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Seedling production of endangered species by the micropropagation: the case of *Alstroemeria plantaginea* Mart. ex Schult. & Schult.f.

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ABSTRACT

Campos Rupestres Ferruginosos present specific environmental conditions and harbor plant species considered endangered, such as *Alstroemeria plantaginea* Mart. ex Schult. & Schult.f., an endemic species found in rocky outcrop areas. This study aimed to improve a protocol for the micropropagation of *Alstroemeria plantaginea* by evaluating the effect of temperature regimes on *in vitro* germination, the impact of light quality, BAP concentration, and explant type on shoot multiplication and elongation, as well as the influence of the carbon source on rooting and plant acclimatization. Seeds collected from native plants were treated with NaOCl, and two temperature regimes were tested (constant 24 ± 1°C and alternating 20°C to 30°C) to induce germination. During the multiplication and elongation stages (which occur simultaneously), two explant types were used: individualized stems and shoot clumps, testing BAP concentrations (0.5 and 1.0 mg L^{-1}) and light quality (white LED, blue LED, and red LED). For rooting, carbon sources (30 g L^{-1} glucose and 30 g L^{-1} sucrose) were evaluated, and acclimatization was assessed according to the carbon source used in the rooting phase. Germination occurred only under the alternating temperature regime (20–30°C), reaching 13.8%. The concentration of 0.5 mg L^{-1} BAP and blue LED light favored bud formation and leaf development in shoot clump explants. The carbon sources had no significant effect on explant rooting; however, those rooted in the presence of sucrose showed higher survival during acclimatization. In the end, it was possible to produce micropropagated plantlets of the species within 150 days.

Index terms: *Campo Rupestre Ferruginoso*, *in vitro* cultivation, Canga ecosystem, light spectrum quality.

INTRODUCTION

Alstroemeria plantaginea Mart. ex Schult. & Schult. f. is a herbaceous species that occurs in high-altitude grasslands and develops mainly in rocky outcrop areas, such as the Campos Rupestres of the Brazilian states of Minas Gerais, São Paulo, and, rarely, Bahia. It is considered endemic to these regions and categorized as "endangered" (EN) (Assis & Mello-Silva, 2002; Dhiman & Kashyap, 2022; Drummond et al., 2008, SIBBR, 2020). The species has tuberous roots, spherical seeds, and red-orange flowers that bloom between December and April, granting it ornamental potential and ecological importance, especially in attracting pollinators (Finot et al., 2016).

In addition to its ornamental and ecological value, *A. plantaginea* holds potential for the restoration of degraded areas in Ferruginous Rocky Fields (Campos Rupestres Ferruginosos), ecosystems rich in biodiversity and highly threatened by anthropogenic activities (Messias et al., 2012). However, sexual propagation is limited by factors such as physiological dormancy, seasonality, and low germination rates, as well as the scarcity of information on the species' reproductive biology and seed technology (Aros et al., 2023).

In this context, micropropagation emerges as a promising alternative for threatened species, allowing for rapid and large-scale multiplication, independent of seed production (Leite et al., 2024;

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Thakur & Karnosky, 2007). However, the success of this technique depends on the optimization of several factors at each stage of the *in vitro* culture process.

Although there are studies related to the propagation of other *Alstroemeria* species, this work is the first to propose a specific micropropagation protocol for *A. plantaginea*, representing a novel contribution to the conservation and propagation of this endemic species. Considering the challenges involved, the propagation of native species presents several difficulties, from low germination rates to the need for appropriate adjustments regarding explant type, growth regulators, light quality, and carbon source, among others, all of which have a direct impact on the final outcome of seedling production (Silva et al., 2021; Barbosa et al., 2021; Souza et al., 2021; Souza et al., 2022).

Temperature is a critical factor, especially during germination, as it influences the rate of water absorption by seeds and the activation of biochemical reactions essential for breaking dormancy (Carvalho & Nakagawa, 2012). Nevertheless, studies evaluating the effect of temperature alternation on the germination of *Alstroemeria* seeds are scarce, which justifies the investigation of this parameter for the species under study.

During the cultivation stages, growth regulators such as BAP (6-benzylaminopurine) have been widely used for shoot induction, but the response may vary depending on the concentration used and the cultivation stage, requiring specific adjustments for each species (Souza et al., 2021). In addition to growth regulators, spectral quality directly influences physiological processes, especially photosynthesis, making it important to evaluate the effects of different LEDs on plant growth during the various stages of cultivation (Hung et al., 2015).

The carbon source is another important adjustment in the culture medium that supports seedling production, primarily influencing the elongation and rooting of plant material. It serves as a source of energy and carbon, potentially impacting root formation and plant survival after transfer to the *ex vitro* environment (Barbosa et al., 2021). However, comparative studies between sucrose and glucose for native species, especially those belonging to the Ferruginous Rocky Field ecosystem are still scarce.

Considering the ecological relevance of *A. plantaginea* and the vulnerability of the *Campo Rupestre Ferruginoso*, this study may serve as a foundation for the conservation and application of micropropagation techniques to other endemic species from these environments.

Therefore, this study aimed to improve a micropropagation protocol for *Alstroemeria plantaginea* by evaluating the effect of temperature regimes on *in vitro* germination, the impact of light quality, BAP concentration, and explant type on shoot multiplication and elongation, and the influence of the carbon source on rooting and plant acclimatization

MATERIAL AND METHODS

Origin of plant material

The seeds used were collected from native *Alstroemeria plantaginea* plants in *Campo Rupestre Ferruginoso* in 2021, at the Research and Innovation Unit belonging to Gerdau (GERDAU Açominas S.A.), located in the municipality of Ouro Branco, Minas Gerais, Brazil (SisGen registration no. ACF88E2).

In vitro germination

The seeds were previously stored for 30 days at low temperatures (6 to 10 °C) to overcome dormancy (Aros et al., 2023; Volpi et al., 2024), then scarified using sandpaper and immersed in warm water (40 °C) for 5 minutes under constant agitation. Subsequently, the seeds were immersed in 70% ethanol for 30 seconds, followed by immersion in a sodium hypochlorite (NaOCl, Clarix®) solution with an active chlorine concentration of 1.0–1.25% for 10 minutes. After this process, the seeds were rinsed three times with deionized and autoclaved water and inoculated under aseptic conditions into test tubes (25 × 150 mm) containing 10 mL of MS culture medium (Murashige & Skoog, 1962).

The culture medium was supplemented with 30 g L^{-1} of sucrose (Synth Ltda) and 6 g L^{-1} of agar (Merck S.A.). The pH of the medium was adjusted to 5.8 ± 0.05 prior to the addition of agar. The culture medium was autoclaved at 121 °C and 1.0 kgf cm^{-2} pressure for 20 minutes.

The experiment was conducted in a completely randomized design, evaluating seed germination under two temperature regimes. The first was at 24 ± 1 °C with constant 12 hour light exposure in

a growth room, and the second was an alternating regime of 20 °C in the dark for 12 hours and 30 °C in the light for 12 hours in a B.O.D. germination chamber. One hundred seeds were used per treatment, each placed individually in a test tube, with each seed considered a replicate.

After 60 days, tissue oxidation, fungal and/or bacterial contamination, germination percentage, and germination speed index were evaluated (Sarwar et al., 2024).

Effect of light quality and BAP concentration on *in vitro* multiplication and elongation using shoot clumps

The seedlings considered established in the *in vitro* germination stage produced shoot clumps because of tillering. These were subcultured into test tubes (25 × 150 mm) containing 10 mL of MS culture medium, supplemented with 30 g L^{-1} of sucrose (Synth Ltda), 6 g L^{-1} of agar (Merck S.A.), 0.4 g L^{-1} of activated charcoal (Synth Ltda), 0.05 mg L^{-1} of α-naphthaleneacetic acid (NAA, Sigma®), and 0.5 mg L^{-1} of 6-benzylaminopurine (BAP, Sigma®), according to each treatment (Leite et al., 2024).

The experiment was conducted in a completely randomized design in a factorial arrangement (2 × 3), evaluating two BAP concentrations (0.5 and 1.0 mg L^{-1}) and three spectral light qualities (white LED, blue LED, and red LED, Figure 1), totaling 6 treatments with 15 replicates, with each explant considered an experimental unit.

At 30 days, the number of leaves, number of shoots, number of elongated shoots, number of roots, oxidation, vigour, and fungal and/or bacterial contamination were evaluated.

Effect of light quality and BAP concentration on the *in vitro* multiplication and elongation of individualized explants

Individualized stems containing only one apical bud from the seedlings considered established in the *in vitro* germination stage were subcultured following the same methodology described in section 2.3.

Effect of carbon source on *in vitro* rooting

For rooting, MS culture medium was used, supplemented with 6 g L^{-1} agar, 0.4 g L^{-1} activated charcoal, 0.05 mg L^{-1} BAP, 0.1 mg L^{-1} NAA, and

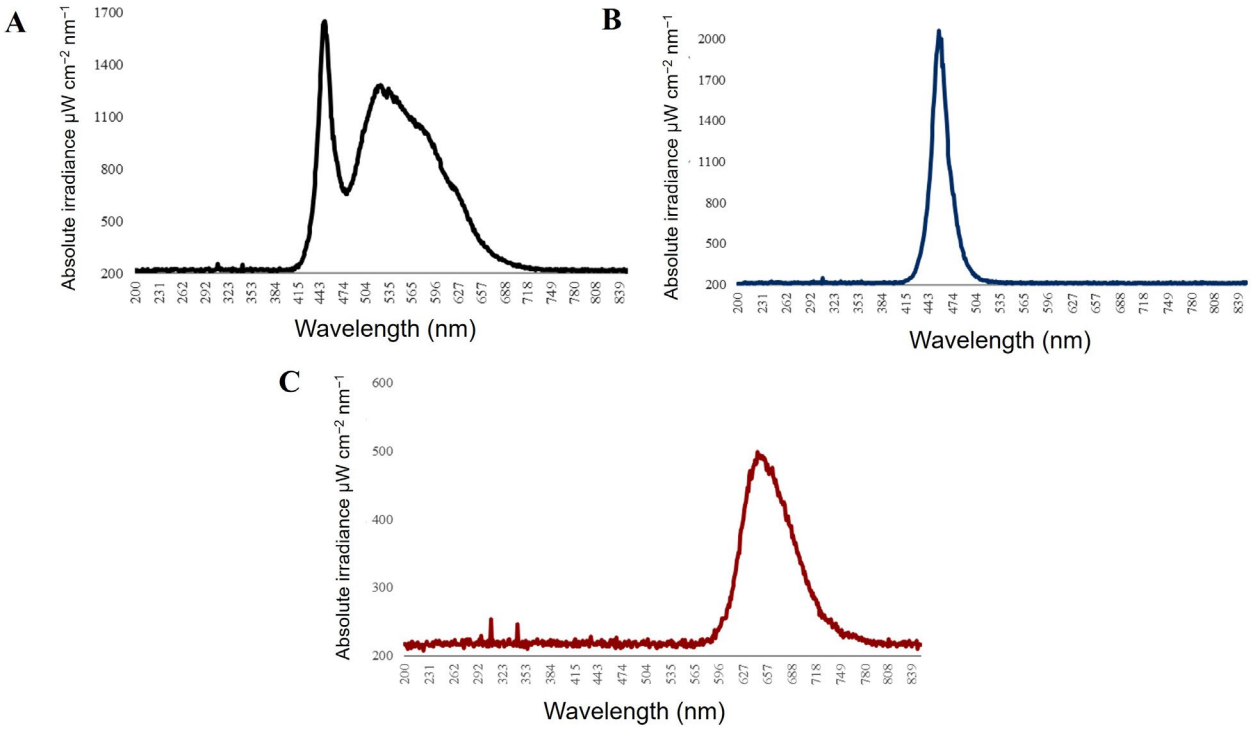

Figure 1: Absolute radiance as a function of wavelength (nm) of the light emitted during the *in vitro* cultivation of *Alstroemeria plantaginea*. (A) White LED lamp, (B) Blue LED lamp, and (C) Red LED lamp.

0.1 mg L^{-1} indole-3-butyric acid (IBA, Sigma®) (Souza et al., 2021; 2022). Two carbon sources were evaluated, both at a concentration of 30 g L^{-1}: sucrose and glucose.

The experiment was conducted in a completely randomized design with 20 replicates per treatment. After 30 days, the number of shoots, number of elongated shoots, and number of roots were evaluated.

Acclimatization

The shoots rooted *in vitro* on culture media containing sucrose and glucose were transplanted into polyethylene cups filled with a commercial substrate composed of decomposed pine bark and fine vermiculite in a 2:1 v/v ratio. These cups were placed in trays and covered with plastic film, creating a mini-incubator system (Leite et al., 2024). They were maintained in a climate-controlled room at 24°C (±1°C) with a 16 hour photoperiod.

After ten days, the plastic cover was completely removed, and in 30 days, survival was evaluated according to the explant's origin (sucrose or glucose).

Data analysis

Data were submitted to the Shapiro-Wilk test ($P > 0.05$) to assess normality and to Bartlett's test ($P > 0.05$) to analyze homogeneity of variances. After these checks, data were analyzed by analysis of variance (ANOVA, $P < 0.05$), followed by Tukey's test for mean comparison ($P < 0.05$). For the survival variable, a descriptive analysis was performed. The R software (R CORE TEAM, 2024) and RStudio were used with the support of the easyanova package (Arnhold, 2013).

RESULTS

The alternating temperature treatment (20–30 °C) proved to be more effective for seed germination, reaching 13.8% germination after 60 days and a germination speed index of 0.4, with germination observed up to 100 days of cultivation. In contrast, seeds maintained at a constant temperature of 24 ± 1 °C did not germinate, even after 100 days of cultivation. These results indicate that alternating temperature is a crucial factor for inducing seed germination in the species under study.

To optimize the multiplication and elongation stages, which occur simultaneously in this species, two types of explants were used: shoot clumps derived from seed germination and individualized shoots (stems). The selection of these explants aimed to increase the efficiency of *in vitro* plant multiplication.

Multiplication of individualized stems showed low shoot formation in most treatments. Only the concentration of 0.5 mg L^{-1} BAP resulted in the formation of a small number of shoots (Figure 2A), while the other treatments did not promote multiplication, although the explants remained viable. Nevertheless, all explants exhibited vegetative growth, with a notable increase in the number of leaves under blue LED lighting combined with 0.5 mg L^{-1} BAP (Figure 2B). Plant vigour analysis indicated that the combination of red LED and 0.5 mg L^{-1} BAP resulted in the greatest explant development, being the only condition in which a significant difference was observed between the BAP concentrations tested (Figure 2C).

Using shoot clumps as explants resulted in better overall performance compared to individualized stems. The concentration of 0.5 mg L^{-1} BAP induced more pronounced responses, especially under blue LED lighting, which promoted the highest number of shoots and leaves among the treatments tested (Figures 2D and 2E). Regarding plant vigour, red LED lighting combined with 0.5 mg L^{-1} BAP yielded the best results, with a significant difference compared to the other conditions (Figure 2F).

Shoot elongation was observed in the treatment with 0.5 mg L^{-1} BAP. Under this concentration, blue LED lighting stimulated the highest number of elongated shoots compared to the other treatments (Figure 2G). Regarding rooting, all plants cultivated from shoot clumps developed roots regardless of the treatment applied (Figure 2H); however, no significant differences were observed among treatments.

During the rooting stage, the plants were transferred to a culture medium containing either glucose or sucrose as the carbohydrate source. Both sources induced an average of five roots per explant, with no significant difference observed between treatments. Although the number of roots was lower than that observed in previous stages, an interesting aspect was the stimulation of new shoot formation, as shown in Figure 3.

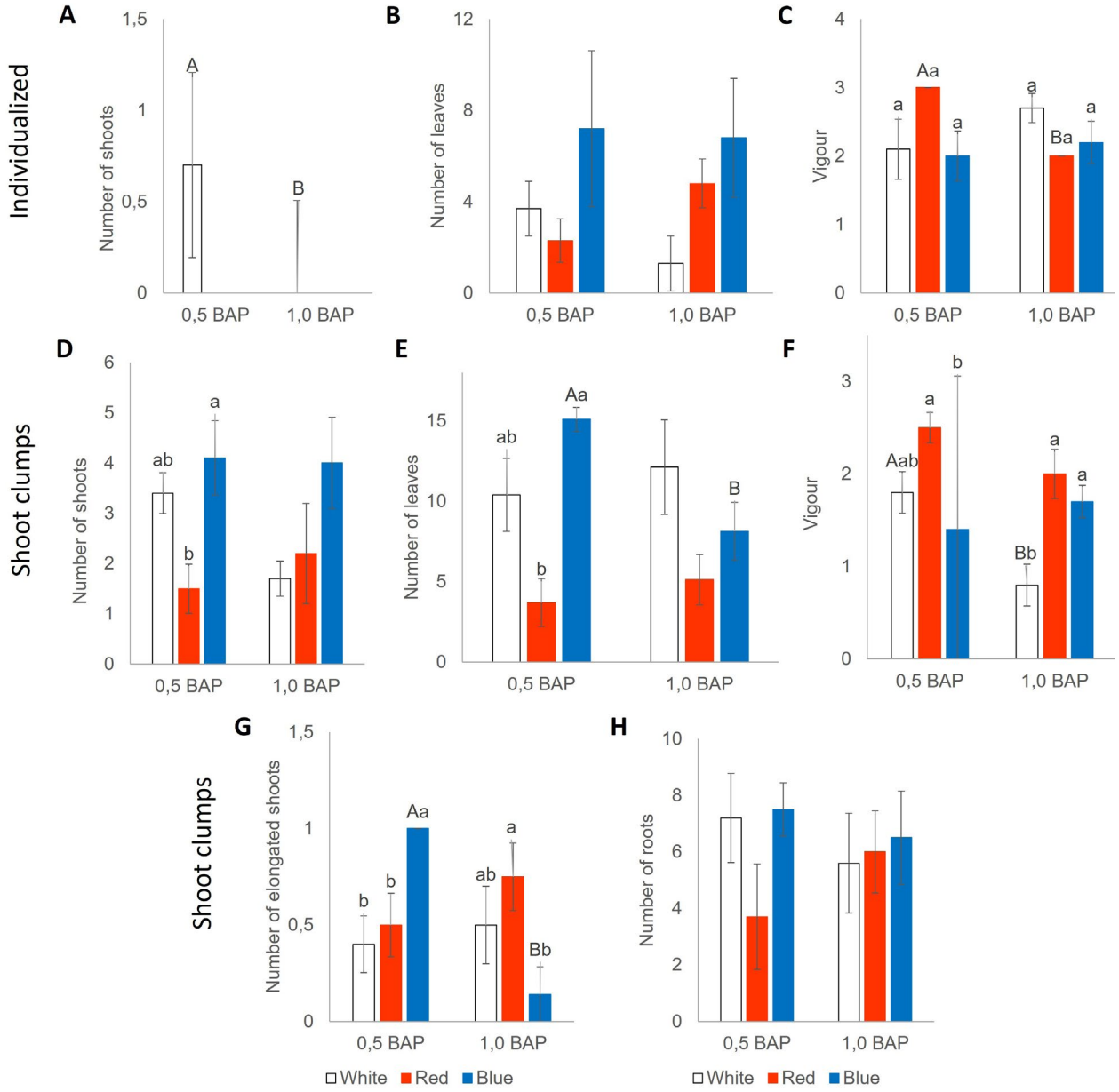

Figure 2: Evaluated characteristics of individualized explants and shoot clumps of *Alstroemeria plantaginea* in relation to spectral quality and BAP concentration (mg L^{-1}) after 30 days. (A and D) Mean number of shoots; (B and E) mean number of leaves; (C and F) mean vigour; (G) mean number of elongated shoots; and (H) mean number of roots. Mean values followed by the same lowercase letter (for BAP concentration) and uppercase letter (for spectral quality) do not differ significantly. Graphs without letters indicate no significant difference between treatments. Error bars represent the standard error of the mean.

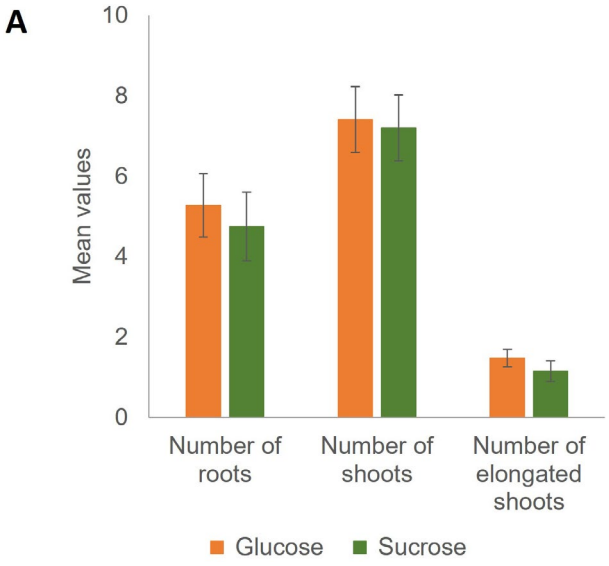

Figure 3: Mean values of number of roots, number of shoots, and number of elongated shoots of *Alstroemeria plantaginea* in relation to the carbohydrate source after 30 days. Error bars represent the standard error of the mean.

The acclimatization stage of *in vitro* rooted plants was conducted considering the carbon source used in the rooting culture medium (sucrose or glucose). Plants rooted in medium with sucrose showed a survival rate of 62%, while those rooted in medium with glucose showed 38%. The employed methodology allowed the production of complete plants, from germination (Figures 4A and 4B) and multiplication (Figure 4C) to rooting and acclimatization (Figure 4D), resulting in plants suitable for field planting after 150 days (Figure 4E).

DISCUSSION

The effectiveness of the sterilization protocol used for the seeds was confirmed by the absence of fungal and/or bacterial contamination (Mazarotto et al., 2023). Staggered germination over time is a common adaptive strategy in various species, allowing their persistence in the environment. In addition the physical protection of seeds, this strategy involves several mechanisms that ensure germination is distributed over time. Although beneficial for species survival in natural environments, this strategy can also pose challenges for seedling production (Duncan et al., 2019; Arana et al., 2016).

Furthermore, germination can be influenced by environmental factors such as temperature, especially in the context of climate change. This temperature sensitivity may compromise the persistence of certain species in their natural habitats (Kildisheva et al., 2020; Wisnoski & Shoemaker, 2022).

Alstroemeria plantaginea, commonly known as Peruvian lily, exhibits a peculiar mechanism for species maintenance in nature. Everyone produces a relatively small number of seeds, with germination occurring gradually and over an extended period, taking up to 100 days as observed in this study. This strategy contrasts with that of other species belonging to the *Campo Rupestre* phytophysiognomy, such as *Paliavana sericiflora*, which shows more uniform germination (Leite et al., 2024).

In addition, the low germination rate and reduced germination percentage of *Alstroemeria* are also notable characteristics of the genus (Guerra et al., 2022; Aros et al., 2023). In the tests conducted, only 6.25% of the seeds germinated after 30 days, increasing to 13.75% in 60 days and reaching 20% after 100 days under alternating temperature conditions. This extended germination period indicates the presence of dormancy mechanisms that regulate seedling emergence timing (Finch-Savage & Leubner-Metzger, 2006; Aros et al., 2023).

The low germination percentages observed in species such as *Hyptis crenata* (9%), *Chamaecrista* sp. (22%), and *Abolboda poarchon* (5%) (Fichino et al., 2016), which inhabit environments similar to that of *Alstroemeria*, suggest the influence of environmental factors on dormancy mechanisms, considering the strong temperature fluctuations in natural habitats (i.e., high temperatures during the day and low at night). Germination occurring only after temperature alternation indicates the presence of physical dormancy or dormancy related to hormonal and metabolic regulation, such as the action of ethylene and secondary metabolites on cell wall permeability (Fichino et al., 2016; Matilla, 2020).

Scarification, by exposing the endosperm, suggests that the restriction to germination may be associated with both physical barriers and physiological mechanisms, as observed in some species of the genus (Guerra et al., 2022; Aros et al., 2023).

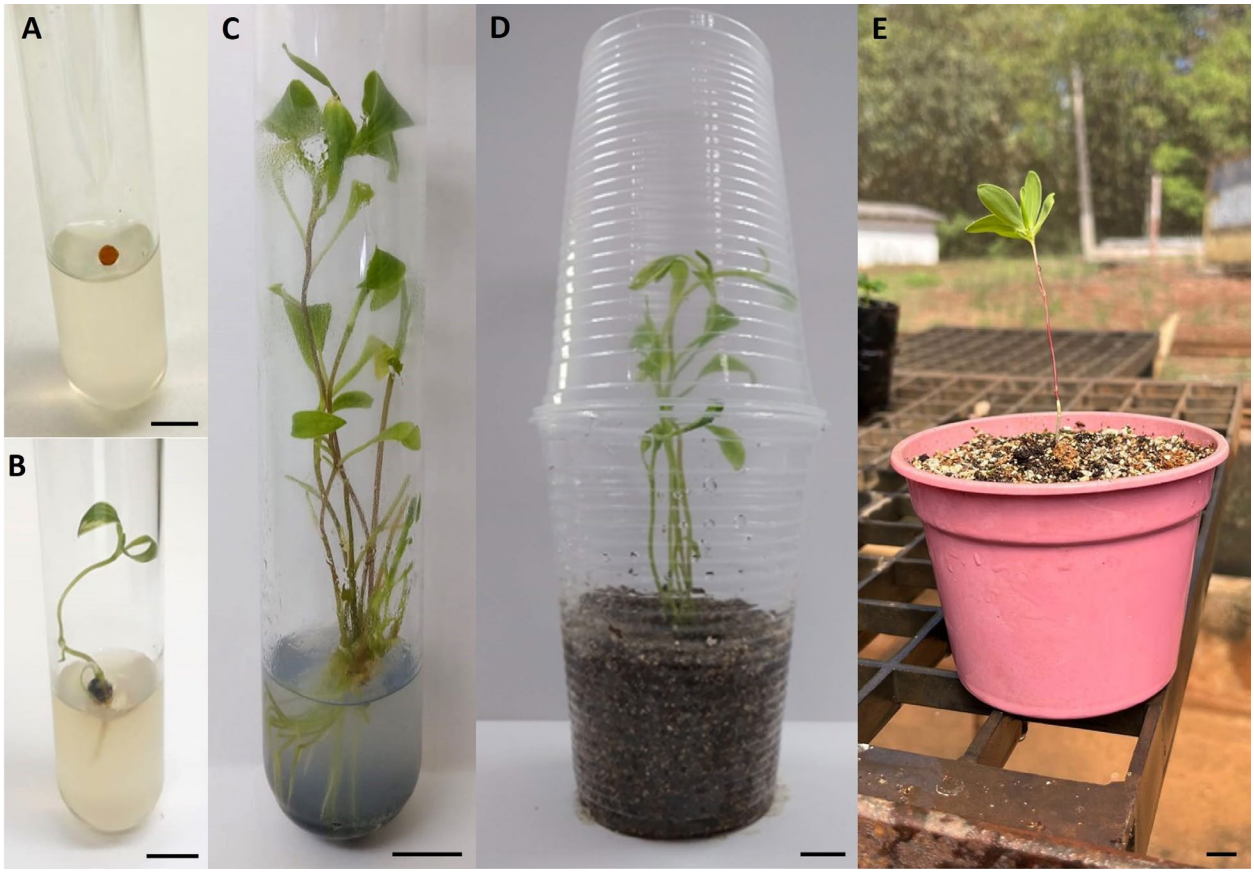

Figure 4: Stages of seedling production of *Alstroemeria plantaginea* through micropropagation technique. (A) *In vitro* seed introduction; (B) germinated plants (60 days); (C) shoot multiplication and elongation (90 days); (D) start of acclimatization in mini-incubator system (120 days); (E) plants grown in pots in the nursery (150 days). Scale bar: 1 cm.

In the *in vitro* multiplication and elongation stage, the results showed that a BAP concentration of 0.5 mg L^{-1} was the most effective for shoot induction, especially when shoot clumps were used as explants. This behavior is consistent with other species studied, including some from the rupestrian field, such as *Cattleya crispata*, *Mimosa calodendron*, and *Vernonia condensata*, where this concentration also favored vegetative growth (Souza et al., 2021; 2022; Almeida et al., 2020).

Furthermore, spectral quality showed a significant influence on the morphological performance of the explants. Blue LED promoted greater leaf emission, number of shoots, elongated shoots, and roots, which can be attributed to the activation of photoreceptors such as cryptochromes, involved in photomorphogenesis and regulation of cell elongation (Hung et al., 2015; Souza et al., 2020). On the other hand, red LED stood out by promoting greater plant vigour, possibly due to its role in photosynthesis and carbohydrate biosynthesis, resulting in higher biomass accumulation and growth (Johkan et al., 2010). These results reinforce the importance of the interaction between phytohormones and light quality in optimizing *in vitro* cultivation.

In the rooting stage, both glucose and sucrose promoted similar results in *A. plantaginea*. This response may be related to the species' ability to efficiently metabolize different carbon sources, since both are readily assimilated by plant cells and serve as energy sources and structural carbon. This lack of specificity may represent a physiological advantage by improving *in vitro* cultivation responses of the species (Monfort et al., 2015; Hesami et al., 2021). Although previous studies have demonstrated the positive effect of sucrose on rooting in *Eucalyptus grandis* × *Eucalyptus*

urophylla and glucose on root development in *Gymnopogon doellii* (Souza et al., 2020; Lopes Paulo et al., 2024), our results indicate that both glucose and sucrose are effective in inducing rooting in *Alstroemeria plantaginea*.

During acclimatization, high seedling survival was observed, especially for those originating from the sucrose treatment. Unlike what was observed in *Paliavana sericiflora* (Leite et al., 2024), where fungal contamination was the main obstacle, in this study excessive water loss emerged as the most relevant limiting factor. To overcome this challenge, the use of substrates with high water retention capacity and gradual moisture management in the mini greenhouse are recommended, allowing a safer transition of seedlings to the *ex vitro* environment.

The improved micropropagation protocol for *Alstroemeria plantaginea* represents a significant advancement for the *ex situ* conservation of endemic species from ferruginous Rupestrian Fields. With efficient *in vitro* seedling production, it is possible to minimize the risks of extinction associated with habitat loss and difficulties in natural regeneration. Furthermore, the techniques established in this study can serve as guidelines for other species from the same phytophysiognomy, broadening conservation and ecological restoration strategies in degraded areas.

CONCLUSIONS

The results of this study demonstrated that alternating temperature (20 °C in the dark for 12 hours and 30 °C under light for 12 hours) was the treatment that enabled the germination of *Alstroemeria plantaginea* seeds.

The *in vitro* multiplication stage was optimized using blue LED lighting and shoot clump explants; the concentration of 0.5 mg L^{-1} BAP promoted the greatest responses in terms of number of shoots, highlighting its importance for inducing growth and cellular differentiation.

The acclimatization stage was effective, allowing the production of high-quality plantlets within 150 days, demonstrating the feasibility of micropropagation for large-scale production of this species.

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