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Micropropagation of *Vellozia seubertiana* (Velloziaceae)

Micropropagação de *Vellozia seubertiana* (Velloziaceae)

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ABSTRACT - *Velloziaceae* is one of the main families in the floristic composition of the Campos Rupestres Montane Savanna ecoregion of the Chapada Diamantina Highland, Bahia, Brazil, and has species with significant ornamental potential and resistance to climate changes. The species *Vellozia seubertiana* stands out for its beautiful flowers and stems covered by leaf sheaths that ensure protection when in contact with fire. However, there is no information on its propagation, conservation, and physiology, which justifies this study. The objective of this work was to establish a micropropagation protocol for *V. seubertiana*. Seeds were disinfected and inoculated in Murashige and Skoog culture medium (MS) with half salt concentration (MS½) for *in vitro* establishment. Plantlets established *in vitro* were used to induce sprouting in MS½ supplemented with 6-benzylaminopurine (BAP; 0.00, 4.44, 8.88, and 17.76 µM) and 1-naphthaleneacetic acid (NAA; 0.00 and 2.22 µM). The shoots obtained were inoculated in MS½ containing activated charcoal (0.0 and 1.0 g L^{-1}) and indole-3-butyric acid (IBA; 0.00 and 2.22 µM) for rooting. The rooted plantlets were acclimated under greenhouse conditions. A mean of 5.7 shoots were generated through organogenesis in medium containing 8.75 µM of BAP and 2.22 µM of NAA. The use of activated charcoal resulted in the highest means for aerial part and root lengths in the *in vivo* rooting phase. Acclimated plants reached 75% survival at 60 days after transplanting to *ex vitro* conditions. The results indicate that micropropagation is a promising technique for the production of *V. seubertiana* seedlings.

Keywords: Campos rupestres. Chapada Diamantina. *In vitro* propagation. Plant growth regulator.

RESUMO - *Velloziaceae* é uma das principais famílias da composição florística dos campos rupestres da Chapada Diamantina e possui espécies com significativo potencial ornamental e resistência a mudanças climáticas. A espécie *Vellozia seubertiana* se destaca pela beleza das flores e por possuir caules cobertos por bainhas foliares que asseguram proteção quando em contato com o fogo. Não há relatos sobre a sua propagação, conservação e entendimento da sua fisiologia, o que justifica a realização deste estudo. Este trabalho teve como objetivo estabelecer um protocolo de micropropagação da espécie *V. seubertiana*. Para o estabelecimento *in vitro*, as sementes foram desinfestadas e inoculadas em meio MS com metade das concentrações salinas (MS ½). Microplantas estabelecidas *in vitro* foram utilizadas para a indução de brotos em meio MS ½ suplementado com BAP (0,00; 4,44; 8,88 e 17,76 µM) e ANA (0,00 e 2,22 µM). Os brotos obtidos foram inoculados em meio MS ½ contendo carvão ativado (0,0 e 1,0 g L^{-1}) e AIB (0,00 e 2,22 µM) para enraizamento. Microplantas enraizadas foram aclimatizadas em casa de vegetação. Obteve-se uma média de 5,7 brotos via organogênese em meio contendo 8,75 µM de BAP e 2,22 µM de ANA. Na fase de enraizamento, a presença do carvão ativado proporcionou as maiores médias para comprimento de parte aérea e comprimento de raiz. As plantas aclimatizadas atingiram 75% de sobrevivência após 60 dias da transferência para a condição *ex vitro*. Conclui-se que a micropropagação é uma técnica promissora para a produção de mudas de *V. seubertiana*.

Palavras-chave: Campo rupestre. Chapada Diamantina. Propagação *in vitro*. Regulador vegetal.

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INTRODUCTION

Chapada Diamantina Highland is in the state of Bahia, northeastern Brazil, and has a great diversity of plant resources that deserves attention. This region is composed of exuberant landscapes, and its flora has high ornamental potential and other uses already known. It explains the extractive removal of large amounts of plants from nature, bringing risks to local plant genetic resources.

Species of the Velloziaceae family have significant degree of endemism in the Campos Rupestres Montane Savanna ecoregion of the Chapada Diamantina Highland (NEVES, 2009). *Vellozia seubertiana* Goethart & Henrard. is among the species in this region; it stands out for its beautiful lilac flowers (NEVES, 2009) and stems covered by leaf sheaths that ensure protection when in contact with fire (ALVES; SILVA, 2011). *V. seubertiana* is endemic to Brazil (MELLO-SILVA, 2015), has herbaceous growth habit, and is found on rocks and in sandy soils (NEVES, 2009).

V. seubertiana is a potential resource for use as ornamental plants; however, the sustainability of this activity requires studies for assisting the management of its natural populations and the development of techniques for *ex situ* conservation and production of quality seedlings.

Plant tissue culture stands out among strategies for seedling production as

an excellent alternative for the ornamental plant sector and *ex situ* conservation of species.

Micropropagation is the most common plant tissue culture technique, which provides production of seedlings with similar characteristics to the parent plants (GRATTAPAGLIA; MACHADO, 1998). It is a technique for asexual reproduction started by small fragments (explants), which enables the production of a large number of healthy seedlings in a small space and short time (GEORGE, 2008), differing from conventional propagation methods.

Researches on other families of ornamental species found in the Chapada Diamantina Highland showed viability of *in vitro* cultivation for Bromeliaceae species (LIMA et al, 2012; LIMA; LIMA-BRITO; SANTANA, 2020), evergreen flowers (ALBUQUERQUE et al., 2016; LIMA-BRITO et al., 2016; LIMA-BRITO et al., 2011; LIMA; LIMA-BRITO; SANTANA, 2021; CARMO; MOURA; LIMA-BRITO, 2020), and Cactaceae species (RESENDE et al., 2021; TORRES-SILVA et al., 2021); however, studies on Velloziaceae species occurring in this region are needed.

Only two studies on *in vitro* propagation of species of the *Vellozia* genus are found in the literature, one for *Vellozia flavicans* Mart. Ex Schult f. (FREITAS NETO, 2009) and other for *Vellozia pyrantha* A. A. Conc. (basionym = *Vellozia sincorana*), which is endemic to the Chapada Diamantina Highland (BORGES, 2015).

Studies focused on establishing efficient protocols for *in vitro* propagation of plants have shown that the addition of plant growth regulators, mainly auxins and cytokinins, to the culture medium is determinant for a successful micropropagation, as these hormone classes control specific stages of the cell cycle and contributes to cell division (KERBAUY, 2019). The combination of auxins and cytokinins drives morphogenesis and promotes the growth of organs, calluses, and cell suspensions (GEORGE, 2008).

Considering the importance of conservation of plant resources in the Campos Rupestres Montane Savanna ecoregion of the Chapada Diamantina Highland, the efficiency of applying micropropagation to meet the demand of the ornamental plant market and the lack of studies on *V. seubertiana*, the objective of this work was to establish a protocol for *in vitro* propagation of plants of this ornamental species.

MATERIAL AND METHODS

Seeds were obtained from dry capsules of the species *Vellozia seubertiana* Goethart & Henrard. (Figure 5b) collected in the City Park of Mucuge, Chapada Diamantina Highland region, Bahia, Brazil (12°59'47"S, 41°22'11"W). The seeds were washed in running water with a drop of a commercial neutral detergent for 10 minutes. In a laminar flow chamber, they were submerged in 70% alcohol for one minute and, then, immersed in 3% sodium hypochlorite with 3 drops of a commercial neutral detergent for 10 minutes. They were then washed four times in sterilized distilled water.

In vitro establishment

The seeds were inoculated in glass test tubes containing 10 mL of Murashige and Skoog culture medium (MS) (MURASHIGE; SKOOG, 1962) with half salt concentration (MS½), supplemented with 30 g L^{-1} of sucrose and solidified with 7 g L^{-1} of agar and 1 g L^{-1} of activated charcoal (BORGES, 2015). After 75 days, the plantlets germinated *in vitro* were used to induce sprouting.

In vitro multiplication

Aerial parts of plantlets of *V. seubertiana* were inoculated in glass test tubes containing 10 mL of MS½ supplemented with 30 g L^{-1} of sucrose, 7 g L^{-1} of agar, and different synthetic cytokinin concentrations (0.00, 4.44, 8.88, and 17.76 µM of 6-benzylaminopurine - BAP) combined with auxin concentrations (0.00 and 2.22 µM of 1-naphthaleneacetic acid - NAA). The aerial part was used as explant due to the absence of responses from other explant types subjected to the same multiplication conditions (data not shown).

A completely randomized experimental design was used, in a 4×2 factorial arrangement consisted of four BAP concentrations (0.00, 4.44, 8.88, and 17.76 µM) and two NAA concentrations (0.00 and 2.22 µM). Each treatment consisted of five four-sample replications, totaling 20 tubes per treatment (one plant per tube).

The following variables were evaluated after 60 days of cultivation: percentage of explants with shoots (%ES), number of shoots per explant (NSE), mean length of shoots (MLS), dry weight of shoots (DWS), and survival of initial explants (%S).

In vitro rooting

Shoots with heights of approximately 2 cm, from *in vitro* multiplication, were individualized and inoculated in 250-mL flasks containing 50 mL of MS½ supplemented with 30 g L^{-1} of sucrose, 7 g L^{-1} of agar, and combinations of concentrations of auxin (0.00 and 2.22 µM of indole-3-butyric acid - IBA) and activated charcoal (0.0 and 1.0 g L^{-1}).

A completely randomized experimental design was used, in a 2×2 factorial arrangement consisted of combinations of IBA (0.00 and 2.22 µM) and activated charcoal concentrations (0.0 and 1.0 g L^{-1}). Each treatment consisted of five four-sample replications, totaling 20 flasks per treatment.

The following variables were evaluated at 30 days: rooting percentage (%R), number of roots per plant (NR), longest root length (LRL), aerial part length (APL), aerial part dry weight (APDW), number of green leaves (NGL), and number of senescent leaves (NSL).

Acclimation

Plantlets of *V. seubertiana* were removed from the flasks using tweezers, their roots were carefully washed with distilled water to remove culture medium residues and, subsequently, transplanted to 200-mL polyethylene cups, with five holes in the bottom, containing topsoil and vermiculite at the proportion of 1:1. The cups were covered with a transparent polyethylene cup for 10 days (LIMA; LIMA-BRITO; SANTANA, 2020) and then kept in trays with a water layer, in a greenhouse covered with a 70% shade screen, for 60 days.

Each treatment consisted of five four-sample replications. The following variables were evaluated at 60 days after transplanting: percentage of plant survival (%PS), aerial part length (APL), and number of green leaves (NGL).

All *in vitro* experiments were conducted in a growth room with temperature of 26±2 °C, 14-hour photoperiod, and active photosynthetic radiation of 60 µmol m^{-2} s^{-1} obtained through fluorescent lamps (250V). The pH of all culture media used was adjusted to 5.7 before autoclaving at 121 °C for 15 minutes. The tubes and flasks were sealed with polyvinyl chloride (PVC) film.

Statistical analysis

The results were analyzed for normality, using the Shapiro-Wilk test, and no rejection was found. The data were then subjected to analysis of variance and the means of the quantitative and qualitative data were compared by the Tukey's test at 5% probability or regression analysis, respectively, using the statistical program SISVAR (FERREIRA, 2019).

RESULTS AND DISCUSSION

The germination rate of *Vellozia seubertiana* seeds was higher than 80%. The use of plants from seed germination enhances the maintenance of genetic variability, which is important for the initial establishment of *in vitro* collections. A high variability is important when the tissue culture is used as an *ex-situ* conservation strategy. Moreover, maintaining the genetic stability of the parent plant is essential for producing clone seedlings intended to the market, which can be obtained by a direct *in vitro* propagation.

V. seubertiana leaves were used as explants in preliminary studies, but did not show morphogenetic responses under the conditions evaluated (unpublished data). Similarly, a study using leaves of the bromeliad *Orthophytum mucugense* WAND and CONCEIÇAO to start multiplication found no significant responses for percentage of explants with shoots and number of shoots per explant (LIMA et al., 2012). However, leaf explants proved to be promising for *in vitro* multiplication of the bromeliad *Aechmea ramosa* Mart. ex Schult. f. (FARIA et al., 2018), denoting the importance of specific studies.

Regarding the *vitro* multiplication phase, induction of shoots at the base of the extant was found in all treatments. No significant interaction between the plant growth regulators tested was found for percentage of explants with shoots (% ES). The presence of the cytokinin BAP in the culture medium had no effect on %ES. However, although the addition of auxin to the culture medium is not essential for the response of explants in *in vitro* shoot formation, it significantly affected the %ES; the mean %ES found with the NAA concentration of 2.22 µM was significantly higher (58.75%) than that in the treatment without this auxin (Figure 1a).

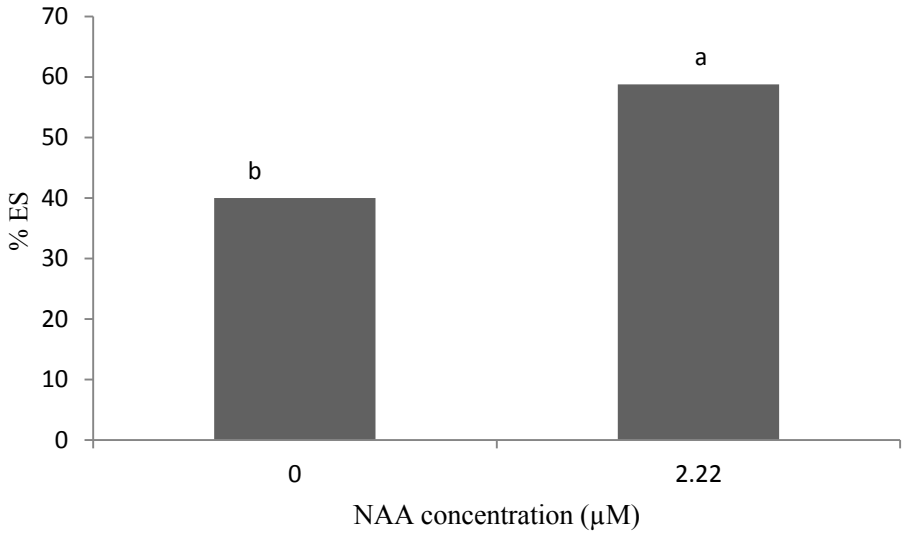

Figure 1. Effect of auxin NAA on percentage of explants with shoots (%ES) in *in vitro* multiplication of *Vellozia seubertiana*.

These results indicate that *V. seubertiana* has enough endogenous auxin for shoot formation; however, the multiplication can be optimized by exogenous supplementation with the plant growth regulator NAA, resulting in hormonal balances more favorable to multiplication.

Considering that only one NAA concentration (2.22 µM) was evaluated and promoted a mean %ES of 58.75%, higher concentrations should be tested to potentialize *in vitro* multiplications. Lima et al. (2012) also found means lower than 60% when using NAA at 0.65 and 1.30 µM concentrations combined with 2.22 µM of BAP for *O. mucugense*. Lima, Lima-Brito, and Santana (2020) found higher %ES (99%) for the bromeliad *Sincoraea mucugensis* (Wand. & A.A. Conc.) LOUZADA & WAND; however, they used higher NAA concentrations (2.60 and 5.20 µM) than that used in the present study and stems as explants.

Lima-Brito et al. (2011) evaluated the species *Syngonanthus mucugensis* Giul. subsp. mucugensis, an evergreen flower of Mucuge, and found %ES of 58% when using stems as explants and no growth regulator, which indicates that the species has similar satisfactory contents of endogenous hormones to the species *V. seubertiana*. These two species have similar growth habit and occur in the same region.

The number of shoots per explant (NSE) was significantly affected by the interaction between BAP and NAA concentrations. Regarding the absence of NAA, the data did not fit to a regression model. However, the regression analysis for NSE with presence of NAA resulted in a quadratic polynomial model, with the highest NSE of 5.45 estimated for the combination of 8.87 µM of BAP and SQI = $\sum(EF's * PF's)$ of NAA; NSE tends to decrease when using higher BAP concentrations (Figure 2).

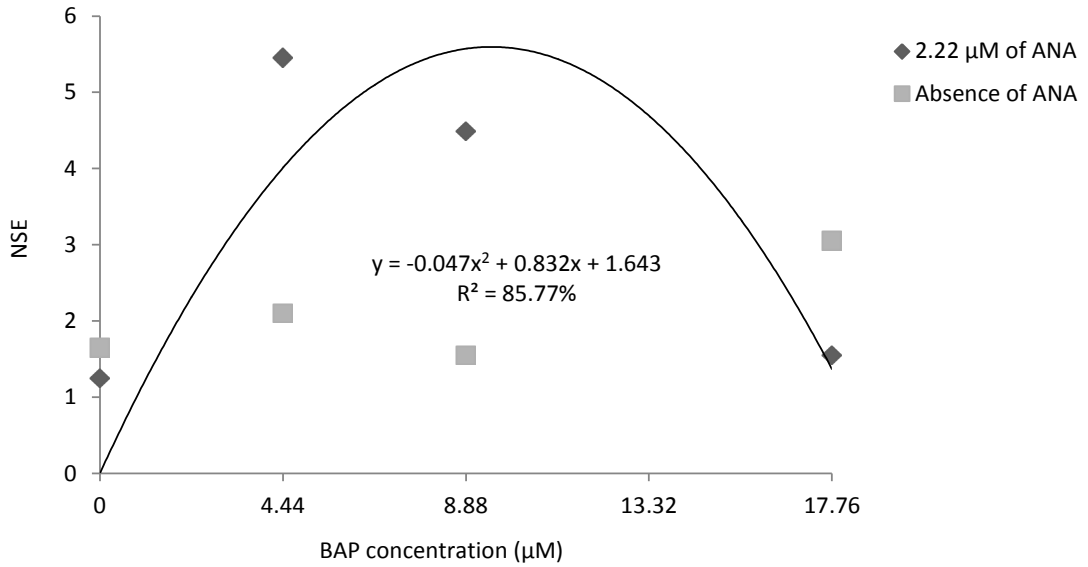

Figure 2. Effect of the interaction between auxin NAA (0.00 and 2.22 µM) and cytokinin BAP on number of shoots per explant (NSE) for *in vitro* multiplication of *Vellozia seubertiana*.

In vitro multiplication experiments intend to improve the cytokinin-auxin balance to establish a protocol with a high shoot production, and the present study is the first on *in vitro* multiplication of the species *V. seubertiana.* The shoot production graph curve decreases after reaching the highest point, which was estimated with the BAP concentration of 8.75 µM, showing that the synthetic cytokinin BAP can have positive effects on *in vitro* multiplication, but it can also have a toxic or inhibitory effect triggered by the use of higher concentrations than the ideal for each species (Figure 4).

Lima, Lima-Brito, and Santana (2020) found similar results for the growth regulator BAP in a study on multiplication of *Sincoraea mucugensis*, with an NSE of approximately 7 for a BAP concentration of 8.92 µM.

Cytokinins are required for *in vitro* multiplication because they induce the breaking of apical dominance and emergence of new axillary buds (ILIEV et al., 2010). Auxin and cytokinin hormones are essential for the advance of the cell cycle. Thus, all plants naturally have auxins and cytokinins, which directly participate in the cell cycle regulation process, more precisely in the transition from the G1 phase to the starting of DNA synthesis. Auxins control cyclins, and one of them is modulated by a cytokinin, forming an active complex that enables the cell cycle evolution; in addition, cytokinins alone participate in the regulation from the G2 to the M phase (KERBAUY, 2019).

The highest NSE found was 5.45, which was higher than those found by Borges (2015) for *V. pyrantha* (basionym = *V. sincorana*), approximately 0.3 when using kinetin and 0.2 when using BAP.

Freitas Neto (2009) evaluated subcultures for *in vitro* multiplication of *V. flavicans* and found NSE lower than 2 in the first subculture and between 6 and 7 in the third subculture when using BAP at concentrations of 2.22 and 4.44 µM,

which confirms that this cytokinin is efficient for *in vitro* production of *Vellozia* shoots.

Garcia et al. (2021) found that increases in BAP concentration increase the NSE in the bromeliad species *Aechmea miniata* (Beer) Baker and *Aechmea blanchetiana* (Baker) L. B. Smith, with the highest NSE of 14.5 for *A. miniata* and 10.5 for *A. blanchetiana* after 225 days when using 13.32 µM of BAP.

Dry weight and length of shoots were not affected by the NAA and BAP concentrations in the *in vitro* multiplication treatments.

The mean survival of initial explants was higher than 95% in all treatments, denoting that the plant aerial part is a promising initial explant for multiplication of *V. seubertiana,* as it provides high sprouting rate and can be used for new subcultures, which probable explains its use in other studies evaluating *Vellozia* species (FREITAS NETO, 2009; BORGES, 2015). Similarly, whole-plant explants from seed germination have been used for rosette species, such as bromeliads (DIAS et al., 2020).

Regarding the *in vitro* rooting phase, roots were found in all plantlets. No interaction was found between activated charcoal and NAA, which had isolated effects. The highest means of aerial part length (3.65 cm) and longest root length (3.60 cm) were found using activated charcoal, which statistically differed from the treatments without this additive (Figures 3a-b). Similar results were reported by Lima et al. (2012), who found positive effect of charcoal on these variables when studying *in vitro* rooting of *O. mucugense*.

The use of activated charcoal in the culture medium for *in vitro* rooting promotes some benefits. Its color provides a dark environment, with no luminosity, which is a favorable condition for plant rooting (GEORGE, 2008). Another benefit is the adsorption of elements that can inhibit rhizogenesis; such elements may be present in the medium itself or released by the plant during cultivation (GRATTAPAGLIA; MACHADO, 1998).

The variables evaluated in the *in vitro* rooting treatments were not affected by the auxin IBA, except number of green leaves (NGL). This can be explained by the presence of sufficient endogenous levels for the formation of adventitious roots in the shoots, since all rooted.

The negative effect of this auxin on NGL (Figure 3c) can be attributed to leaf toxicity caused by hormonal unbalance, exceeding the limits tolerated by the species; NGL was 7.82 when using IBA and 11 when it was not used. Additionally, synthesized auxins naturally participate in several processes in plants, including senescence (KERBAUY, 2019), and increases in auxin levels by supplementation may have contributed to increases in senescence of *in vitro* leaves. The plants free from supplementation with auxin presented higher number of green leaves (Figure 3c) and lower number of senescent leaves (data not showed).

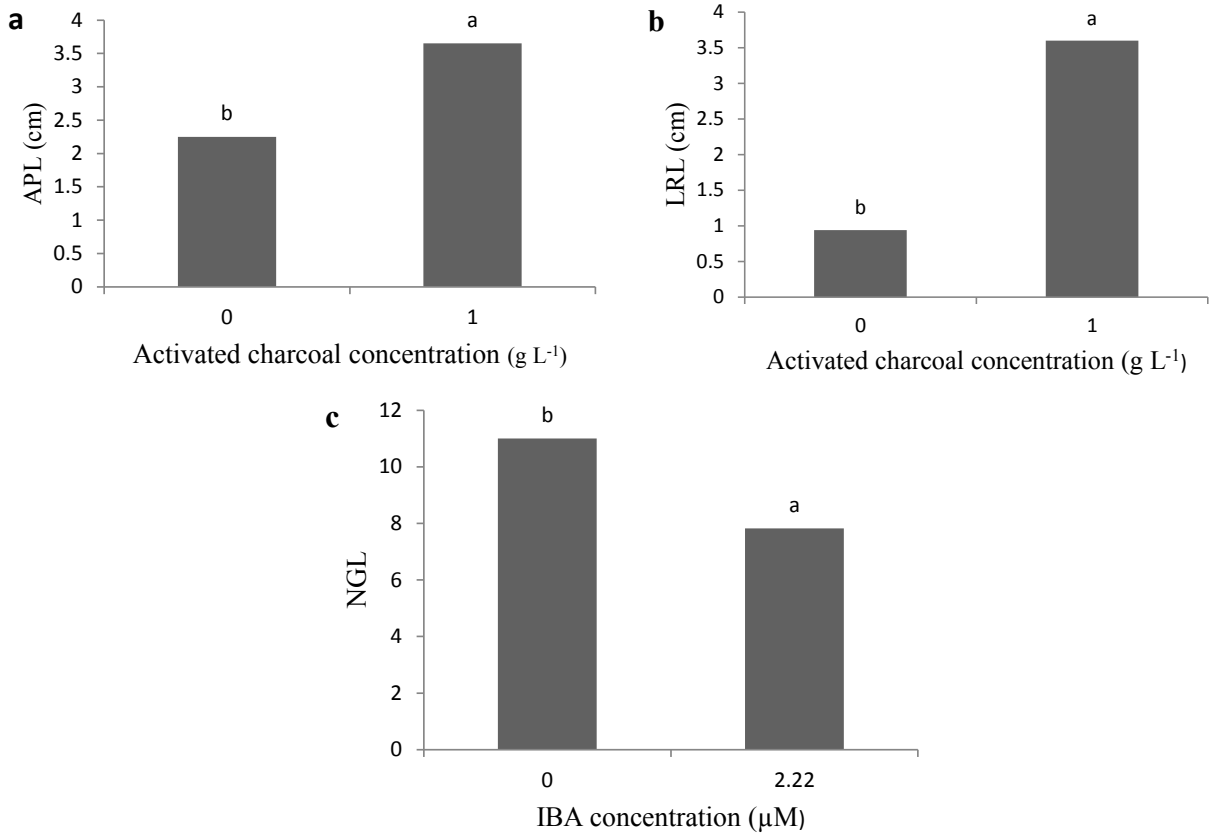

Figure 3. *In vitro* rooting of *Vellozia seubertiana*: isolated effect of activated charcoal on aerial part length - APL (a) and longest root length - LRL (b), and isolated effect of IBA on number of green leaves - NGL (c).

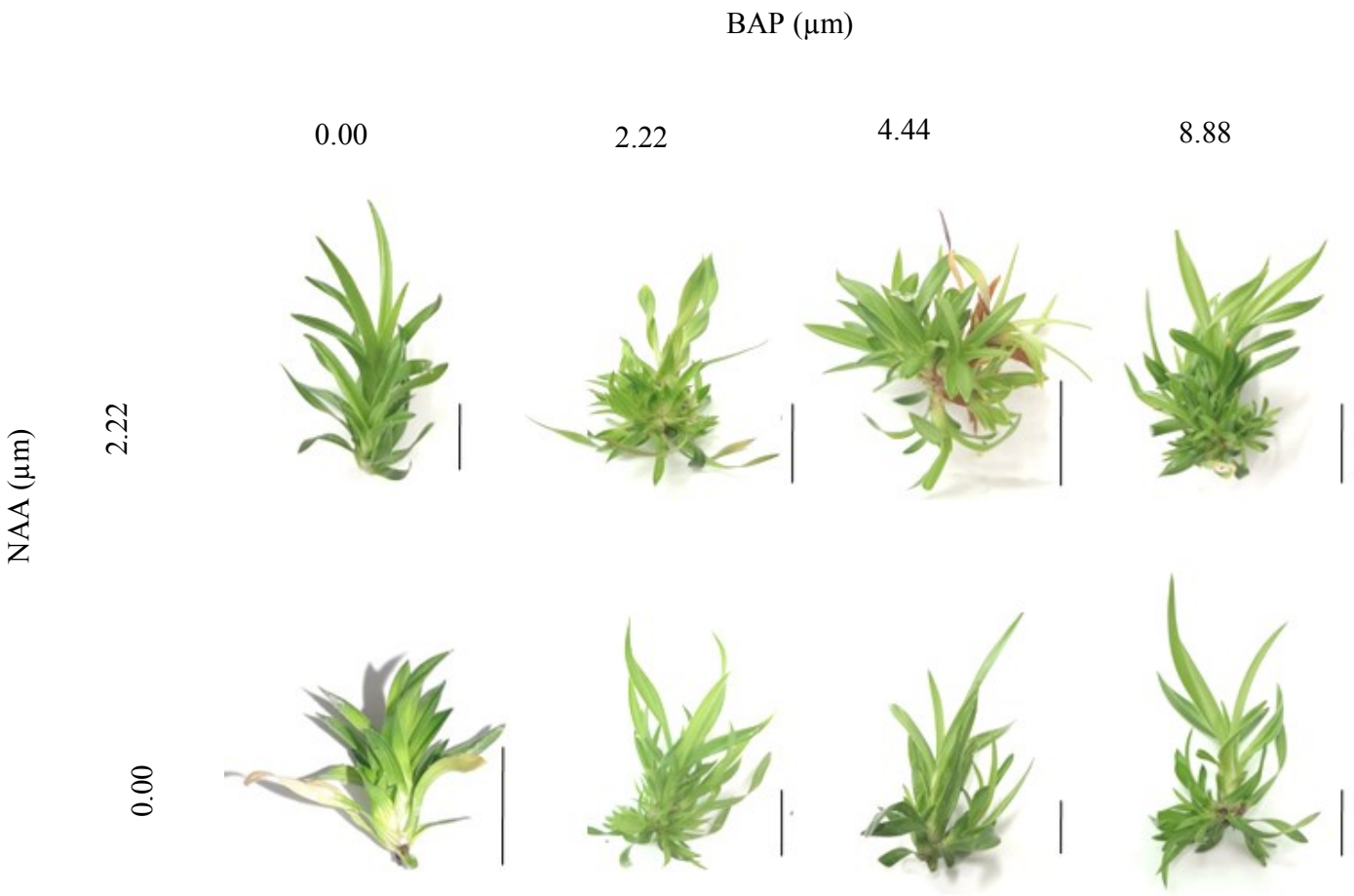

Figure 4. Regeneration of *in vitro* shoots of *Vellozia seubertiana*, in MS½ culture medium supplemented with NAA and BAP, grown for 60 days (Bar: 1 cm).

The auxin and activated charcoal had no effects on the variables analyzed in the acclimation phase. The percentage of plant survival (%PS) at 60 days after transplanting to the *ex -vitro* environment varied from 50% to 75% among the treatments tested. It denotes that, although significant differences were found for the variables analyzed in *in vivo* rooting, these differences did not affect the adaptation of *V. seubertiana* to the external environment conditions evaluated. It is emphasized that all the plantlets were already rooted when transplanted to the substrates, denoting their capacity for *in vitro* rooting, which is essential for a successful acclimation.

These results differed from those of Lima, Lima-Brito, and Santana (2020), who found that the culture medium containing 1 g L^{-1} of activated charcoal had a positive effect on acclimation of *Sincoraea mucugensis*.

Freitas Neto (2009) found changes in leaf cuticle thickness during acclimation of *V. flavicans:* very thin in samples grown *in vitro*, very thick in plants grown in the field, and intermediate in acclimated plantlets, which denotes that this species easily adapts to growing conditions. Variation in leaf cuticle thickness is an anatomical adaptation of plants to avoid water loss and, consequently, dehydration under water deficit conditions.

The good adaptation of *V. seubertiana* to the acclimation can be attributed to fast mechanisms of adaptation to external environments, as described by Freitas Neto (2009) for *V. flavicans*, which is connected to the rusticity of these species in their local of natural occurrence, the Campos Rupestres Montane Savanna ecoregion, where they present high tolerance to water stress and, in many cases, protected stomata and an efficient metabolism against dehydration (RAPINI, 2008).

The results found denote that the period for obtaining seedlings of *V. serbertiana* from seeds through organogenesis is approximately 230 days (Figure 5). The present study is unprecedented for the species *Vellozia seubertiana* and is the first description of a complete *in vitro* propagation protocol for the genus *Vellozia*; therefore, it will be useful to guide future studies for other species of the family Velloziaceae.

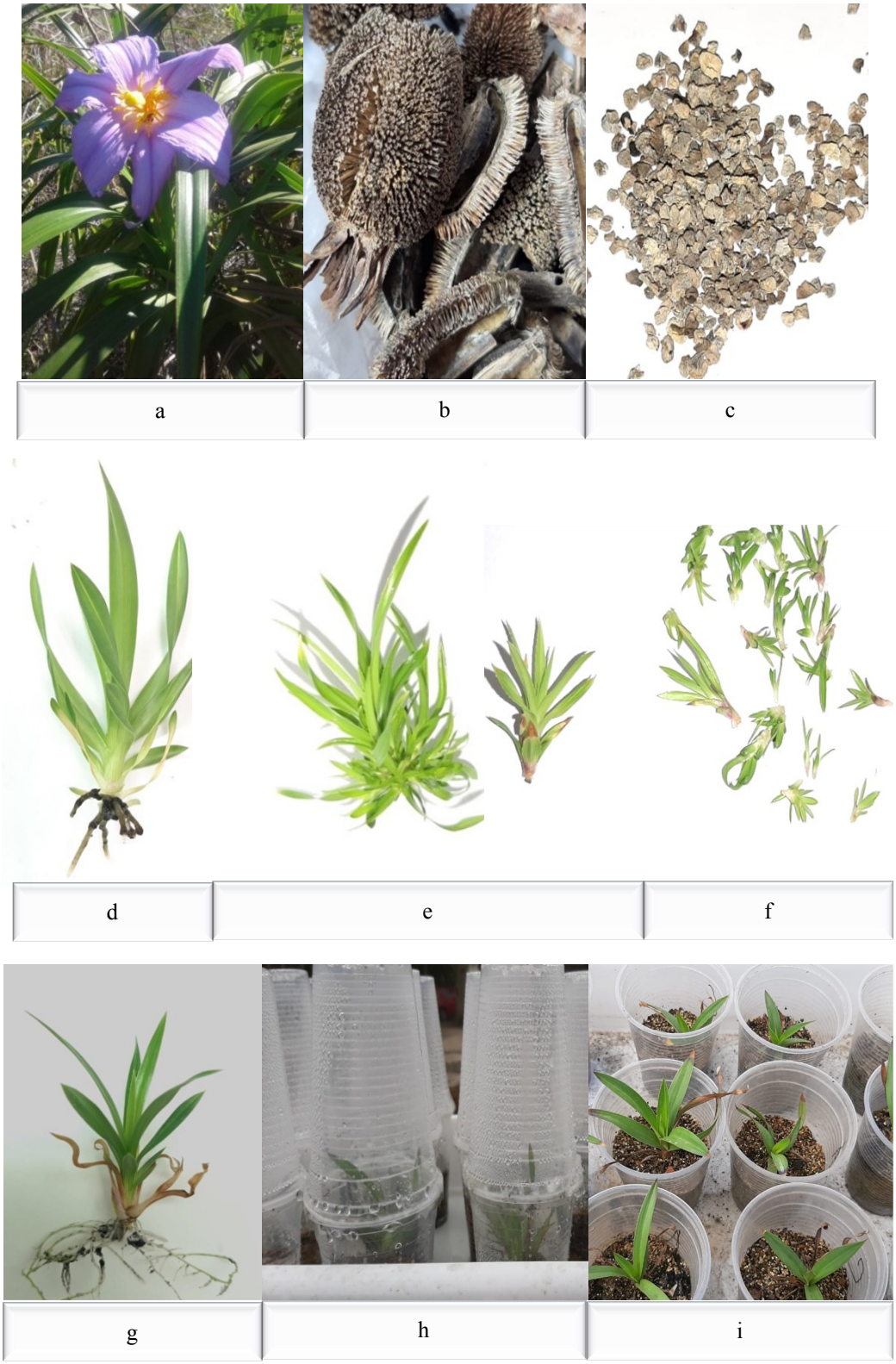

Figure 5. Plant in the field (a), capsules (b), and seeds (c) of *Vellozia seubertiana*; schematic representation of the micropropagation protocol via organogenesis: initial *in vitro* growth form plantlets germinated *in vitro* (d), multiplication in culture medium containing 8.88 µM of BAP and 2.22 µM of NAA (e), shoots isolated from the parent plant (f), rooting (g), acclimation (h), and acclimated seedlings at 60 days after transplanting to *ex vitro* conditions (i).

CONCLUSION

Micropropagation is a promising technique for the production of seedlings of the species *Vellozia seubertiana*; the period for obtaining seedlings from seeds is approximately 230 days. According to the results found, it is recommended to use MS½ culture medium supplemented with 30 g L^{-1} of sucrose, 7 g L^{-1} of agar, and 1 g L^{-1} of activated charcoal is recommended for the *in vitro* establishment, and addition of 8.75 µM of BAP to the culture medium for the *in vitro* multiplication phase. The MS½ medium favored the rooting, and the acclimated plants presented 50% to 75% survival under *ex vitro* conditions.

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