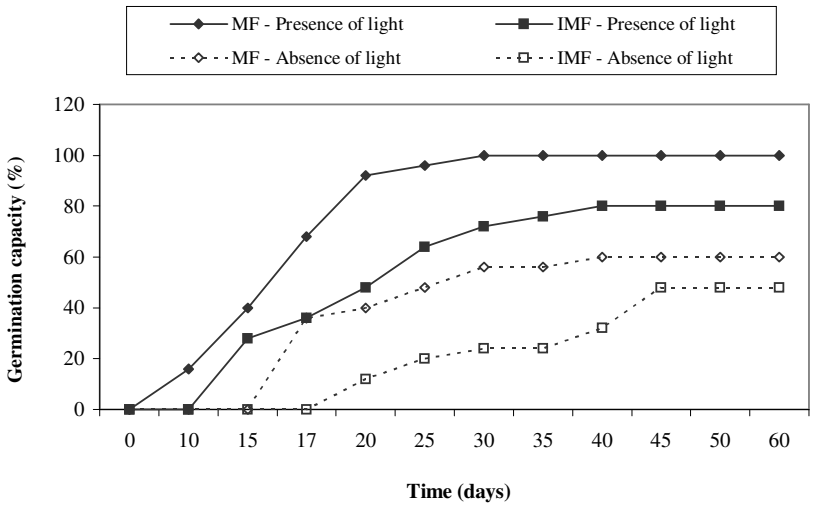

Figure 1 - *In vitro* cumulative germination of *N. variegata* (Arr. Cam.) Mez. seeds originated from mature (MF) and immature fruits (IMF) in the absence and presence of light.

Micropropagation of adventitious shoots and plant development

The multiplication of the shoots occurred in all the treatments, whereas the NAA and BAP combination in the medium provided a higher shoot production compared to the association of NAA and KIN (Table 1). The maximum multiplication ratio was observed for the NAA (0.5 µM) + BAP (4.4 µM) treatment, with an average of 60.58 adventitious shoots per explant after five sub cultivations (Fig. 2). Similar results were obtained with other bromeliads such as those reported by Barboza et al. (2004) that obtained greater amount of buds from 'Smooth Cayene' pineapple in culture medium containing NAA + BAP in comparison to medium supplemented with NAA + KIN. Other pineapple micropropagation works reported greater ratios of proliferation using BAP and cytokinin in concentrations which varied from 4.4 to 9.9 µM in combination with NAA (Hirimburegama and Wijesinghe 1992; Macêdo et al. 2003). For the micropropagation of *Cryptanthus sinuosus* (L.B. Smith), an extinct endemic Bromeliaceae from Brazil, the best results were obtained in MS medium without plant regulators, or whenever 2.2 µM of BAP and 0.05 µM of NAA was added (Arrabal et al. 2002), whereas other combinations seemed inhibitory for the shoot development. Although the addition of NAA and BAP might seem to favor shoot formation in bromeliads, it should be pointed out that, depending on the endogenous levels of these regulators in the explants, the responses obtained could be undesirable indicating the need for optimization and protocol adjustment for each target species (Mercier and Kerbauy 1997; Hirimburegama and Wijesinghe 1992; Karp 1995). The effect of different combinations of the phytoregulators, NAA, BAP and KIN, in the number of roots of micropropagated caroá shoots after five sub cultivations can be observed in Table 1. The media supplemented with 0.5 NAA presented the best results in the formation of roots during the multiplication phase, except for the medium with 2.2 µM of BAP. The largest number of roots was obtained with the NAA (0.5 µM) + KIN (2.2 µM) treatment, which provided an average of 37.03 roots per plant; a significantly superior value compared to the other combinations (Table 1 and Fig. 2). Kukulczanka and Czastka (1989), propagating various species from the Bromeliaceae family, *in vitro*, in RM medium, obtained rooted shoots using NAA + KIN, while the combination NAA + BAP promoted the formation of a greater number of adventitious shoots; a result similar to the one obtained in this work.

Table 1 - Number of adventitious shoots and of roots in the micropropagation of caroá [*N. variegata* (Arr. Cam.) Mez.] after five sub cultivations carried out every 35 days.

Number of shoots				
NAA (µM)	**BAP (µM)**		**Kinetin (µM)**	
	2,2	4,4	2,2	4,4
0.05	15.825 aA*	18.975 bA	6.257 Bb	10.725 bAB
0.5	11.729 bC	60.575 aA	28.525 Ab	21.375 aB
Control				5.902
Number of roots				
NAA (µM)	**BAP (µM)**		**Kinetin (µM)**	
	2,2	4,4	2,2	4,4
0.05	4.075 aB	1.400 bB	8.971 Ba	13.050 bA
0.5	3.000 aC	4.200 aC	37.025 aA	17.500 aB
Control				6.536

*Averages followed by the same lower case letters in the column and capital letters in the lanes do not differ statistically among themselves by the Tukey test at 5% probability.

As far as the morphology of the shoots was concerned, all the treatments provided normal shoots, without any apparent abnormality. However, the presence of BAP, although provided a greater ratio of multiplication, induced the formation of small shoots and few roots, making the separation difficult during the transplantation (Fig. 3). On the other hand, the presence of kinetin provided the formation of well defined shoots with roots. In many reports regarding Bromeliaceae micropropagation, this problem was observed in combinations of BAP with dosages greater than or

equal to 4.4 µM and NAA with dosages superior or equal to 2.5 µM (Macêdo et al. 2003; Barboza et al. 2004).

In order to evaluate the real efficiency of a micropropagation protocol for a determined species or variety, the system should be evaluated as a whole, not observing only the multiplication ratios, but also the time needed to obtain the entire plant, as well as the effect of the composition of the culture medium used in the period of acclimatization.

Acclimatization

The percentage of shoot survival and plants varied from 70 to 100%, whereas the ones originated from MS medium containing NAA (0.5 µM) + KIN (2.2 µM), presented higher ratios in almost all treatments (Fig. 2). The smallest ratio of survival was detected in the substrate containing coconut fiber and Plantmax®, regardless of the origin of the plants (culture medium).

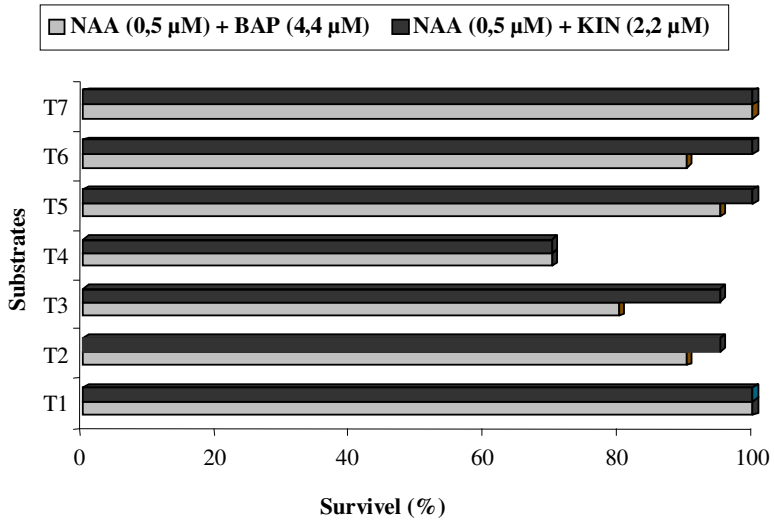

Figure 2 - Survival of micropropagated shoots of caroá [*N. variegata* (Arr. Cam.) Mez.] 150 days of acclimatization in different substrates: washed sand (T1); Ecoterra® (T2); coconut fiber (T3); coconut fiber + Plantmax® (T4); Plantmax® (T5); vermiculite (T6); vermiculite + Plantmax® (T7).

At 150 days of acclimatization, there was a significant increase in the number of leaves of the shoots developed in the Ecoterra® (T2), Plantmax® (T5) and vermiculita + Plantmax® (T7) substrates in comparison to the other treatments, regardless of the origin of the plants (culture medium). However, the largest growth ratio of this variable was observed in the T7 substrate (9,81%), opposite of what was registered for the substrates in T3, T4 and T6 (Table 2). The report of a negative growth, such as the one observed in these treatments, occurred due to the presence of dry leaves in the shoots and the lack of emission of new leaves, since the leaves considered in the evaluation of the variable were green ones. The presence of coconut fiber in two of these treatments indicated a negative effect of this component in this result which could be explained by the high C/N ratio of this substrate, demanding, therefore, the enrichment with N for better initial development of the plants (Carrijo et al. 2004). It would be worth mentioning that the substrate that registered the higest mortality of plants in the initial phase of acclimatization contained coconut fiber.

The growth ratio regarding the height of the plant surface, considering the *in vitro* culture medium in which these plants were produced is shown in Table 3. It was noticed that Plantmax® favored better development of shoots (35.59%) originated from the medium enriched with BAP (T5), whereas Ecoterra® substrate (T2) promoted better growth in the plants originated from the medium with KIN (37,72%) that presented roots (Fig. 3). Ecoterra® was a substrate with high organic matter content, which could have favored the adherence and better root development, not always functional,

when taken from laboratory conditions. Plantmax®, however, presented the most adequate properties for the acclimatization of shoots without roots, probably due to its capacity of water absorption and retention, good aeration and draining, avoiding the accumulation of humidity and shoot base rot. Substrate is the factor that mostly affects the rooting and plays an important role, especially in the species of difficult rooting. According to Couvillon (1998), an ideal substrate retains enough water to avoid the dryness of the base of the stake and once saturated, has adequate porous space to facilitate the rooting and avoid the development of the diseases. On the other hand, the adequate substrate should be available in good quantity and be easy to handle and cheap (Faria et al. 2001).

These results showed significant differences between the growth ratios observed in the best treatments, regardless of the presence or absence of roots, and demonstrated the possibility to acclimatize caroá shoots without the need of an *in vitro* stage of rooting. This implied in the reduction of work and cost in the process as a whole, considering that the *in vitro* multiplication ratio in the NAA (0.5 µM) + BAP (4.4 µM) medium were much superior than those registered in the medium enriched with KIN.

Table 2 - Number of leaves from micropropagated caroá [*N. variegata* (Arr. Cam.) Mez.] shoots from the initial period (0 days) and final (150 days) from acclimatization and growth ratio in different substrates.

Substrate	Inicial (1st day)	Final (150 days)	Growth rate (%)
T1	7.575 aA	8.000 bA	1.84
T2	7.750 aB	9.703 aA	7.78
T3	7.375 aA	6.543 cB	-3.91
T4	6.750 aA	6.571 cA	-0.89
T5	7.325 aB	8.897 aA	6.70
T6	6.975 aA	5.842 cB	-5.74
T7	7.175 aB	9.500 aA	9.81

Averages followed by the same lower case letters in the columns and capital letters in the lanes do not differ statistically among themselves by the Scott-Knott and Tukey test, respectively, at 5% probability. T1 = washed sand, T2 = Ecoterra®, T3 = coconut fiber, T4 = coconut fiber + Plantmax®, T5 = Plantmax®, T6 = vermiculite, T7 = vermiculite + Plantmax®.

Table 3 - Height (cm) of plant surface of micropropagated caroá shoots [*N. variegata* (Arr. Cam.) Mez.] originally multiplied in the presence of NAA (0.5 µM) + BAP (4.4 µM) and NAA (0.5 µM) + KIN (2.2 µM) during the initial period (0 days) and final (150 days) of acclimatization and growth ratio in different substrates.

Substrate	NAA + BAP			NAA + KIN		
	Initial (0 days)	Final (150 days)	Growth rate (%)	Initial (0 days)	Final (150 days)	Growth rate (%)
T1	1.950 aB	2.795 bA	12.75	2.360 aB	3.371 bA	12.62
T2	2.465 aB	3.956 aA	17.08	2.045 aB	5.342 aA	37.72
T3	1.815 aA	1.775 cA	-0.74	2.310 aA	2.621 cA	4.30
T4	1.735 aB	2.207 cA	8.35	2.615 aA	2.728 cA	1.42
T5	1.725 aB	4.300 aA	35.59	2.030 aB	3.840 bA	23.67
T6	1.460 aB	2.789 bA	24.08	2.240 aB	3.380 bA	14,70
T7	2.065 aB	4.010 aA	24.76	1.945 aB	3.525 bA	21.92

Averages followed by the same lower case letters in the columns and capital letters in the lanes do not differ statistically among themselves by the Scott-Knott and Tukey tests, respectively, at 5% probability. T1 = washed sand, T2 = Ecoterra®, T3 = coconut fiber, T4 = coconut fiber + Plantmax®, T5 = Plantmax®, T6 = vermiculite, T7 = vermiculite + Plantmax®.

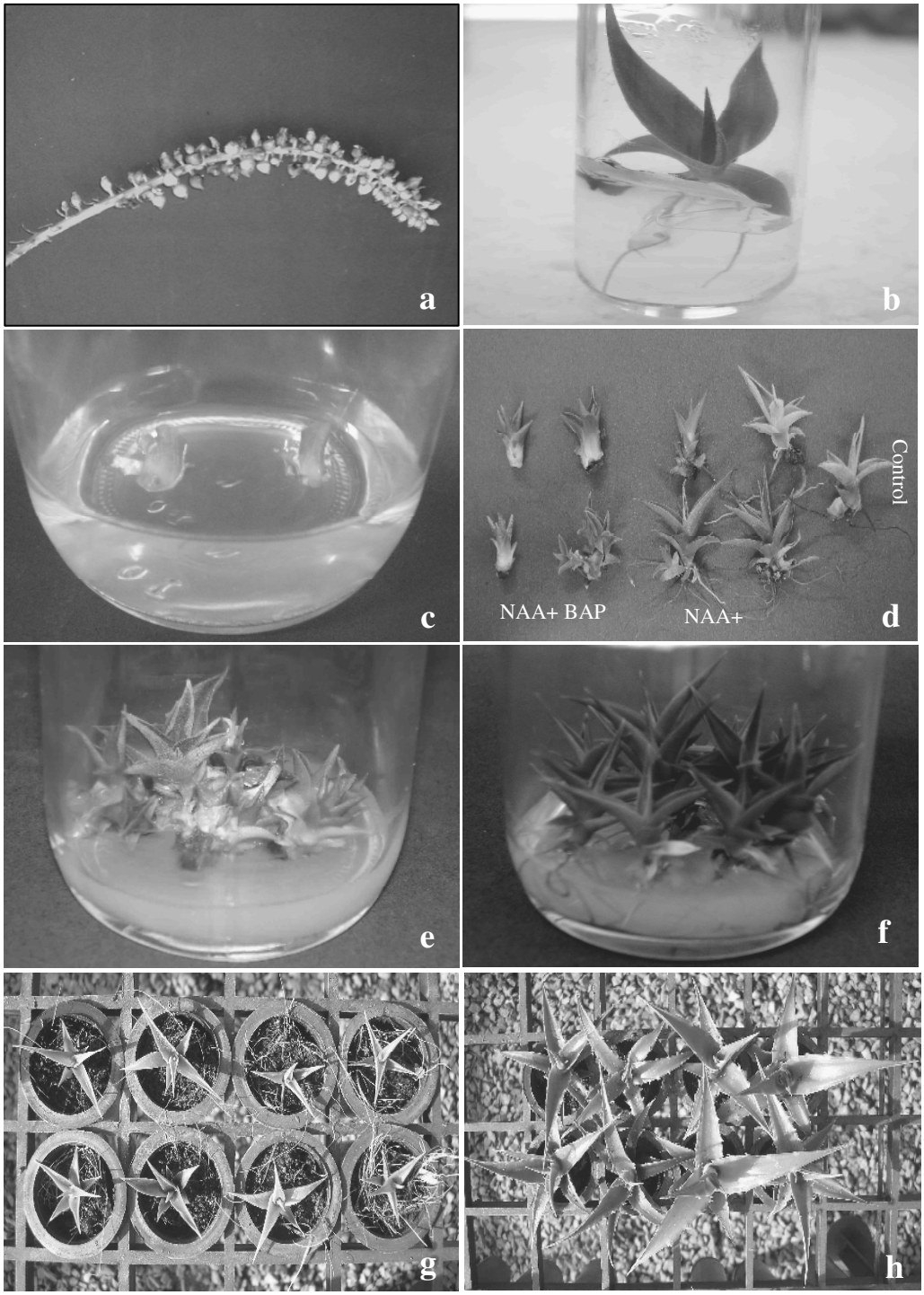

Figure 3 - Stages of germination, micropropagation and acclimatization of caroá [*N. variegata* (Arr. Cam.) Mez.]: (a) fruits during inflorescence; (b) plantlet after 60 days of inoculation in culture medium; (c) sub cultivated plantlet in culture medium; (d) adventitious shoots originated from media with and without regulators; (e) small shoots without roots originated from micropropagation medium with NAA (0.5 µM) + BAP (4.4 µM); (f) developed plants from the medium supplemented with NAA (0.5 µM) + KIN (2.2 µM); (g) plants at 150 days of acclimatization originated from culture media mentioned earlier in the coconut fiber substrate and (h) Ecoterra®.

***In vitro* conservation**

Plants were maintained *in vitro* conservation conditions to the Pineapple and Bromeliad Germplasm Bank (temperature of 22 ± 1ºC, photoperiod of 12 h and light intensity of 22 $\mu E.m^{-2}.s^{-1}$). Decrease in the growth ratios was monitored with the use of controls cultivated under the incubation conditions in the growth chambers (temperature of 27 ± 1ºC, photoperiod of 16 h and light intensity of 22 $\mu E.m^{-2}.s^{-1}$). Incubated caroá plants in lower temperatures presented a rate of growth inferior to the controls, demonstrating the effect of the temperature on the growth. This result was similar to the *in vitro* conservation of pineapple, another plant from the Bromeliaceae family (Souza et al. 2006a). Despite the differences in response regarding time and *in vitro* conservation of different bromeliads, some varieties were being preserved for a period of one or two years (Souza et al. 2006b).

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RESUMO

Neoglaziovia variegata é uma Bromeliaceae nativa da Caatinga, usada para extração de fibras na Região Nordeste do Brasil. A atividade antrópica coloca esta espécie entre as ameaçadas. O objetivo deste trabalho foi estabelecer uma propagação e conservação *in vitro* de caroá. Foram cultivadas sementes em meio MS na presença ou ausência de luz. Plântulas germinadas *in vitro* foram multiplicadas em meio MS suplementado com as combinações de 0,05 e 0.50 µM de NAA e 2.2 e 4.4 µM de BAP e KIN. Foram obtidas as melhores porcentagens de germinação com sementes incubadas na presença de luz. A taxa de multiplicação mais alta foi obtida no tratamento NAA (0,5 µM) + BAP (4,4 µM) e, o número de raízes, com NAA (0.5 µM) + KIN (2.2 µM). Aclimatização das plantas apresentou resultados diferenciados em relação aos substratos testados. A conservação foi estabelecida.

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