



Sterilization and gas exchange on *in vitro* cultivation of *Sinningia rupicola* (Mart.) Wiehler: production of seedlings from an endangered species

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Received: 19 August 2025 / Accepted: 20 November 2025 / Published online: 25 February 2026 / Editor: Alfred Huo
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Abstract

The Ferruginous Rupestrian Grassland is an ecosystem that harbors endemic and endangered species, such as *Sinningia rupicola*. The low germination rate and restricted habitat of this species increase its risk of extinction, making the development of conservation strategies essential. Micropropagation is a recommended technique in such cases and, although effective for seedling production, *in vitro* cultivation requires careful measures such as strict contamination control during inoculation, as well as process optimizations that may induce morphophysiological changes favoring acclimatization. In this context, sterilization methods, the use of permeable membranes, and mini-incubator system emerge as promising strategies to ensure the production and survival of these plants, mitigating anthropogenic impacts and preserving biodiversity. This study aimed to establish *in vitro* seed culture according to exposure time to sodium hypochlorite, to evaluate the effect of gas exchange on the multiplication, elongation, and rooting of *Sinningia rupicola*, and to assess the effectiveness of the mini-incubator system in acclimatizing the seedlings. During the *in vitro* establishment stage, exposure to active chlorine for 15 min resulted in the lowest contamination percentage, while 5 and 10-min treatments yielded the highest germination rates (55.0%). In the multiplication stage, the treatment with three membranes (3Mem) showed the best vigor, with an average value of 1.04. Regarding the clump diameter variable, the treatments without membranes (0Mem) and with one membrane (1Mem) had the best results in both the multiplication and elongation stages. In the elongation stage, concerning shoot length, treatments 1Mem and 3Mem showed the best performances, with averages of 4.3 cm and 3.95 cm, respectively. The use of 3Mem also resulted in higher levels of photosynthetic pigments across all evaluated stages. A survival of 85.8% was achieved during acclimatization, and all seedlings transferred to the greenhouse survived after 45 d, demonstrating the effectiveness of the *in vitro* cultivation conditions and the mini-incubator system in producing micropropagated plants.

Keywords Ferruginous Rupestrian Grassland · Micropropagation · Mixotrophism · Acclimatization

Introduction

The Brazilian territory comprises a wide diversity of natural areas, some of which are exclusive to the country. Among these formations are the Rupestrian Grasslands, which are

composed of plant species that develop on rocky outcrops in highland regions (quartzitic, granitic, or ferruginous substrates). Their core area is located along the Espinhaço Mountain Range, in the states of Minas Gerais and Bahia (Ataíde *et al.* 2011; Silveira *et al.* 2016). Ferruginous Rupestrian Grassland located in the Iron Quadrangle, between the Atlantic Forest and the Cerrado biomes, occurs on iron ore deposits, which hold significant importance for the global economy (Arruda 2010). In addition, this area harbors high biodiversity and is considered one of the most diverse ecosystems in the world, home to rare and endemic species that are at varying degrees of extinction risk, primarily due to intense anthropogenic activities. (Giulietti *et*

Editor: Alfred Huo.

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al. 1997; Madeira et al. 2008; Messias et al. 2012; Leite et al. 2017).

Sinningia rupicola (Mart.) Wiehler, a member of the Gesneriaceae family, is found in areas restricted to the Iron Quadrangle region (Araujo et al. 2005; Ranieri et al. 2012). This herbaceous species reaches heights of 20 to 40 cm and flowers between October and March, being pollinated by hummingbirds (Araujo et al. 2005). The aerial stem is produced once a year, while for the rest of the year the plant remains in its tuberous form. Its seeds are considered small and lightweight, which facilitates greater dispersal range. However, they exhibit low germination capacity, which may be related to their limited nutritional reserves (Ranieri et al. 2012).

The low germination rate of *Sinningia rupicola* seeds, combined with its restricted occurrence in a highly specific and fragile phytophysiology that is continuously losing habitat, makes the species even more vulnerable (Messias et al. 2012; Santos et al. 2021). Currently, *Sinningia rupicola* is classified as "Endangered" (EN) on the Official National List of Threatened Flora Species (Ministério do Meio Ambiente 2014) and on the Brazilian Red List of Flora (CNCFlora 2020).

The conservation of endemic and threatened species is essential for maintaining and balancing the ecosystems in which they are found, especially in the face of increasing anthropogenic pressures and climate change. In this context, the large-scale production of seedlings to support restoration and rehabilitation projects becomes an important strategy. Among the existing plant propagation techniques, micropropagation is frequently recommended to mitigate damage in various *habitats*, reducing the extinction risk of threatened species due to its numerous advantages (Righeto et al. 2012). Moreover, when carried out *in vitro*, propagation allows for the year-round production of seedlings, making it one of the most viable techniques for the multiplication of endangered plant species (Souza et al. 2022; Leite et al. 2025).

Micropropagation is a technique that enables cultivation under controlled phytosanitary, nutritional, and environmental conditions, allowing for long-term storage and application in the conservation of native species through the formation of germplasm banks (Xavier et al. 2013; Keret et al. 2018). On the other hand, if not properly executed, it can lead to contamination, physiological and morphological disorders, and unsatisfactory rooting of the plants (Xiao et al. 2011).

Contamination by microorganisms present in explants and seeds must be eliminated using disinfecting agents prior to inoculation (Chaves et al. 2005). Among the most used substances for surface sterilization of explants on *in vitro* culture are ethanol and sodium hypochlorite. However, the concentrations and exposure times vary depending on the

species and type of explant, requiring optimization for effective disinfection (Chaves et al. 2005; Souza et al. 2011).

Furthermore, to ensure success in the stages of *in vitro* cultivation, physical, chemical, and biological factors must be considered and adjusted to provide optimal conditions that maximize both the production and quality of the propagated seedlings (Kania and Giacomelli 2000). Gas exchange during *in vitro* cultivation must be tailored to the specific requirements of each species, creating a favorable environment, as it can affect photosynthesis and plant morphogenesis, and consequently, *ex vitro* survival (Ferreira et al. 2016). The sealing of flasks and tubes is used to prevent fungal and bacterial contamination and to avoid dehydration of the culture medium and propagules. However, this sealing can reduce the plant's autotrophic capacity, necessitating the addition of carbohydrates to the culture medium, and may negatively impact survival during the acclimatization stage due to difficulties in transitioning from a heterotrophic to an autotrophic metabolism (Dutra et al. 2009; Xiao et al. 2011; Pinheiro et al. 2013; Parveen and Shahzad 2014; Ferreira et al. 2016).

During the acclimatization stage, optimal conditions must also be provided to prevent plant mortality, and one technique that can enhance this stage is the use of a mini-incubator system (Brondani et al. 2012; Brondani et al. 2018). Therefore, the need to establish and improve seedling production techniques for native species particularly those with low seed propagation capacity and endangered status, such as *Sinningia rupicola* becomes evident. Research aimed at this goal is fundamental for the conservation of the species and the maintenance of biodiversity in its endemic region.

To date, no standardized micropropagation protocol has been established for *Sinningia rupicola*, focusing on optimizing key aspects such as sterilization and gas exchange to achieve maximum efficiency and *ex vitro* survival. Thus, the present study aimed to evaluate the *in vitro* establishment of seeds according to the exposure time to sodium hypochlorite, the effect of gas exchange on the multiplication, elongation, and rooting of *Sinningia rupicola*, and the effectiveness of the mini-incubator system during the acclimatization of *in vitro*-produced seedlings.

Materials and Methods

Origin of Plant Material, *In Vitro* Germination and Establishment The seeds were collected from wild *Sinningia rupicola* plants in 2021 (SisGen: ACF88E2), in a Ferruginous Rupestrine Grassland area within the Research and Innovation Unit owned by Gerdau (GERDAU Açominas S.A.), located in the municipality of Ouro Branco, Minas Gerais, Brazil (Fig. 1). *Sinningia rupicola* seeds were rinsed under running water for 5 min and subsequently immersed in a

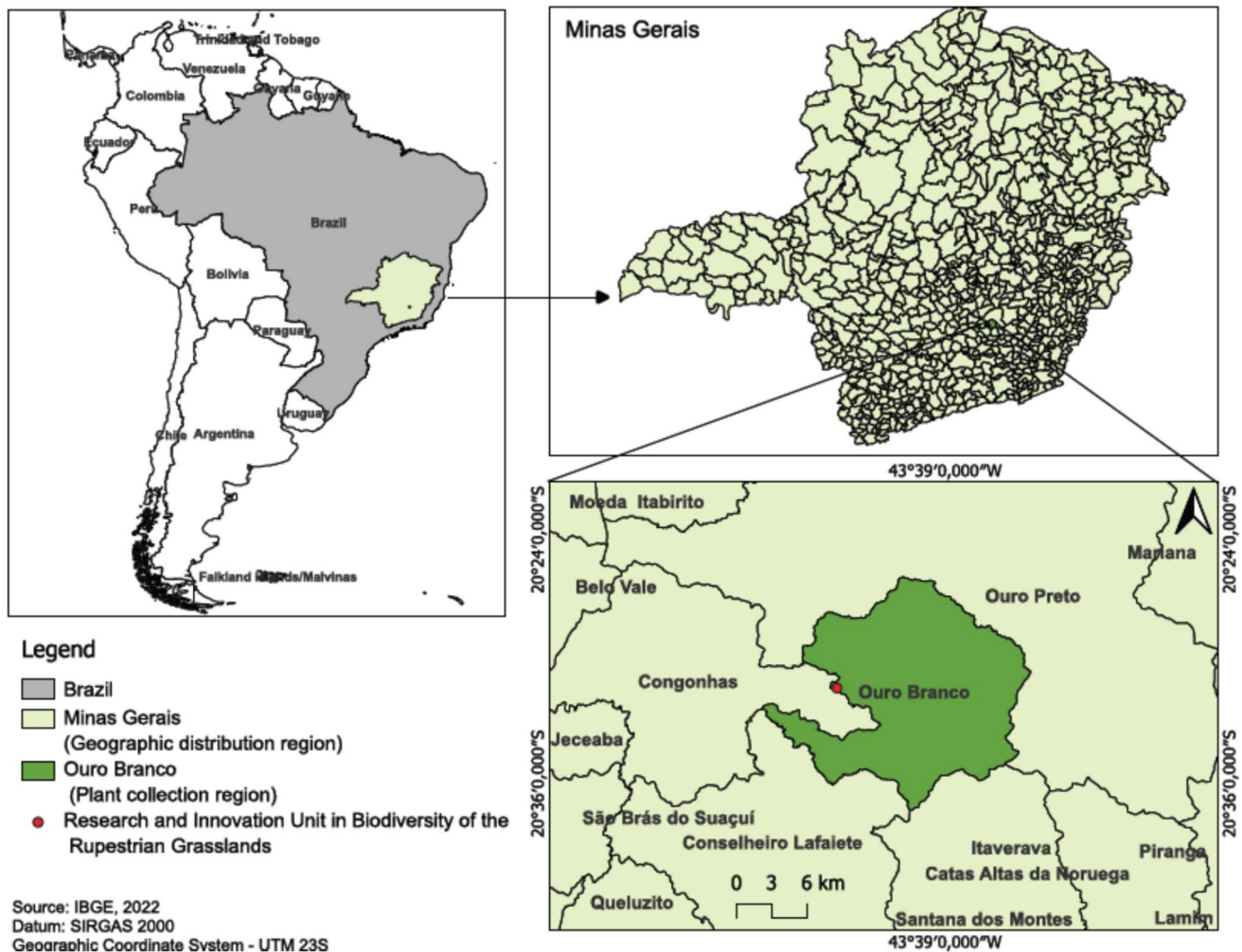


Figure 1. Geographic distribution map and collection site area of *Sinningia rupicola* (Mart.) Wiehler.

sodium hypochlorite (NaOCl) solution at 2.0 to 2.5% (v/v) (Clarix®, Viamão, Brazil) for 5, 10, and 15 min inside a horizontal laminar flow hood (VECO, Campinas, Brazil), with the surface previously sterilized with 70% alcohol (Coperalcool®, Barueri, Brazil) and UV light (LUCMAT GL254nm, 30 W, São Paulo, Brazil) for 15 min. After each treatment period, the seeds were rinsed five times with deionized and autoclaved water and, under aseptic conditions, 10 seeds were inoculated per test tube (25 × 150 mm) containing 10.0 mL of Murashige and Skoog (MS; Murashige and Skoog 1962) culture medium supplemented with 30.0 g L⁻¹ sucrose (Synth Ltda, Diadema, Brazil) and 6.0 g L⁻¹ agar (Merck S.A., Rio de Janeiro, Brazil). The pH of the culture medium was adjusted to 5.8 (± 0.05) prior to agar addition, and the medium was autoclaved in autoclave Digitale 20L (BS Equipamentos Ltda, Varginha, Brazil) at 121 °C and 1.0 kgf cm⁻² pressure for 20 min.

The inoculated seeds were cultivated in a growth chamber at 24 °C (± 1 °C), with a 16-h photoperiod (cool white,

fluorescent lamps, HO Sylvania T12, 20 W, São Paulo, Brazil) and an irradiance of 40 μmol m⁻² s⁻¹ (measured with a LI-COR® LI-250A light meter, Lincoln NE). After 30 d, germination percentage, fungal and bacterial contamination were evaluated. Seedlings that germinated without signs of contamination were considered established and remained in the growth chamber for an additional 30 d before being used in subsequent experiments. The experiment was conducted in a completely randomized design with three treatments and 20 replicates per treatment, with each test tube considered an experimental unit.

Effect of Gas Exchange on *In Vitro* Multiplication To evaluate the effect of gas exchange during the multiplication stage, established shoots measuring 0.5 cm in length were used. Four shoots were subcultured per 250.0 mL glass jar containing 40.0 mL of MS culture medium supplemented with 30.0 g L⁻¹ sucrose (Synth Ltda), 0.5 mg L⁻¹ 6-benzylaminopurine (BAP – Sigma®), 0.05 mg L⁻¹ α-naphthaleneacetic

acid (NAA – Sigma®), and 6.0 g L⁻¹ agar (Merck S.A., Rio de Janeiro, Brazil). The jars were sealed using three different gas exchange systems: (a) polypropylene caps without membranes (0Mem); (b) polypropylene caps with a single 1.0 cm diameter hole covered with a 1.0 cm² membrane (1Mem); and (c) polypropylene caps with three 1.0 cm diameter holes, each covered with a 1.0 cm² membrane (3Mem). The membranes were composed of three layers of microporous surgical tape (Cremer®, Blumenau, Brazil) and one layer of polytetrafluoroethylene (PTFE – Amanco®, Joinville, Brazil) tape measuring 0.05 mm ± 0.01 mm, as described by Saldanha *et al.* (2012).

After 30 d, the following parameters were evaluated: tissue oxidation, vigor, fungal and bacterial contamination, number of leaves per explant, cluster diameter (bud cluster), and foliar content of photosynthetic pigments [chlorophyll *a*, chlorophyll *b*, total chlorophyll (*a* + *b*), and carotenoids]. Microbial presence was assessed as either present or absent, while vigor and oxidation were evaluated based on the scales described by Souza *et al.* (2018). The experiment was conducted in a completely randomized design with 80 replicates (20 jars containing 4 shoots each), with each shoot considered an experimental unit.

Effect of Gas Exchange on *In Vitro* Elongation Clusters measuring 5 mm in diameter were subcultured into 250.0 mL glass jars containing 40.0 mL of MS culture medium supplemented with 30.0 g L⁻¹ sucrose (Synth Ltda), 0.05 mg L⁻¹ BAP (Sigma®), 0.5 mg L⁻¹ NAA (Sigma®), and 6.0 g L⁻¹ agar (Merck S.A.). The jars were sealed using the gas exchange systems from the previous experiment (0Mem, 1Mem, and 3Mem).

After 45 d of cultivation, tissue oxidation, vigor, fungal and bacterial contamination, number of leaves per explant, cluster diameter (mm), average shoot length (cm), and foliar content of photosynthetic pigments [chlorophyll *a*, chlorophyll *b*, total chlorophyll (*a* + *b*), and carotenoids] were evaluated. The experiment was conducted in a completely randomized design with 20 replicates, considering each jar containing 4 clusters as the experimental unit.

Effect of Gas Exchange on *In Vitro* Rooting Shoots measuring 1.0 cm in length from the elongation experiment were isolated and inoculated into 250.0 mL jars containing 40.0 mL of MS culture medium supplemented with 30.0 g L⁻¹ sucrose (Synth Ltda), 0.05 mg L⁻¹ BAP (Sigma®), 0.1 mg L⁻¹ NAA (Sigma®, Barueri, Brazil), 0.1 mg L⁻¹ indole-3-butyric acid (IBA – Sigma®), and 6.0 g L⁻¹ agar (Merck S.A.). The jars were sealed using the gas exchange systems from previous experiments (0Mem, 1Mem, and 3Mem). After subculturing and sealing, the jars remained in the growth chamber for 60 d. At the end of the experiment, tissue oxidation, vigor, fungal and bacterial contamination,

number of leaves per explant, average shoot length (cm), number of roots, and foliar content of photosynthetic pigments [chlorophyll *a*, chlorophyll *b*, total chlorophyll (*a* + *b*), and carotenoids] were evaluated. The experiment was conducted in a completely randomized design with 80 replicates (20 jars containing 4 shoots each), considering each shoot as an experimental unit.

Acclimatization *In vitro*-produced *Sinningia rupicola* seedlings were rinsed under running water to remove the culture medium and subsequently planted in 200.0 mL polypropylene containers (Copobras S.A., Guarulhos, Brazil) with drainage holes at the bottom, filled with 100.0 mL of a substrate composed of decomposed pine bark (CarolinaSoil®, Santa Cruz do Sul, Brazil) and vermiculite (BrasilMinerios S.A., São Luis de Montes Belos, Brazil) in a 2:1 (v/v) ratio, arranged in trays and sealed with plastic film. After planting and adding 5.0 mL of water per seedling, the plants were cultivated in a mini-incubator system (Brondani *et al.* 2012; Brondani *et al.* 2018). The seedlings were kept in a growth chamber at 24 °C (±1 °C) with a 16-h photoperiod (white LED lamps, SuperLed, 6500k, 9 W, OuroLux®, São Paulo, Brazil) and an irradiance of 37 μmol m⁻² s⁻¹ (measured with a USB-650 RED TIDE radiometer, Ocean Optics Inc., Dunedin, FL) and were irrigated every 48 h. After 10 days, the containers were uncovered, and survival and fungal contamination were assessed at 30 d. At the end of the acclimatization period, the seedlings were transferred to a greenhouse covered with shade cloth allowing 50% light transmission and irrigated every other d. After 45 days, seedling survival was evaluated, completing the *in vitro* seedling production stages (Fig. 2).

Analysis of Photosynthetic Pigments Content At the end of the multiplication, elongation, and rooting experiments, 25.0 mg of fresh leaf tissue from each treatment was sampled. The samples were immersed in 5.0 mL of DMSO solution (Sigma®, Barueri, Brazil) for 48 h in the dark (Lichtenthaler 1987). Absorbance was measured in triplicate using a 10 mm path length quartz cuvette in a Genesys 10UV spectrophotometer (Thermo Scientific, São Paulo, Brazil). Readings were taken at wavelengths of 665, 649, and 480 nm, and the concentrations of chlorophyll *a*, chlorophyll *b*, total chlorophyll (*a* + *b*), and carotenoids were calculated based on the equations described by Wellburn (1994).

Data Analysis The data were analyzed for homoscedasticity and normality using Hartley's test ($P > 0.05$) and the Shapiro-Wilk test ($P > 0.05$), respectively. The results were subjected to analysis of variance (ANOVA, $P < 0.05$), followed by Tukey's multiple comparison test ($P < 0.05$). Data analysis was performed using R software version 4.0.3 (2020), employing the easyanova package (Arnhold 2013).

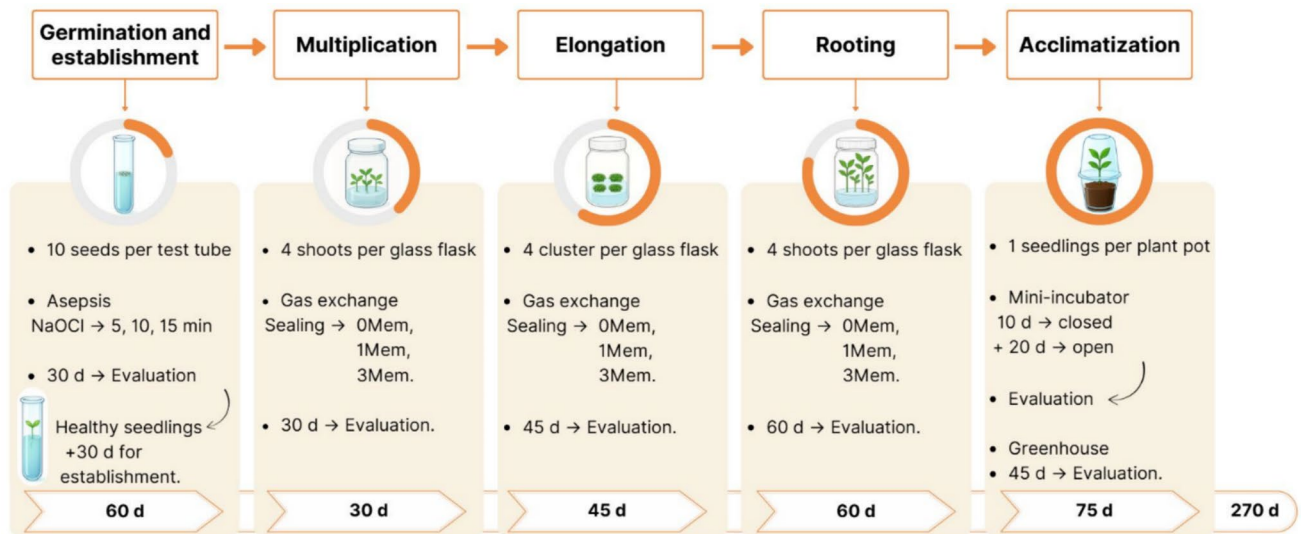


Figure 2. Flowchart of the seedling production stages of *Sinningia rupicola* (Mart.) Wiehler.

Results

***In vitro* Germination and Establishment** The different exposure times of *Sinningia rupicola* seeds to active chlorine (2.0 to 2.5%) showed significant differences in germination percentage and contamination levels. The 15-min treatment differed significantly from the others, exhibiting the lowest average contamination rate (17.8%), followed by the 5-min

(20.7%) and 10-min (21.4%) treatments (Fig. 3a). However, in terms of germination, the 5- and 10-min treatments showed the best results, with similar performance between them, reaching 55.0%, which statistically differed from the 15-min treatment that showed 37.6% germination (Fig. 3b).

Effect of Gas Exchange on *In Vitro* Multiplication Tissue oxidation was not observed in any of the treatments, with scores equal to 1.0. Vigor, cluster diameter, chlorophyll *a*,

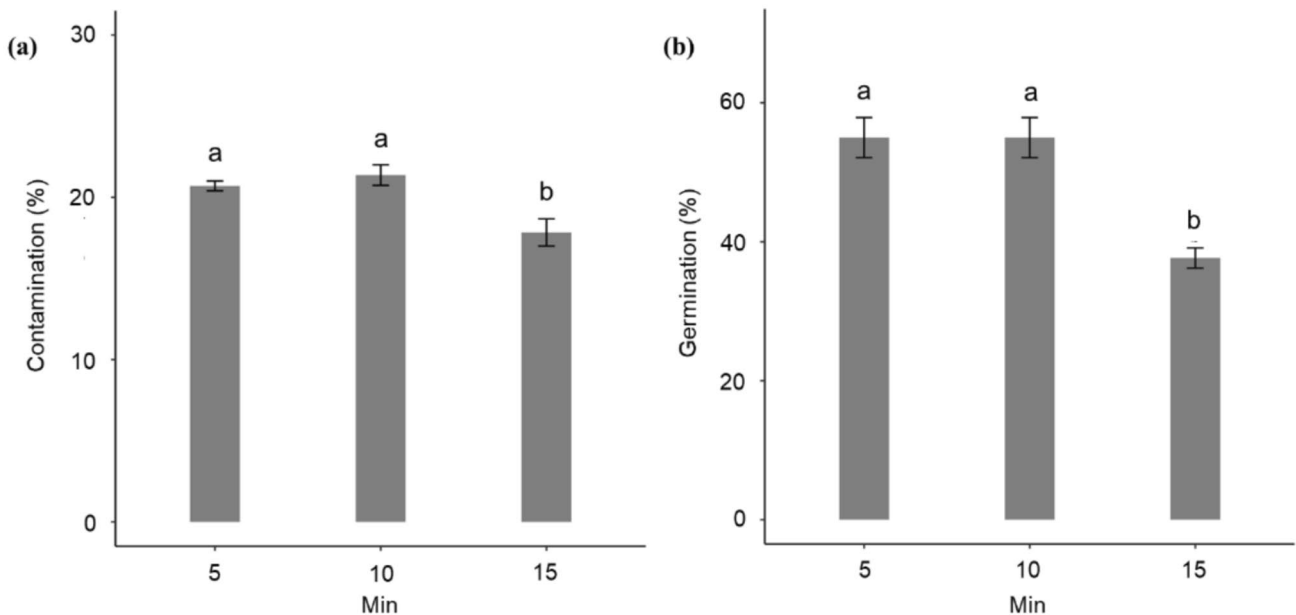


Figure 3. Characteristics evaluated 30 d after *in vitro* inoculation of *Sinningia rupicola* (Mart.) Wiehler seeds regarding exposure time to sodium hypochlorite (5, 10, and 15 min). (a) Fungal and bacterial

contamination; (b) *In vitro* germination. Means followed by the same letter do not differ significantly according to Tukey's test at a 5% significance level. Error bars represent the standard error of the sample.

b, total ($a + b$), and carotenoids showed significant differences among treatments (Figure 4a–f). For vigor, evaluated using the scale of Souza *et al.* (2018), the treatment with three membranes (3Mem) showed the best result, with a mean vigor value of 1.04, statistically differing from 1Mem, which had a mean of 1.3, while 0Mem presented a mean of 1.16 (Fig. 4a).

Fungal and bacterial contamination were absent in all three treatments, and the number of leaves per explant did not differ statistically, with means of 4.9, 5.9, and 4.8 for treatments 0Mem, 1Mem, and 3Mem, respectively. Regarding cluster diameter, treatments 0Mem and 1Mem showed the best results, with means of 6.25 mm and 5.97 mm, respectively, statistically differing from treatment 3Mem with 4.54 mm (Fig. 4b).

In the photosynthetic pigment analyses, treatment 3Mem showed the highest concentrations of chlorophyll *a* ($0.1557 \mu\text{g}\cdot\text{mg}^{-1}$), chlorophyll *b* ($0.1535 \mu\text{g}\cdot\text{mg}^{-1}$), total chlorophyll ($a+b$) ($0.3093 \mu\text{g}\cdot\text{mg}^{-1}$), and carotenoids ($0.0290 \mu\text{g}\cdot\text{mg}^{-1}$), differing statistically from the conventional treatment 0Mem,

which presented the lowest pigment concentrations: chlorophyll *a* ($0.0756 \mu\text{g}\cdot\text{mg}^{-1}$), chlorophyll *b* ($0.1144 \mu\text{g}\cdot\text{mg}^{-1}$), total chlorophyll ($a+b$) ($0.1901 \mu\text{g}\cdot\text{mg}^{-1}$), and carotenoids ($0.0103 \mu\text{g}\cdot\text{mg}^{-1}$). The 1Mem treatment showed intermediate results between the other treatments (0.1014 , 0.1255 , 0.2270 , and $0.0167 \mu\text{g}\cdot\text{mg}^{-1}$, respectively) (Fig. 4c–f).

Effect of Gas Exchange on *In Vitro* Elongation Tissue oxidation was not observed in any of the treatments, with scores equal to 1.0. There was no significant difference in vigor among the 0Mem, 1Mem, and 3Mem treatments, with means of 1.0, 1.0, and 1.1, respectively. Fungal and bacterial contamination were absent in all three treatments. The number of leaves per explant did not differ statistically, with means of 6.2, 5.8, and 6.2 for treatments 0Mem, 1Mem, and 3Mem, respectively.

Cluster diameter, average shoot length, chlorophyll *a*, chlorophyll *b*, total chlorophyll ($a + b$), and carotenoids showed significant differences among treatments (Fig. 5a–f). The cluster diameter, treatments 0Mem and 1Mem showed

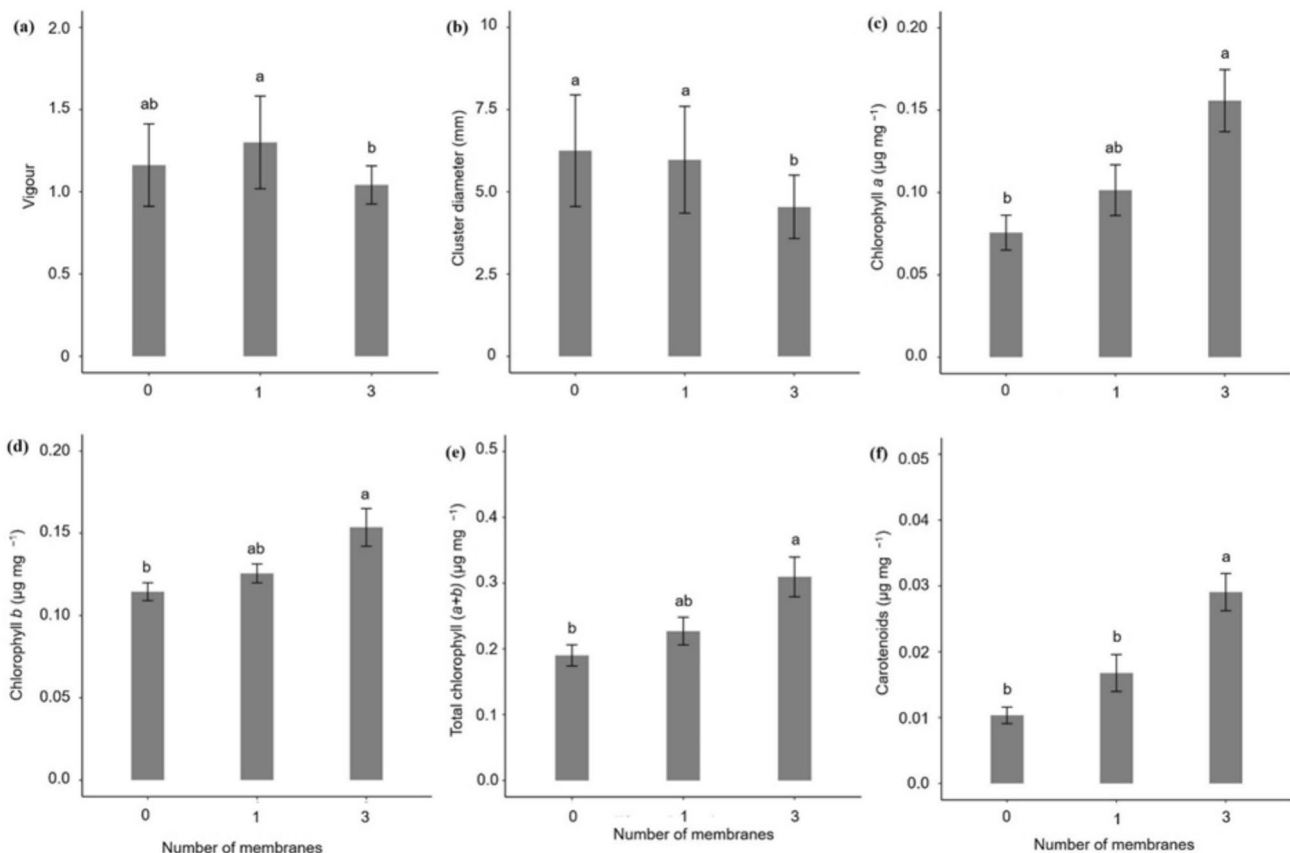


Figure 4. Characteristics evaluated at 30 d of *in vitro* multiplication of *Sinningia rupicola* (Mart.) Wiehler in relation to the number of membranes used for gas exchange (0Mem, 1Mem, and 3Mem), which showed significant differences. (a) Vigour (according to rating scale: 1 = excellent; 2 = good; 3 = poor, Souza *et al.* 2018); (b) Cluster

diameter; (c) Chlorophyll *a*; (d) Chlorophyll *b*; (e) Total chlorophyll ($a+b$); (f) Carotenoids. Means followed by the same letter do not differ significantly according to Tukey's test at a 5% significance level. Error bars represent the standard error of the sample.

better results, both with a mean of 60 mm, statistically differing from treatment 3Mem, which had a mean of 53.5 mm (Fig. 5a). Regarding shoot length, the best results were observed in treatments 1Mem and 3Mem, with average lengths of 4.3 cm and 3.95 cm, respectively, statistically differing from treatment 0Mem, which had an average length of 2.9 cm (Fig. 5b).

In the photosynthetic pigment analyses, treatment 3Mem showed the highest concentrations of chlorophyll *a* ($0.1662 \mu\text{g}\cdot\text{mg}^{-1}$), chlorophyll *b* ($0.0588 \mu\text{g}\cdot\text{mg}^{-1}$), total chlorophyll (*a* + *b*) ($0.2251 \mu\text{g}\cdot\text{mg}^{-1}$), and carotenoids ($0.0429 \mu\text{g}\cdot\text{mg}^{-1}$), statistically differing from treatments 1Mem (0.1230 , 0.0456 , 0.1686 , and $0.0307 \mu\text{g}\cdot\text{mg}^{-1}$ for chlorophyll *a*, *b*, total chlorophyll (*a* + *b*), and carotenoids, respectively) and 0Mem (0.1058 , 0.0349 , 0.1407 , and $0.0334 \mu\text{g}\cdot\text{mg}^{-1}$ for chlorophyll *a*, *b*, total chlorophyll (*a* + *b*), and carotenoids, respectively) (Fig. 5c–f).

Effect of Gas Exchange on *In Vitro* Rooting Tissue oxidation did not differ statistically, with scores of 1.0, 1.1, and 1.2 for treatments 0Mem, 1Mem, and 3Mem, respectively. No

significant differences in vigor were observed among treatments 0Mem, 1Mem, and 3Mem, with means of 1.02, 1.01, and 1.05, respectively. Fungal and bacterial contamination were absent in all three treatments. The number of leaves per explant did not differ statistically, with means of 4.8, 5.4, and 5.1 for treatments 0Mem, 1Mem, and 3Mem, respectively. Average shoot length was not significantly different among treatments, with means of 1.55 cm, 1.35 cm, and 1.26 cm for 0Mem, 1Mem, and 3Mem, respectively. The number of roots also did not differ significantly among treatments 0Mem, 1Mem, and 3Mem, with means of 1.1, 1.3, and 1.7, respectively, but all produced complete plants suitable for the next stage of seedling production.

In vitro rooting, only chlorophyll *a*, *b*, and total chlorophyll (*a* + *b*) showed significant differences among treatments. Treatment 3Mem showed the highest concentrations of chlorophyll *a* ($0.1615 \mu\text{g}\cdot\text{mg}^{-1}$), chlorophyll *b* ($0.0569 \mu\text{g}\cdot\text{mg}^{-1}$), and total chlorophyll (*a* + *b*) ($0.2185 \mu\text{g}\cdot\text{mg}^{-1}$), differing statistically from treatment 0Mem, which had the lowest pigment concentrations (0.0812 , 0.0217 , and $0.1029 \mu\text{g}\cdot\text{mg}^{-1}$, respectively). Treatment 1Mem presented

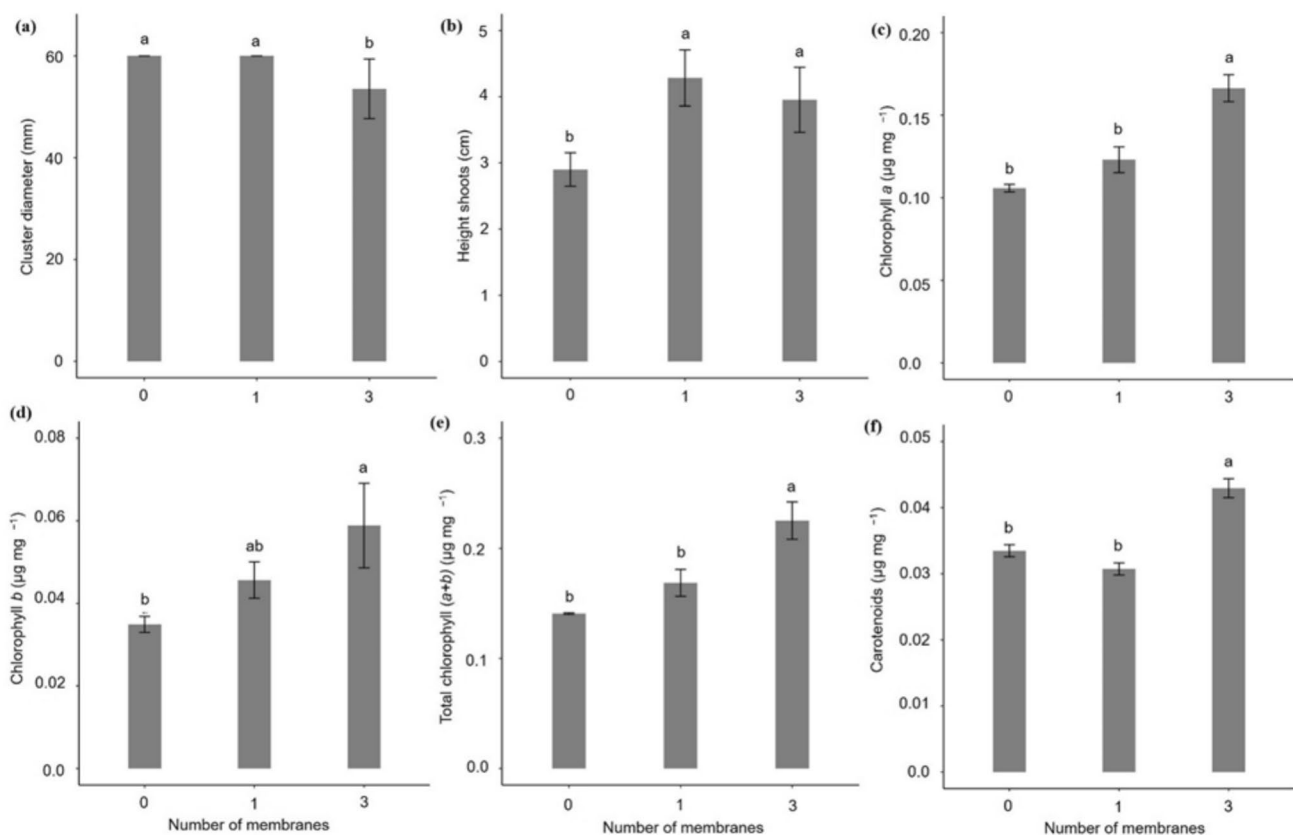


Figure 5 Characteristics evaluated at 45 d of *in vitro* shoot elongation of *Sinningia rupicola* (Mart.) Wiehler in relation to the number of membranes used for gas exchange (0Mem, 1Mem, and 3Mem), which showed significant differences. (a) Cluster diameter; (b) Shoot length;

(c) Chlorophyll *a*; (d) Chlorophyll *b*; (e) Total chlorophyll (*a* + *b*); (f) Carotenoids. Means followed by the same letter do not differ significantly according to Tukey's test at a 5% significance level. Error bars represent the standard error of the sample.

intermediate results between the treatments (0.1326, 0.0361, and 0.1688 $\mu\text{g}\cdot\text{mg}^{-1}$) (Fig. 6a–c).

Acclimatization Out of a total of 255 acclimatized seedlings, 36 showed fungal contamination followed by senescence, and 219 survived, resulting in an acclimatization rate of 85.8%. After 45 days under nursery conditions, the *in vitro* produced plants exhibited 100% survival.

Discussion

For endangered species with propagation difficulties, it is essential to study techniques that enable mass seedling production, aiding in the restoration and preservation of these species. Micropropagation is an alternative for producing seedlings of species with natural propagation challenges, as it can provide large quantities of seedlings in a short time (Righeto *et al.* 2012). However, adjustments are necessary according to the specific requirements of each species to ensure the efficiency of the technique in seedling production (Oliveira *et al.* 2013).

During the *in vitro* establishment stage, one of the main challenges is fungal and/or bacterial contamination, both exogenous and endogenous, which can hinder tissue culture and limit establishment (Xavier *et al.* 2013). Aseptic methodologies involving immersion in sodium hypochlorite solutions are commonly used. For instance, Silva *et al.* (2019) observed in *Melanoxylon brauna* *in vitro* germination that a 25-min exposure to 2.5% active chlorine NaOCl reduced contamination and consequently favored higher normal seedling production. For *Mimosa calodendron*, the best results were observed with immersion times of 5 and 10

min in 2.0 to 2.5% active chlorine NaOCl solution, while 15 min resulted in higher oxidation, non-responsive seeds, and lower germination (Souza *et al.* 2022). These studies demonstrate that exposure time to the disinfectant, here NaOCl, must be evaluated and tailored for each target species.

In this study, exposing *Sinningia rupicola* seeds to sodium hypochlorite for 5 and 10 min resulted in higher germination percentages, with 5 min showing the lowest contamination among these treatments. Conversely, the 15-min treatment, despite lower fungal contamination, showed the lowest germination percentage, likely due to prolonged cellular tissue exposure to NaOCl. Silva *et al.* (2019) reported that extended exposure to disinfectants can increase seed coat permeability and leach essential plant growth regulator required for germination, thereby impairing seed germination. Therefore, 5 min of exposure to sodium hypochlorite at 2.0 to 2.4% active chlorine can be considered the most effective aseptic treatment for inoculation and *in vitro* germination of *Sinningia rupicola* seeds (Fig. 7a).

Gas exchange systems that prevent pathogen entry into culture vessels during *in vitro* cultivation are important; however, sealing reduces CO_2 availability inside the vessels, which can induce morphophysiological changes in plants (Ferreira *et al.* 2016; Batista *et al.* 2018; Souza *et al.* 2020). Such changes include deficient epicuticular wax formation and impaired stem-root connections, increased intercellular spaces and stomatal density, altered stomatal closure mechanisms, and reduced leaf mesophyll differentiation, all potentially leading to photosynthesis and transpiration rate deficiencies (Dutra *et al.* 2009; Pinheiro *et al.* 2013).

The plant's response to gas exchange during *in vitro* cultivation may vary by species. For *Sinningia rupicola*, in the multiplication stage using different gas exchange systems,

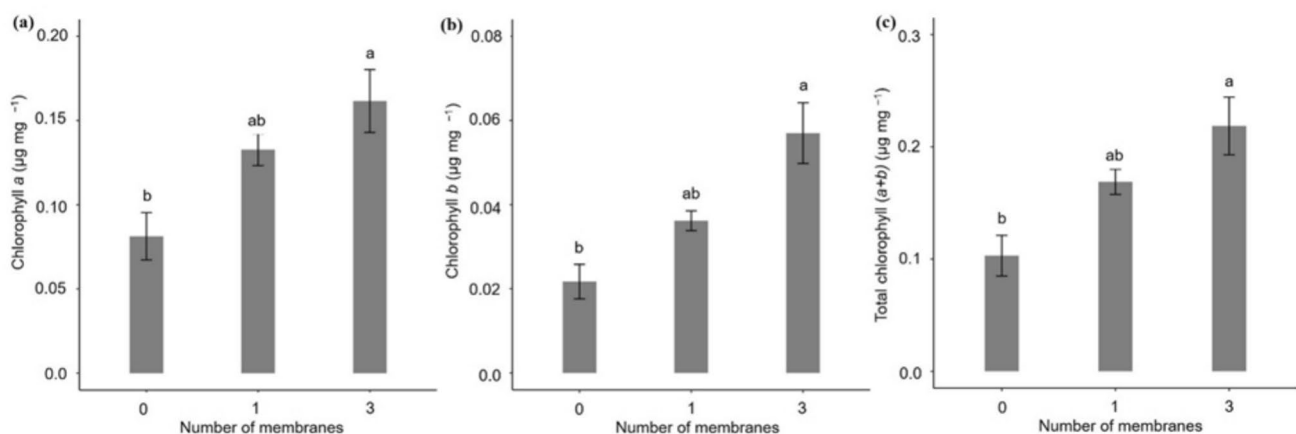
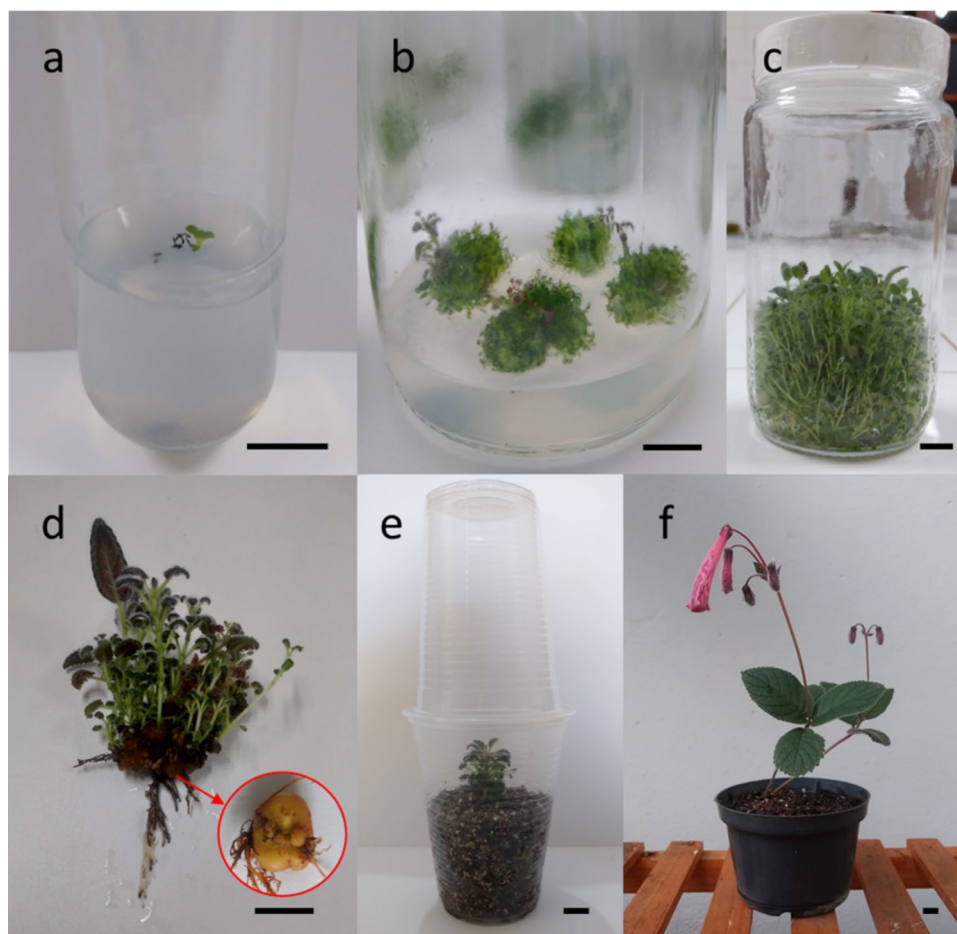


Figure 6. Characteristics evaluated at 60 d of *in vitro* rooting of *Sinningia rupicola* (Mart.) Wiehler in relation to the number of membranes used for gas exchange (0Mem, 1Mem, and 3Mem), which showed significant differences. (a) Chlorophyll a; (b) Chlorophyll b;

(c) Total chlorophyll (a+b). Means followed by the same letter do not differ significantly according to Tukey's test at a 5% significance level. Error bars represent the standard error of the sample.

Figure 7. Stages of micropropagation of *Sinningia rupicola* (Mart.) Wiehler. (a) Seeds inoculated *in vitro* and germinated plantlets. (b) Shoot clusters after 30 d of culture in multiplication medium. (c) Shoot cluster 45 d of culture in elongation medium. (d) Rooted seedling at 60 d, showing detail of the *in vitro* tuber. (e) Beginning of acclimatization in a mini-incubator system. (f) *In vitro* produced seedling established under greenhouse conditions at first flowering after the species' natural regrowth cycle. Scale bar = 1.0 cm.



the treatment with three membranes (greatest gas exchange) yielded the most vigorous shoots after 30 d. This outcome aligns with Martins *et al.* (2015), who found that increased gas exchange with the external environment can produce more vigorous shoots suitable for subsequent *in vitro* culture stages.

The species showed high explant multiplication (Fig. 7b), complicating precise shoot counting; thus, cluster diameter was measured. Treatments 0Mem and 1Mem yielded the largest cluster diameters, averaging 6.25 mm and 5.97 mm, respectively, statistically differing from the 3Mem treatment at 4.54 mm. Although the three-membrane treatment produced higher vigor during multiplication, higher gas exchange did not favor explant multiplication compared to conventional (0Mem) and low gas exchange (1Mem) systems. These findings agree with observations in other species, such as *Kalanchoe delagoensis*, where the one-membrane system resulted in more leaves and seedlings than the three-membrane system (Soares *et al.* 2024). In *Solanum tuberosum*, greater shoot length, fresh mass, and node number occurred under conventional sealing compared to gas exchange treatments (Mohamed and Alsadon 2010). Conversely, Hung *et al.* (2016) found that high gas exchange conditions favored multiplication in species like *Vaccinium*

ashei Reade. These species-specific responses highlight the importance of research on different systems to optimize *in vitro* cultivation conditions (Saldanha *et al.* 2012; Oliveira *et al.* 2021).

Photosynthetic pigments serve as useful indicators for adjusting propagation methodologies. These pigments are crucial components closely related to cell multiplication and plant development (Favetta *et al.* 2017). Despite the 3Mem treatment showing the highest pigment concentrations, statistically different from the conventional 0Mem with the lowest concentrations, it did not correspond to the highest shoot multiplication. Similarly, in elongation and rooting stages, the three-membrane treatment showed higher pigment values; however, as Agathokleous *et al.* (2020) noted, both high and low pigment levels can indicate adaptive stress responses. Sanchez-Zabala *et al.* (2015) suggested higher chlorophyll concentrations as a strategy to increase CO₂ assimilation and mitigate stress. Hence, higher pigment content does not necessarily imply the best treatment.

The 3Mem treatment exhibited medium cracking and volume reduction in the rooting stage, likely resulting from increased gas exchange and subsequent moisture loss. This condition, although expected for systems with higher

permeability, may have intensified desiccation stress, negatively affecting root elongation and overall seedling vigor. Conversely, treatments with fewer membranes, particularly 1Mem, maintained more stable humidity while still allowing gradual gas exchange, promoting a more balanced physiological adjustment. Therefore, intermediate chlorophyll levels and the physical stability observed under 1Mem appear to be more favorable for acclimatization success.

Except for carotenoids during multiplication, the one-membrane treatment (1Mem) consistently exhibited intermediate pigment values, suggesting a balanced physiological condition with reduced stress and more stable *in vitro* growth. This equilibrium likely reflects an optimal balance between gas exchange and humidity retention, providing favorable conditions for multiplication, elongation, and rooting of *Sinningia rupicola*. On *in vitro* elongation stage, cluster diameter was greater in 0Mem (conventional) and 1Mem treatments, both averaging 60 mm, consistent with multiplication stage results. This suggests that conventional and moderate gas exchange (1Mem) systems are effective for explant multiplication even when induced to elongate. The high explant multiplication during elongation (Fig. 7c) could be advantageous by allowing combination of multiplication and elongation stages, reducing total cultivation time for *Sinningia rupicola*. Treatment 1Mem also yielded the longest shoots, averaging 4.3 cm, statistically exceeding the conventional 0Mem system shoots averaging 2.9 cm. Similar outcomes were reported for other species, such as *Vaccinium ashei*, where membrane-based gas exchange systems enhanced shoot length, number, and chlorophyll content (Hung *et al.* 2016). For *Paliavana sericiflora*, endemic to the Ferruginous Rupestrian Grassland, both one- and three-membrane systems promoted shoot growth and new shoot emergence during *in vitro* elongation (Leite *et al.* 2024). *Capsicum annuum* also showed longer shoots under membrane-sealed cultures compared to conventional systems (Batista *et al.* 2017). Forest species like *Eucalyptus dunnii* benefited from gas exchange in elongation and adventitious rooting stages (Souza *et al.* 2019). According to Kozai (2010) and Martins *et al.* (2015), gas exchange systems permitting gas flow between the vessel interior and exterior can enhance growth if CO₂ concentrations are adequate.

Gas exchange systems formed by permeable membranes prevent pathogen ingress without blocking air circulation, increasing CO₂ concentrations and lowering relative humidity inside culture vessels. This can enhance transpiration rates and nutrient uptake, favoring photosynthesis. It also promotes proper stomatal function, increases drought tolerance, and reduces hyperhydricity risk in shoots (Zobayed *et al.* 2001; Erig and Schuch 2005; Dutra *et al.* 2009; Batista *et al.* 2018). These factors further improve acclimatization success and survival rates of micropropagated plants (Kozai and Kubota 2001; Xiao *et al.* 2011; Souza *et al.* 2020).

Seedlings developed tuberous roots (Fig. 7d), characteristic of the species, demonstrating the efficacy of micropropagation for producing plants suitable for acclimatization. Moreover, acclimatization conditions and the mini-incubator system (Fig. 7e) proved efficient for *ex vitro* adaptation of *Sinningia rupicola* seedlings propagated *in vitro*. This system was also effective in acclimatizing other Ferruginous Rupestrian Grassland species such as *Paliavana sericiflora* (Leite *et al.* 2024), and exotic species like *Eucalyptus benthamii* (Brondani *et al.* 2012). Fungal contaminations may have occurred due to the substrate used; therefore, adjustments such as autoclaving or aseptic pre-treatments can be adopted to increase survival rates during acclimatization. Under nursery conditions, *in vitro*-produced plants exhibited 100% survival, enabling healthy *Sinningia rupicola* plants within 270 d. This supports the use of *in vitro* cultivation as a strategy for seedling production, aiding conservation and biodiversity maintenance in the Ferruginous Rupestrian Grassland (Fig. 7f). Although this study focused on morphological and physiological markers, future research should include assessments of clonal fidelity through genetic stability analyses (RAPD, ISSR) to further validate the conservation potential of the established protocol.

In conclusion, the use of sodium hypochlorite (2.0 to 2.5% of active chlorine) for 5 min was the most effective for seed asepsis of *Sinningia rupicola*, promoting *in vitro* germination and establishment. Gas exchange system with one membrane (1Mem) favored explant multiplication and elongation, as well as the development of healthy seedlings during the rooting stage, making them suitable for acclimatization. The mini-incubator system enabled successful *ex vitro* adaptation of plants and was considered efficient for acclimatizing *Sinningia rupicola* seedlings.

Acknowledgements We thank the National Council for Scientific and Technological Development, Brazil ('Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq'), Coordination for Improvement of Higher Education Personnel, Brazil ('Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES – Código de Financiamento 001'), and Foundation for Research of the State of Minas Gerais, Brazil ('Fundação de Amparo à Pesquisa do Estado de Minas Gerais – FAPEMIG', Rede Mineira de Biotecnologia em Multiplicação e Clonagem de Plantas). We also thank the Research and Innovation Unit in Ferruginous Rupestrian Grassland ('Unidade de Pesquisa e Inovação em Campos Rupestres Ferruginosos'), GERDAU Açominas S.A. ('Convênio DICON/UFLA: 008/2020') and Companhia Siderúrgica Nacional S. A. (Acordo de Parceria UFLA: 006/2025). The Article Processing Charge for the publication of this research was funded by the Coordination for the Improvement of Higher Education Personnel - CAPES (ROR identifier: 00x0ma614). For open access purposes, the authors have assigned the Creative Commons CC BY license to any accepted version of the article.

Funding The Article Processing Charge (APC) for the publication of this research was funded by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) (ROR identifier: 00x0ma614).

Declarations

Competing Interest The authors have no competing interests.

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